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EVALUATION OF ANTITUMORAL ACTIVITY OF BONE TARGETED DRUGS/CONVENTIONAL CHEMOTHERAPIES AND IDENTIFICATION OF BIOMARKERS FOR THE SELECTION OF PATIENTS WITH BREAST CANCER FOR THE BONE TARGETED THERAPY IN ADJUVANT SETTING

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1 Introduction

1. 1Breast Cancer

1.1.1 Epidemiology

In 2008, the estimated age-adjusted annual incidence of breast cancer in Europe (40 countries) was 88.4/100 000 and the mortality 24.3/100 000. The incidence increased after the introduction of mammography screening and continues to do so with the aging of the population. The most important riskfactors include genetic predisposition, exposure to estrogens (endogenous and exogenous) and ionising radiation, low parity and history of atypical hyperplasia. The Western-style diet, obesity and consumption of alcohol also contribute to the rising incidence of breast cancer [2]. There is a steep age gradient, with about a quarter of breast cancers occurring before age 50, and <5% before age 35. The estimated prevalence of breast cancer in Europe in 2010 was 3 763 070 cases [3] and is increasing, both as a consequence of increased incidence and of improvements in treatment outcomes. In most Western countries, the mortality rate has decreased in recent years, especially in younger age groups because of improved treatment and earlier detection [4]. However, breast cancer is still the leading cause of cancer-related deaths in European women.

1.1.2 Pathologial classification and clinical parameters

Final pathological diagnosis should be made according to the World Health Organization (WHO) classification [5] and the tumour–node–metastases (TNM) staging system analysing all tissue removed (Table 1). to include number, locationand maximum diameter of tumours removed, the total number of removed and number of positive lymph nodes, and the extent of metastases in the lymph nodes [isolated tumour cells, micrometastases (0.2–2 mm), macrometastases].

Table 1: TNM Classification

Anatomic stage/prognostic groups[a]		
0		
Tis	N0	M0
IA		
T1[b]	N0	M0
IB		
T0	N1mi	M0
T1[b]	N1mi	M0
IIA		
T0	N1[c]	M0
T1[b]	N1[c]	M0
T2	N0	M0
IIB		
T2	N1	M0
T3	N0	M0
IIIA		
T0	N2	M0
T1[b]	N2	M0
T2	N2	M0
T3	N1	M0
T3	N2	M0
IIIB		
T4	N0	M0
T4	N1	M0
T4	N2	M0
IIIC		
Any T	N3	M0
IV		
Any T	Any N	M1

Histological type, grade, immunohistochemical (IHC) evaluation of estrogen receptor (ER) status using a standardized assessment methodology (e.g. Allred or H-score), and, for invasive cancer, IHC evaluation of PgR and HER2 receptor expression are all necessary data for futher clinical therapeutic decisions. HER2 gene amplification status may be determined directly from all tumours by in situ hybridization (fluorescent or chromogenic or silver in situ hybridisation) [6]. Proliferation markers such as the Ki67 labelling index may supply further useful information, particularly if the assay can be standardised [7]. Tumors were divided in surrogate intrinsic subtypes according histology used in the clinical practice and IHC (Table 2).

Tab2: surrogate definitions of intrinsic subtypes of breast cancer according to the 2013 St Gallen Consensus Conference and recommended by the ESMO Clinical Practice Guidelines

Intrinsic subtype	Clinicopathologic surrogate definition	Notes
Luminal A	'Luminal A-like' • ER-positive • HER2-negative • Ki67 low* • PgR high**	*The cut-off point between high and low values for Ki67 varies between laboratories. **Suggested values are 20% for both PgR and Ki67, but laboratory specific cut-off points can be used to distinguish between low and high values for Ki67 and PgR; quality assurance programmes are essential for laboratories reporting these results.

Luminal B	'Luminal B-like (HER2-negative)'
	• ER-positive
	• HER2-negative
	• and either
	  • Ki67 high or
	  • PgR low	

	'Luminal B-like (HER2-positive)' • ER-positive • HER2-positive • any Ki67 • any PgR	
HER2 overexpression	'HER2-positive (non-luminal)' • HER2-positive • ER and PgR absent	
'Basal-like'	'Triple-negative (ductal)' • ER and PgR absent • HER2-negative	There is ~80% overlap between 'triple-negative' and intrinsic 'basal-like' subtype, but 'triple-negative' also includes some special histological types such as (typical) medullary and adenoid cystic carcinoma with low risks of distant recurrence.

1.1.3 Prognostic and predictive factors

The most important prognostic factors in early breast cancer are expression of ER/PgR, HER2 and proliferation markers, number of involved regional lymph nodes, tumour histology, size, grade and presence of peritumoural vascular invasion. Clinical parameters (age, tumour stage, ER expression and histological grade) have been incorporated into scoring systems that permit a relatively accurate estimation of the probability of recurrence and death from breast cancer; [8]. Gene expression profiles such as MammaPrint® (Agendia, Amsterdam, the Netherlands) or Oncotype DX® Recurrence Score (Genomic Health, Redwood City, USA) may be used to gain additional prognostic and/or predictive information to complement pathology assessment and to predict response to adjuvant chemotherapy. This is particularly true in patients with ER-positive early breast cancer; however, their true clinical utility is still being evaluated in large randomised clinical trials such as MINDACT, TAILORx and RxPONDER.

ER/PgR and HER2 are the only validated predictive factors, allowing for selection of patients for endocrine therapies (ETs) and anti-HER2 treatments, respectively. High ER expression is also usually associated with lesser absolute benefit of chemotherapy. After neoadjuvant systemic treatment, the response to treatment and quantity of residual disease are important prognostic factors but need as much standardisation as any of the other biological markers, and no uniform guidelines exist for the evaluation of response to neoadjuvant treatment, although some guidance is provided by the FDA recommendation for accelerated drug approval in neoadjuvant treatment of breast cancer [9].

1.1.4 Antitumoral Treatment

Adjuvant systemic treatment

After surgery, the decision on systemic adjuvant treatment is based on predicted sensitivity to particular treatment methods and their use and individual risk of relapse. According to the 2011 and 2013 St Gallen guidelines, the decision on systemic adjuvant therapies should be based on the surrogate intrinsic phenotype determined by ER/PgR, HER2 and Ki67 assessment with the selective help of first-generation genomic tests when available (such as MammaPrint® or Oncotype DX®) for luminal cases with unclear chemotherapy indications [10,11]. All luminal cancers should be treated with Endocrine Therapy. Most luminal A tumours, except those with highest risk of relapse (extensive nodal involvement), require no chemotherapy, whereas luminal B HER2-negative cancers constitute a population of the highest uncertainty regarding chemotherapy indications.

Indications for chemotherapy within this subtype depend on the individual risk of relapse, taking into account the tumour extent and features suggestive of its aggressiveness (grade, proliferation, vascular invasion), presumed responsiveness to ET and patient preferences. Features associated with lower endocrine responsiveness include low steroid receptor expression, lack of PgR expression, high tumour grade and high expression of proliferation markers.

Luminal B HER2(+)tumours are treated with chemotherapy, ET and trastuzumab; no randomised data exist to support omission of chemotherapy in this group; however, in cases of contraindications for chemotherapy or patient refusal, it is acceptable to offer the combination of targeted agents (ET and trastuzumab). Triple-negative tumours benefit from adjuvant chemotherapy, with the possible exception of low-risk ‘special histological subtypes’ such as medullary or adenoidcystic carcinomas. HER2 (non-luminal) cancers, apart from selected cases with very low risk, such as T1aN0, are treated with chemotherapy plus trastuzumab.

Trastuzumab may routinely be combined with non-anthracycline-based chemotherapy and ET; concomitant use with anthracyclines is not routinely recommended outside of clinical trials, although may be considered in selected patients treated in experienced centres. For most patients, the use of a sequential anthracycline-based followed by taxane-trastuzumab-based regimen is the preferred choice. RT may be delivered safely during trastuzumab, ET and nonanthracycline- based chemotherapy. If chemotherapy and RT are to be used separately, chemotherapy usually precedes RT.

Endocrine therapy

ET is indicated in all patients with detectable ER expression, defined as ≥1% of invasive cancer cells, irrespective of chemotherapy and/or targeted therapy [12]. The choice of medication is primarily determined by patient's menopausal status. Other factors include (minor) differences in efficacy and side effect profile. Permenopausal patients are treated with Tamoxifen

Premenopausal patients. Tamoxifen 20 mg/day for 5–10 years. In patients becoming postmenopausal during the first 5 years of tamoxifen, a switch to letrozole, an aromatase inhibitor (AI), seems to be particularly beneficial [13]. The value of addition of ovarian suppression [by gonadotropin-releasing hormone (GnRH) agonists or ovarian ablation] is not well-defined, in particular in chemotherapytreated patients, who frequently develop ovarian failure as a consequence of cytotoxic treatment [14] failure is not well-established and contradictory data exist.

Postmenopausal patients. Aromatase Inhibitors (AIs) (both non-steroidal and steroidal) and tamoxifen are valid options. AIs allow for prolongation of the DFS, with no significant impact on OS (1%–2%, depending if upfront or sequential strategy) [15-16].

The use of tamoxifen is associated with increased risk of thromboembolic complications and endometrial hyperplasia(including endometrial cancer). Caution should be exercised in patients with conditions predisposing to these sequelae and appropriate diagnostic tests carried out in those presenting with symptoms suggestive of these complications. Although there are no unequivocal data on their detrimental effects, patients on tamoxifen should be advised to avoid the use of strong and moderate CYP2D6 inhibitors or, if such drugs cannot be replaced, a switch to alternative treatment, i.e. AIs, should be considered [17]. Patients undergoing ovarian suppression and AI users are at increased risk of bone loss and should be advised to assure adequate calcium plus vitamin D3 supply and to assess periodically the bone mineral density [by dual energy X-ray absorption (DEXA) scan].

Chemotherapy

Chemotherapy is recommended in the vast majority of triple negative, HER2-positive breast cancers and in high-risk luminal HER2-negative tumours. The benefit from chemotherapy is more pronounced in ER-negative tumours [18]. In ERpositive tumours, chemotherapy at least partially exerts its effect by induction of ovarian failure [19]. Most frequently used regimens contain anthracyclines and/or taxanes, although in selected patients CMF may still be used. Four cycles of AC (doxorubicin, cyclophosphamide) are considered equal to six cycles of CMF, whereas six cycles of three-drug anthracyclinebased regimens are superior[20].

The addition of taxanes improves the efficacy of chemotherapy, independently of age, nodal status, tumour size or grade, steroid receptor expression or tamoxifen use, but at the cost of increased non-cardiotoxicity [20]. Sequential rather than the concomitant use of anthracyclines and taxanes is superior. Overall, chemotherapy regimens based on anthracyclines and taxanes reduce breast cancer mortality byabout one-third [20]. Non-anthracycline, taxane-based regimens (such as four cycles of TC) may in selected patients (such as those at risk of cardiac complications) be used as an alternative to four cycles of anthracycline-based chemotherapy [21]. Chemotherapy is usually administered for 12–24 weeks (four to eight cycles), depending on the individual recurrence risk and the selected regimen.

HER2-directed therapy

Trastuzumab combined with chemotherapy in patients with HER2 overexpression/amplification approximately halves the recurrence risk, compared with chemotherapy alone; this translates into ~0% absolute improvement in 3-year DFS and 3% increase in 3-year OS [22]. Trastuzumab is approved in patients with node-positive disease and in N0 patients with tumours >1 cm, although—due to relatively high failure risk even in patients with N0 tumours <1 cm—it should also be considered in this patient group, in particular in ERnegative disease [23]. In most studies, trastuzumab was administered for 1 year, although in the FinHER trial a similar improvement was obtained with only 9 weeks of treatment. Due to its cardiotoxicity, trastuzumab should not be routinely administered concomitantly with anthracyclines. Combination with taxanes is safe and has been demonstrated to be more effective than sequential treatment [24]. Trastuzumab may also be safely combined with RT and ET.

Bisphosphonates

Some data suggest a beneficial anticancer effect of bisphosphonates, especially when used in a low-estrogen environment (women undergoing ovarian suppression or postmenopausal), although study results are equivocal and such a treatment cannot be

routinely recommended in women with normal bone mineral density. In patients with treatment-related bone loss, bisphosphonates decrease the risk of skeletal complications [25,26].

1.1.5 Follow up

The aims of follow-up are to detect early local recurrences or contralateral breast cancer, to evaluate and treat therapy-related complications (such as menopausal symptoms, osteoporosis and secondary cancers). Ten-year survival of breast cancer exceeds 70% in most European regions, with 89% survival for local and 62% for regional disease [27]. patients with node-positive disease tend to have higher annual hazards of recurrence than patients with node-negative cancers. In the first years the risk of recurrence is higher in patients with ER-negative cancers, but after 5–8 years after diagnosis, the annual hazards of recurrence drop below the level of ER-positive tumours. Relapses of breast cancer may occur as late as >20 years after the initial diagnosis, particularly in patients with ER/PgR-positive disease .Guidelines recommend regular visits every 3 to 4 months in the first 2 years, every 6 months from years 3–5and annually thereafter. Ipsilateral (after BCS) and contralateral mammography is recommended every 1 to 2 years. An MRI of the breast may be indicated for young patients, especially in the case of dense breast tissue and genetic or familial predispositions. In asymptomatic patients, there are no data to indicate that other laboratory or imaging tests (e. g. blood counts, routine chemistry tests, chest X-rays, bone scans, liver ultrasound exams, CT scans or any tumour markers such as CA15-3 or CEA) produce a survival benefit .However, routine blood tests are usually indicated to follow-up patients on ET due to the potential side-effects of these drugs namely in the lipid profile .

1.2 Bone metastases

1.2.1 Phisiopathology of bone metastases

Cancer patients mainly do not die for the primary tumor, but rather for the formation of metastases.

Many of the most common cancers such as breast, prostate and lung commonly metastasize to the bone, indeed more than 50% of patients with prostate cancer or advanced breast show bone metastases.

Radiographically 80% of bone metastases derived from this tumor are osteolytic, 20% are osteoblatic at the time of diagnosis. The 5-year survival of patients with lesions

exclusively bone is 37% while in the presence of extraskeletal metastases that survival is reduced to 13%. Osteoblatic metastases are associated with a better prognosis. Bone metastases are usually accompanied by a significant bone pain, pathological fractures, nerve compression syndromes and hypercalcemia: these complications are called Skeletal related events (SRE) .

The bone is an ideal microenvironment for the development of metastases following hypothesis "seed and soil " proposed by Stephen Paget in 1889 [28]: a metastasis settles in a particular organ if the cells of the primary tumor (seed) are in the favorable site (soil) conditions in terms of chemokines, growth factors and development, sufficient for their arrest and their growth in that site; furthermore, according to this hypothesis, bone microenvironment has many factors and properties that allow cancer cells an important development.

Bone is a supportive connettive tissue consisting of cells spread in an abundant extracellular matrix, consisting of fibers and amorphous substance of glycoproteic origin; this is calcified and also formed from minerals. Furthermore bone is a dynamic tissue which has a structural support, protective, mechanical and trophic functions as it serves as a repository of minerals, particularly calcium ions that play an important role in various cellular activities**.** It is composed of various cell types: in addition to stromal cells, hematopoietic and endothelial cells, osteoclasts and osteoblasts are involved in the development and regulation of bone remodeling. Osteoclasts are derived from progenitor cells of the monocyte-macrophage line and are responsible for bone resorption. These cells adhere to bone matrix via integrin surface and, once activated, they degrade it [29,30]. They resorb bone creating an acidic and isolated microenvironment between the plasma membrane and the bone surface that determines the solubilization of minerals. The free organic matrix is subjected to enzymatic degradation by lysosomal proteases released by osteoclasts (as cathepsin K). The products of the degradation of the organic matrix are endocited and esocited from the opposite side of the cell.

MCSF and RANKL are two essential growth factors for osteoclastogenesis . While MCSF is essential in the early stages of osteoclastogenesis, RANKL is critically involved in the maturation and activation of osteoclasts. MCSF is produced by stromal cells and osteoblasts and binds to its receptor c-fms expressed on the surface of the macrophage precursors and stimulates proliferation [31-33]. RANKL is expressed by osteoblasts and stromal cells and interacts with the receptor RANK localized on the membrane of the

monocyte - macrophage precursors and induces differentiation into osteoclasts and their activation [34-38].

Different cytokines produced locally as well as systemic calciotropic hormones, including parathyroid hormone (PTH), the 1,25 dihydroxyvitamin D3 and prostaglandins, indirectly stimulates osteoclastogenesis by increasing the expression of RANKL on bone marrow stromal cells and osteoblasts . In addition, other cytokines such as IL -1 and TNF- α are able to act directly on osteoclasts [39-40].Osteoblasts are cells of mesenchymal derivation delegated to the synthesis and mineralization of bone matrix. For osteoblast differentiation, mesenchymal stem cell (MSC) first undergoes proliferation, it becomes the commitment and therefore differentiate in pre-osteoblast (which produces alkaline phosphatase) and later in a mature osteoblast which produces an increasing amount of osteocalcin and calcified matrix. Runx2 and Osteorix are two transcription factors that determine the expression of many genes associated with osteoblast differentiation. The commitment of MSCs into osteoblast line is controlled by three morphogenetic pathway: the BMP, HH and Wnt pathway [41-44]. Once formed the matrix , numerous osteoblasts become trapped in bone lacunae and thus they become osteocytes. They are not a inert cell for bone metabolism; osteocytes, indeed, could participate in the exchange of minerals from the bone, then intervening in the homeostatic regulation of the concentration of calcium in the body and, working as mechano sensors, can modulate the bone resorption in response to different stimuli [45,46]. Bone matrix is constituted by the organic matrix reinforced by the deposition of calcium salts. The type I collagen constitutes about 90-95 % of the organic matrix while non- collagenous proteins constitute the remaining 5-10 %. The crystalline salts deposited in the matrix are primarily calcium and phosphate in the form of hydroxyapatite. The proteins can be divided into non- collagenous proteins of cell adhesion, proteoglycans, γ - carboxylated and growth factors .Each of the adhesion proteins as osteopontin, bone sialoprotein (IBSP), vitronectin and type I collagen facilitate interactions with integrins that are expressed by hematopoietic stem cells and specialized cells of the bone , as well as osteotropic tumor cells .

As a consequence of bone remodeling, growth factors stored in the bone such as IGF, FGF, PDGF , TGF-β and BMP, are released into the medullary cavity and act on metastatic cancer cell growth [47-49].

1.2.2 Metastasis process

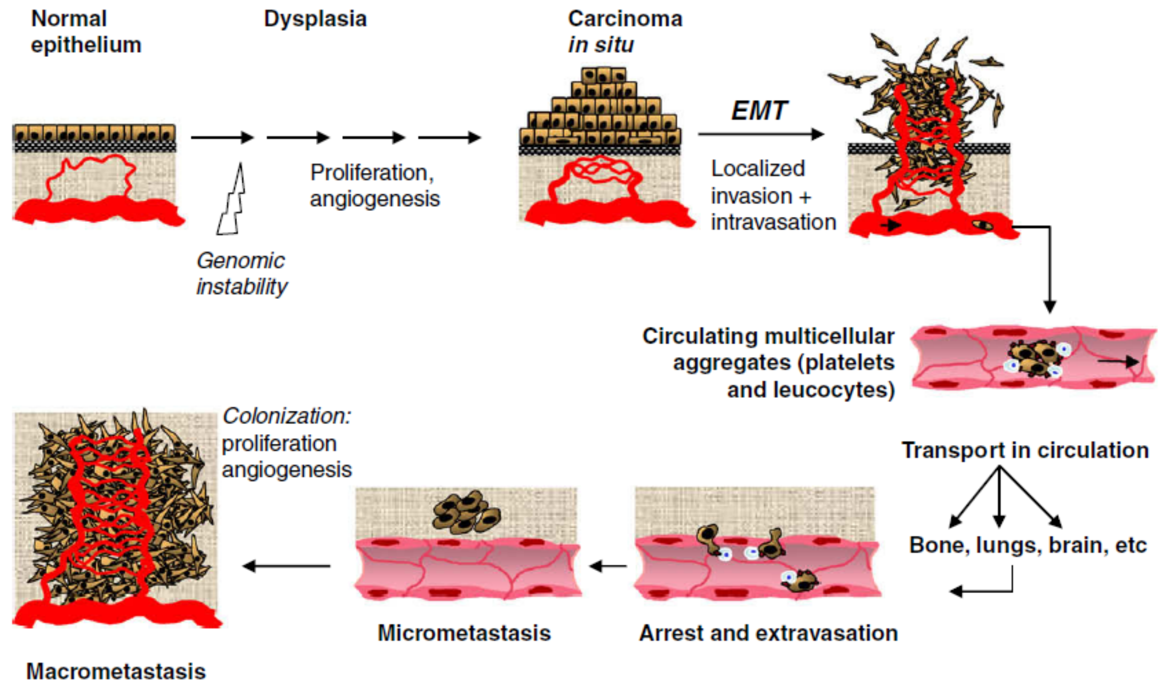
It has long been recognized that primary cancers spread to distant organs with characteristic features [50], and the skeleton is one of the most common organs to be affected by metastatic cancer. Breast and prostate cancer are osteotropic tumors, i.e., carcinomas that have a special predilection to form bone metastases. At postmortem examination, about 70% of patients with these tumros had metastatic bone disease. Together, breast and prostate cancer probably account for more than 80% of cases of metastatic bone disease [51, 52].

At time of diagnosis, most patients with breast and prostate [53] cancer do not have clinicopathologic signs of overt distant metastases. Thus, after resection of the primary tumor and all positive lymph nodes, these patients are in complete clinical remission. However, disseminated tumor cells (DTCs) may already be present in bone marrow (BM) [54-55], a clinical situation called minimal residual disease (MRD). Most DTCs have a limited life span and disappear in time, indicated by the clinical findings that a significant fraction of breast cancer patients with DTCs in BM never develop distant metastases [54]. However, DTCs with an indefinite proliferative potential that have acquired the abilities of metastasizing to, surviving in, and colonizing the bone/BM, can eventually result in the development of an overt bone metastasis. Only this subpopulation of DTCs can, therefore, be regarded as true metastasis-initiating cells (MICs). The clinical courses of patients with breast and prostate cancer with a first recurrence in bone are relatively long, with a median survival of 24 and 40 months. This is in marked contrast to those with first recurrence of breast cancer in the liver (3 months). However, involvement of the skeleton in metastatic disease is a major cause of morbidity, characterized by severe pain and high incidence of SREs.

1.2.3 local invasiveness and EMT

The first phase in metastasization process is the acquisition of motility and invasiveness; capabilities that are not compatible with normal tissue. Cancer cells must therefore shed many of their epithelial characteristics, detach from epithelial sheets, and undergo a drastic alteration, a process called the “epithelial-mesenchymal transition” (EMT). Achievement of this invasive phenotype is reminiscent of events of early embryonic development. The importance of this process during embriogenesis is highlighted by the fact that a disfunction in EMT process determines the developmental arrest at the stage of blastula blastula [56].

Fig 1 Metastases



Buijs JT1, van der Pluijm G. Osteotropic cancers: from primary tumor to bone. Cancer Lett. 2009 Jan 18;273(2):177-93.

In malignancy, genetic alterations and the tumor environment can both induce EMT in tumor cells. The important steps that facilitate metastasis seem to be reversible, 7 and cannot be explained solely by irreversible genetic alterations, indicating the existence of a dynamic component to human tumor progression. In cancer, although the PI3K/Akt pathway is the primary inducer of epithelial-mesenchymal transition, the Wnt/B-catenin, Notch, Ras, integrin-linked kinase, and integrin signaling pathways are also involved [28-30].

The epithelial cells that form the epithelia have phenotypic and morpho-functional features:

• They organize to form laminar structures where neighboring cells are adherent to each other by means of junctions systems. This allows the maintenance of the structural characteristics (integrity, stiffness, etc) and functional epithelium;

• The epithelium is polarized , which means that the surfaces on the basal and apical side have different "specilizations", adhere to different substrates and have different functions;

• These cells are poorly mobile, movements are limited only within the epithelium.

Mesenchymal cells instead form structures of different shape and density, unorganized and among them there are only points of focal adhesion and junctional devices as stable between the epithelial cells. Mesenchymal cells are also equipped with high mobility that allows migration or as single cells or as chains of cells. When the epithelial-mesenchymal transition is completed, the epithelial cells has lost some epithelial markers that are replaced by mesenchymal markers.The reduction of cadherins expression (proteins involved in cell-cell adhesion), in particular of E-cadherin, seems to be the key event that allows the realization of the entire process. The formation and stabilization of the clusters of E-cadherin, at the level of the junctions of adhesion between cells, require the chains, in particular the β-catenin, which binds to the cytoplasmic portion of E-cadherin. Furthermore actin filaments (F-actin) stabilize and immobilize E-cadherin clusters at the level of the adherent junctions [57-59]. When E-cadherin levels decrease till becoming limiting, there is a loss of intercellular junctions and of the sequestration of E-cadherin β-catenin-mediated. This means that the β-catenin accumulates and traslocates in the nucleus where, by binding to LEF/TCF transcription factors, activates target genes EMT related as vimentin, and the regulators Twist and Snail [60-62].A part from cadherin, some other proteins involved in tight junction formation are down regulated as ZO-1 (a protein of the zonula occludens), that interact with different trans membrane proteins as ocludina and claudin [63].The reduction of cadherins expression is related to cellular migration increase and with formation of metastases. In presence of N cadherin, for example, FGF-2 causes the activation of the microtuble-associated protein kinase-extracellular signal-regulated kinase (MAPK-ERK) and this pathway, inducing transcription of matrix metalloproteinase 9 (MMP-9), increases dramatically the invasiveness of breast cancer cell. Matrix metalloproteinases are important EMT markers; they are members of the family of neutral endopeptidases Zn dependent that selectively degrade the extracellular matrix. They are expressed in several tumors and they are involved in different phases of metastases development: expansion and escape of single cancer cell from primary tumor, their passage through the blood vessels, survival of into the circulation, and exit of tumor cells from the blood vessels at sites secondary [64]. MMPs are able to degrade the growth factors in an active form and cleave proteins bound and exposed on the surface of the cell as the E-caderina. Fibronectin and vimentin are other two important mesenchymal markers and are respectively, a cytoskeleltal proein, and a protein released in the matrix.

The consequent acquisition of a mesenchymal invasive phenotype by cancer cells causes the break of the basal lamina and the invasion of the underlying stromal compartments. The acquisition of an invasive mesenchymal phenotype do not depend only to somatic mutations and other epigenetic alterations in the cacner cells, but some changen in stromal environment are also necessary for the neoplastic progression [65-66].

In the tumor, indeed, both genetic alterations and tumoral microenvironment can induce EMT in cancer cells [67]. The cancer cells are able to activate the local stromal cells, such as fibroblasts , smooth muscle cells and adipocytes and recruit progenitors of endothelial, mesenchymal and inflammatory cells. The activation of stromal cells leads to the secretion of growth factors and proteases that promote further proliferation and cancer cell invasion [68]. The EMT also enhances angiogenesis. The production of pro-angiogenic factors , including VEGF -A and MMPs , is induced mainly by Snail [69].

Among the factors that cause the most epithelial-mesenchymal transition in cancer are the TGF- β [70-72], FGF , EGF , HGF and IGF. The EMT is also activated by some extracellular signals arising from the interaction of cancer cells with extracellular matrix components such as collagen and hyaluronic acid.

This leads to the activation, at a intracellular level, of different effector proteins such as Ras , Rho, MAPK , Rac and Src that cause a change in the organization of the cytoskeleton and disassembly of different junctional complexes .

Two of the main targets of Ras and MAPK are Slug and Snail, two transcription factors that inhibit the expression of genes that have an E -box in the promoter region such as the E-cadherin and the proteins that constitute the occluding junctions (occludine and claudine). Recently it has been discovered that Elf5, a key regulator of cell fate in the development of alveolar gland mammaria, 64 directly represses the transcription of Snail2 , key transcription factor nell'EMT [73-74].In carcinogenesis, TGF-β plays a key role but with different effects; in the early stages it inhibits cell proliferation but subsequently promotes the formation of metastasis inducing EMT [74]. The signaling of the TGF-β is one of those best characterized. It is based on SMAD-dependent mechanisms where SMAD2 and SMAD3 , once phosphorylated and activated , bind to SMAD4 and are translocated into the nucleus where they activate the co-repressor SIP -1, cha acts as Snail and Slug , inhibiting the expression of genes that contain E-box sequences at the level of the promotor. Furthermore this mechanism induces the autocrine production of TGF- β , which further increases the EMT process [75-79]. After epithelial-mesenchymal transition,

cancer cells must go through a multistep process to metastasize to bone, which involves dislodgement from a primary site, survival in the circulation, binding to the resident cells in bone, and survival and proliferation in the bone and bone marrow. The dissemination of cancer cells may take place early in disease progression with tumor cells preferentially engaged in the bone marrow, and a subset of cells surviving and evolving into clinically apparent disease. These cells then enter a period of dormancy in which they either stop proliferating, or proliferate at a reduced rate before showing evidence of metastasis; a process that can sometimes exceed 10 years. However, in some situations, there is at least 1 further and crucial event that takes place, the trigger that reactivates tumor cell dormancy. However, the mechanisms that facilitate this process remain not completely known. Cancer cells have preferential site where grow and finally form a metastases. This concept of selective homing of cancer cells in a specific organ happens mainly according to 3 mechanisms:

- Selective growth: cells leave the primary tumor in a ubiquitous manner but they can grow selectively only in specific organs with the necessary growth factors and miocroenvironment.
- Selective adhesione: Cancer cells can attach only to the surface of endothelial cells of specific organs
- Chemotaxis: cancer cells reach the specific organ by chemoattraction due to the realse of soluble growth factors secreted by the organ where metastasis will be formed.

1.2.4 Blood and lymphatic dissemination

After leaving of cells from the primary tumor site, they are released into the blood and lymphatic circulation, and from there they spread throughout the body.

Despite this, the event that leads to the development of metastases is very rare and many cancer cells are not able to cross the capillary bed of the pulmonary circulation. In the blood circulation, tumor cells can interact with platelets and leukocytes with the formation of aggregates which increase the resistance of cancer cells and inhibits the immuno-mediated clearance. This process facilitate the cancer cells stop in the capillaries of the various organs and promotes the extravasation. Once the tumor cells have left circulation, the activated platelets are a source of factors that are able to induce angiogenesis, stimulating tumor proliferation, and indirectly increase osteoclast activity in the bone environment [80-82].Angiogenesis is not only important in the development of metastasis

and invasiveness of the tumor but also in the early pre-invasive stages where nutrients and oxygen are supplied to the tumor through the neo formed vessels. These vessels are also a way for cancer cells to spread in the body. The angiogenic inducing factors are VEGF, FGF1 and FGF2. Interestingly, the PGF (placental growth factor) has been implicated in the induction of angiogenesis in the disease state but not in the normal conditions.Two Other factors, VEGF-C and VEGF-D are the major inducers of lymphangiogenesis and are overexpressed in colon and breast tumors [83-85].

A vertebral venous system with thin walls and lack of valves that can communicate freely exists , in which a part of the blood origining from pelvis and from thoracic site is released. This system would explain the predilection of prostate and breast cancer to metastasize to the level of the axial portion of the skeleton; the tumor cells from the thorax and the pelvis, indeed, avoid the polmonar circulation and they can spread freely.

1.2.5 Diffusion and colonization of secondary tissues

The arrival of cells in a secondary organ is therefore not a random process. The first contact between the "seed " and "soil " consists in the interaction between circulating tumor cells in the blood and lymph vessels and the endothelium of a specific organ. In particular, with regard to bone metastases tumor cells must reach, colonize and grow in the bone marrow. The combination of specific chemoattrattive and adhesion molecules in the bone marrow endothelium promote migration and retention of circulating tumor cells [86]. This phenomenon also depends on the presence of receptors for cytokines and growth factors, localized on the surface of cancer cells .

CCR-7 and CXCR4 are the most important receptors and they are the expressed predominantly by prostate and breast cancer cells , which interact with the Chemokines like monocyte chemoattractant protein 1 (MCP -1) and stromal cell- derived factor 1 (SDF-1), that are chemoattrattive cytokines and chemokines expressed constitutively by endothelial cells , osteoblasts and other stromal cells of the bone marrow. The SDF-1/CXCR4 axis plays a key role in the development of metastases; in normal tissue levels of CXCR4 are low, meanwhile in breast cancer they are higher. SDF-1 in the bone marrow is abundantly produced by osteoblasts in particular during the process of bone remodeling and its production is increased by factors such as PTH , PDGF , IL-1, VEGF and TNF- α . SDF-1 also recruits osteoclast precursors by inducing chemotaxis , the activity of MMP -9 and the transmigration of collagen. The activation of the SDF-1/CXCR4 pathway not only

regulates the homing and migration of tumor cells in the bone but also the adhesion, invasion, and the rearrangement of of cancer cells cytoskeleton [87-90].

The osteopontin, bone sialoprotein and the type I collagen are the predominant components of mineralized bone: these proteins mediate the local adhesion , motility , survival and growth by interacting with integrins and adhesion molecules expressed by different types of cells. The integrin αvβ3 is the receptor for vitronectin (another molecule of the extracellular matrix) and is an essential component for the adhesion of osteoclasts to bone. This integrin is expressed at high levels on the surface of cells of breast carcinoma and seems to cooperate with the bone sialoprotein and the MMP- 2 and -9 in the invasion of bone. The αvβ1 integrin mediate the binding to the vascular cell adhesion molecule 1 (VCAM-1) or to fibronectin promoting a regulation of the expression of cytokines and growth factors in the stromal cells of the bone marrow increasing tumor growth and resistance to chemotherapy. CD44 is an adhesion molecule that does not belong to the integrin family; it is a receptor of glycosaminoglycans ialuronated and osteopontin . It is expressed by various cancer cells and has a well-defined role in skeletal metastasis. A portion of the cells that are spread in the bone marrow stroma may reactivate certain epithelial properties through a mesenchimal-epithelial transition (MET) and xpressing some epithelial markers . This indicates that the malignant progression is based on the dynamic processes that can not be explained only by the onset of irreversible genetic alterations but rather by temporal transitional states that are affected strongly by the tumoral microenvironment [91-96]. Recent studies have shown that ADAMTS1 and MMP1, two metalloproteases, synergistically promote the invasion of breast cancer: the two metalloproteases cut the ligands of EGF (AREG, TGF-α and HB-EGF) from the surface of tumor cells and, consequently, the expression of OPG by osteoblasts thus is reduced. ADAMTS1 and MMP1 also increase the production of RANKL. In addition to the expression of molecules involved in homing to bone , the tumor cells of breast and prostate acquire the ability to express bone matrix proteins such as osteonectin , osteopontin and bone sialoprotein. The acquisition of the typical properties of bone cells by tumor cells is a process that is termed "osteomimicry " [97-100] and improves the homing, adhesion, proliferation and survival in the bone microenvironment. The classical hypothesis according to which the tumor cells begin to interact with the microenvironment in which the metastasis develop only when they reach the microenvironment itself appears to be too semplicistic [101] after the discovery of the "premetastatic niche " [102] . It has been shown indeed, that the hematopoietic stem cells (HSC) that reside in the bone marrow at the level of two niches, the osteoblastic and the vascular niceh [103-105], are mobilized

by factors secreted by the primary tumor. HSCs begin to produce growth factors (eg. VEGF), chemokines and other molecules that prepare the different metastatic sites before the arrival of the tumor cells .There are many factors that are primarily derived from the endocrine system that may affect the functions of osteoclasts and osteoblasts both directly and indirectly leading to the formation of bone metastases .

1.2.6 Types of bone metastases

Bone metastases can be classified in two different types: osteolytic and osteoblastic lesions

1.2.6.1 Osteolytic metastases

The osteolytic metastasis occurs mainly in patients with solid tumors such as breast, prostate, lung , kidney and thyroid [106] .

In breast cancer the dominant lesion is lytic and destructive although there is also a local bone formation that probably represents an attempt to repair the bone loss [107]. This increase in bone formation in patients with osteolytic bone metastases is reflected in an increase in serum levels of the enzyme alkaline phosphatase (an enzyme localized at the surface of osteoblasts involved in bone mineralization), used as a marker for determination of osteoblastic activities 8.Both serum alkaline and idrossiprolin in urine are cheap and non invasive markers of, respectively, bone formation and bone resorption. Recently other more specific and sensitive markers identified to assess response in bone have been identified: they include bone-specific alkaline phosphatase . Regarding bone resorption the evaluation of products of collagen degradation as CTX and NTX were quite used [108] . These markers may be useful for planning and evaluating the use of a preventive treatment with inhibitors of bone resorption.

Many in vivo studies have shown that osteolysis is associated with increased osteoclast activity and a reduction in the activity of osteoblasts with a direct effect of cancer cells on bone tissue [109].

Osteolytic metastasis occurs following a complex interaction between tumor cells and the bone microenvironment that gives rise to a "vicious cycle" [110] . Bone homeostasis is regulated by direct interaction between osteoblasts and osteoclasts, in particular, by the axis of the RANK/ RANKL/ OPG. RANKL, expressed on the surface of osteoblasts and bone marrow stromal cells, induces the recruitment, activation, and osteoclasts differentiation by binding to its receptor RANK localized on the surface of the osteoclasts precursors [110] .

The process is controlled by the production, by osteoblasts and other cell types in the bone microenvironment , of Osteoproteogerin(OPG). OPG is a " decoy receptor " able to bind RANKL limiting its biological activity and thus inhibiting the osteoclasts differentiation , mainly by blocking the stages of fusion and differentiation of osteoclasts and their bone resorption activity [111]. Once activated, the osteoclasts begin the process of bone resorption by the secretion of proteases and the formation of an acidic environment between the plasma membrane and the bone surface. The tumor cells that reach the bone microenvironment secrete factors that influence the process of bone resorption. The peptide PTHrP (tumor -produced parathyroid hormone-related protein) is the most important mediator in the activation of osteoclasts in metastatic breast cancer [112]. It has a 70 % homology with the first 13 amino acids of the thyroid hormone (PTH), it binds to the same PTH receptor 114 showing a similar biological activity [113-114]. 50-60% of breast tumors primitive produce PTHrP but its expression appears to be higher in the bone microenvironment (90% of bone metastases from breast cancer expresses PTHrP) with respect to the site of the primary tumor and metastases to other sites (only 17% of bone metastases in different anatomical sites expresses PTHrP) [113-116]. PTHrP stimulates RANKL production by osteoblasts and inhibits the OPG production increasing osteoclastogenesis. The signal activated in osteoclast precursors following the binding of RANKL to RANK leads to increased expression of some transcription factors such as AP1 (activated by JUN N-terminal kinase) and NF -kB (activated by the inhibitor of kB kinase IKK) leading to the maturation of the osteoclasts progenitors of [117-118]. The newly formed osteoclasts, then, begin the process of bone resorption.

The induced osteolysis by osteoclasts is related to the release by the matrix of bone growth factors such as TGF- β and IGF-1 and to an increase in the concentration of extracellular calcium. These growth factors , and in particular TGF- β, bind to their receptors on the surface of tumor cells and induce mechanisms of signal transduction mediated by Smad proteins and Mapk [119-120]. This leads to an increase in the proliferation of cancer cells and to an increase of the production of PTHrP which in turn increases the production of RANKL by osteoblasts closing this vicious cycle [110].

Apart PTHrP, the expression of RANKL on osteoblasts and stromal cells is increased by other factors produced by tumor cells , such as IL - 1, IL- 6, IL -8 , IL- 11 and PGE2 (Fig.3).

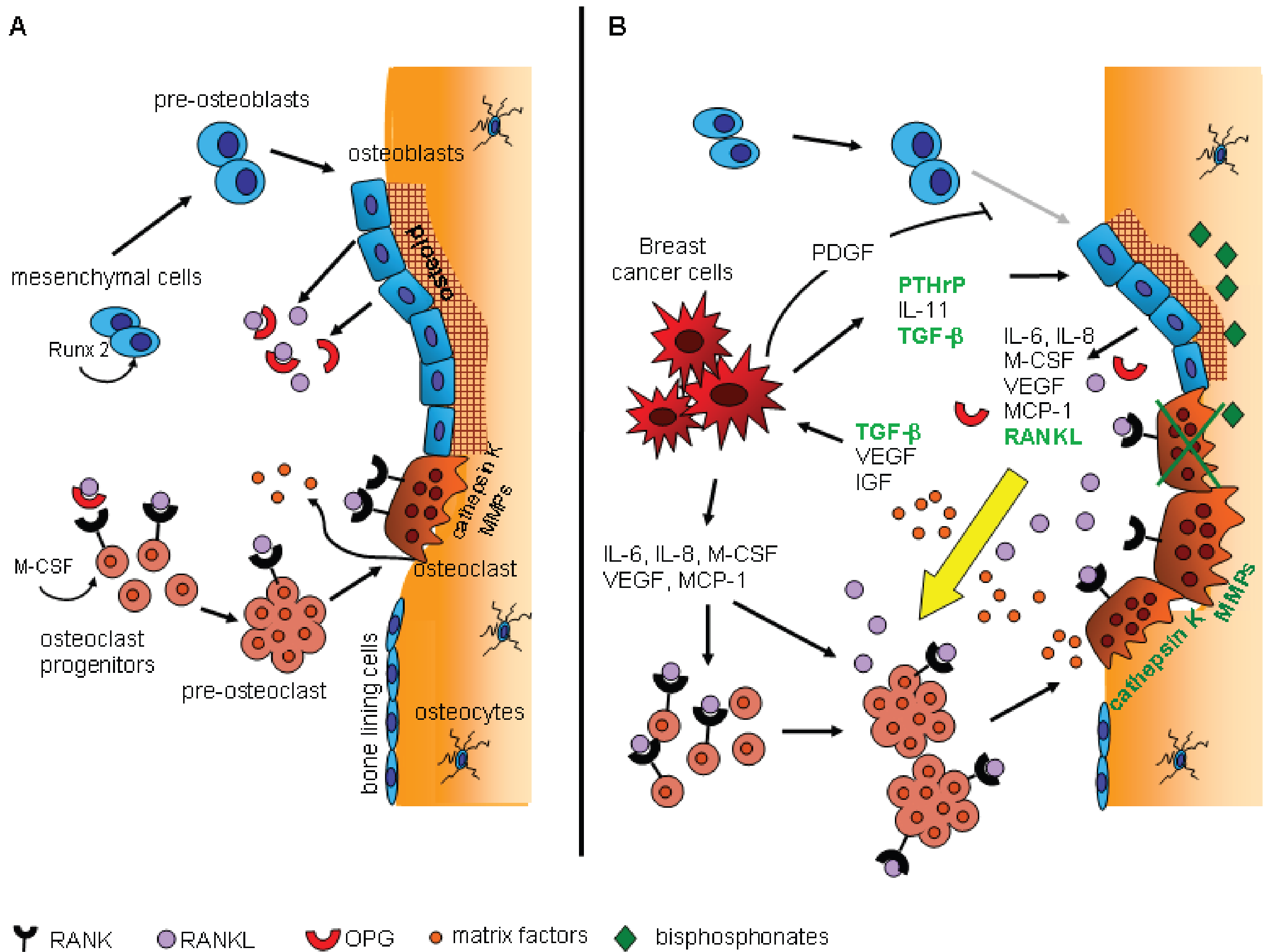


Fig. 3 Osteolytic lesions

Some of these factors not only stimulate osteoclasts by RANKL but also in a independent manner. The IL-8, indeed, binds directly to the CXCR1 receptor localized on the surface of the osteoclasts precursors .

Also COX-2, overexpressed in bone metastases from breast cancer, can activate osteoclasts directly or via RANKL increasing the production of IL-8 and PGE2 .

The factors produced by tumor cells recruit and activate T cells that act supporting osteoclastogenesis in two ways: by producing TNF-α and TRAIL (which inhibits the effect of OPG).Recently it has been discovered that a high expression of Jagged 1 in the cells of breast cancer promotes the bone metastasis by activating the Notch pathway in bone cells of the support. Jagged 1 is overexpressed in metastatic tumor cells and is further activated by the cytokine TGF- β resulting from the osteolisis of osteolytic lesions .

The Jagged 1 -expressing tumor cells have a growth advantage in the bone microenvironment by promoting the expression and release of IL-6 by osteoblasts and increasing osteolysis by stimulating the maturation of osteoclasts [121].

Jagged 1 is not expressed only by tumor cells but also by bone cell [122-123].

that regulate hematopoietic stem cell niche through the Notch pathway [124]. (Fig.4)

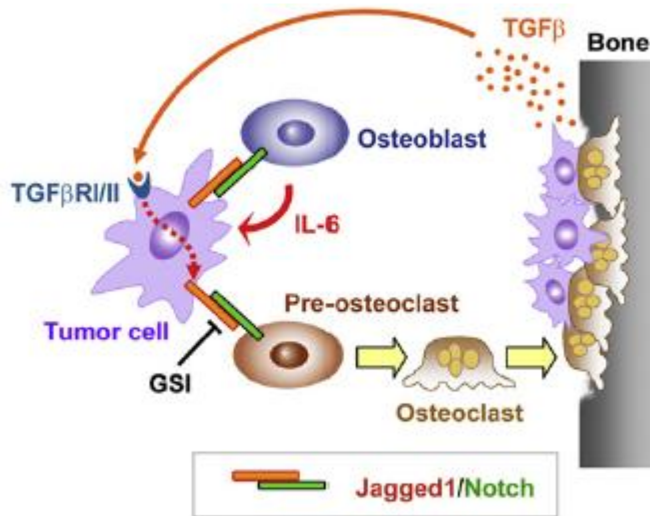


Fig.4 Jagged1/Notch

1.2.6.2 **Osteoblastic lesions**

Although osteoblastic metastases occur mainly in bone lesions from prostate cancer , 15-20% of patients with breast cancer develop this type of metastases [124]. We should note that in metastases from breast cancer, however, there is a prevalence of metastases characterized by a mixed lytic and osteoblastic component.

In osteoblastic metastases there is a loss of bone homeostasis in favor of bone formation compared to bone resorption; however,the osteoid deposited and subsequently mineralized is of poor quality, and this leads to pathological fractures as a quite common event [106].

The formation of osteoblastic metastases depends on a hyperstimulation of osteoblasts or by an inhibition of osteoclasts (or both) by cancer cells.

The mechanisms that underlie the formation of osteoblastic metastases are not well defined but it is thought that the massive production of bone matrix in the region surrounding the deposit of tumor cells is due to the abundant production and secretion of growth factors that induce the recruitment the proliferation and differentiation of osteoblast progenitors by metastatic tumor cells [124]. Among the major factors involved in the development of osteoblastic metastasis we highlight the BMPs (BMP2, BMP3, BMP4, BMP6 and BMP7), members of the superfamily of TGF- β, produced mainly by tumor cells. These bone morphogenic proteins stimulate osteoblast differentiation by activating transcription factors

such as Runx-2 [126-128]. and also indirectly induce angiogenesis. The pattern of expression of BMPs play an important role in the etiology of osteoblastic metastases arising mainly from prostate cancer. It was seen that the primary tumor and the metastases have different patterns of expression of BMPs ; BMP6 is expressed at high levels in both the primary tumor and in metastases, whereas BMP7 is expressed at high levels only at the level of bone metastasis. The endothelin-1(ET-1) is another very important factor; it is a vasoactive peptide of 21 amino acids produced by cancer cells [129-130]. The pathophysiological role of ET- 1 in the development of metastasis has been demonstrated in preclinical models for breast and prostate [131-132]. ET-1 binds to its receptor, Endothelin A receptor (ETA), that is expressed by tumor cells and also by bone cells (osteoblasts and osteoclasts), suggesting that the activity is paracrine and autocrine [133]. ET-1 increases the activity of cancer cells and enhances the mitogenic effect of other growth factors such as IGF-1, PDGF and EGF [134]; it also leads to bone formation by stimulating osteoblasts and inhibiting the resorption mediated by osteoclasts .Also the Platelet-derived growth factor (PDGF) and fibroblast growth factors (FGFs) produced by many types of tumor cells are implicated in the formation of osteoblastic metastases. PDGF is a dimeric polypeptide that has 3 isoforms AA, BB and AB . The BB isoform osteotropic is a powerful factor that contributes to the development of osteoblastic metastases by promoting the migration and proliferation of osteoblasti [106]. The FGFs , both the acid (FGF1) that the basic (FGF2) form stimulate the formation of new bone in vivo. Both increased osteoblast proliferation while only FGF2 suppresses the formation of osteoclasts .

VEGF is also involved in bone growth directly by stimulating the differentiation and activation of osteoblasts, and indirectly promoting angiogenesis.

Some Serine proteases such as protease urockinase (uPA) and the prostate-specific antigen (PSA), appear to be involved in metastasis formation osteoblastic [135]. uPA , produced by tumor cells , is synthesized as a precursor (pro-uPA) but subsequently undergoes a proteolytic cleavage that leads to its activation. The carboxy- terminal proteolytic domain uPA (ATF) contains 2 domains: a growth factor domain (GDF) , so called because it is structurally similar to EGF and a Kringle domain. This domain is essential for the activation and proliferation of osteoblasts . Moreover , uPA cleaves and activates TGF- β , which regulates the differentiation of osteoclasts and osteoblasts and promotes the growth of cancer cells stesse ; hydrolyzes and proteins that bind IGF increasing the concentration of IGF libero .PSA is a serine protease of the kallikrein family , marker known to be used

for the assessment of the progression of prostate cancer. PSA cleaves the protein IGFBP -3 that binds IGF-1, IGF-1 making it available to the binding with its receptor and stimulate the osteoblastic proliferation [136]. PSA can also hydrolyze PTHrP reducing bone resorption by osteoclasts in order to make the predominant response osteoblastica .

As seen at the beginning of the paragraph, the bone microenvironment more ofter develop metastases that have mixed characteristics between those osteolytic and osteoblastic.

1.2.7 Complications of Bone Metastases:

About 25% of patients with bone metastases are asymptomatic and the diagnosis is only made when tests are carried out for other reasons or during primary tumour staging. In the remaining 75%, bone metastases are responsible for different clinical complications defined as skeletal-related events (SREs) such as pathologic fractures, spinal cord compression, hypercalcaemia, bone marrow infiltration and severe bone pain requiring palliative radiotherapy [137]. Such complications are often devastating for patients and substantially reduce their functional independence and quality of life, decrease survival rates and increase healthcare costs [138].

A study evaluated the pattern of metastatic disease in 180 triple-Breast cancer patients who were compared with other subgroups. The risk of developing bone metastases within 10 years of the diagnosis was 7%-9% for all subgroups[139]. Some clinical trials have evaluated the bisphosphonates efficacy in decreasing SREs in patients with breast cancer and bone metastases [140-141]. The median time to the first SRE was 13.9 months among bisphosphonate-treated women and 7.0 months in the placebo group (P = 0.001) [141]. The SREs that occurred in the control group were radiation to bone, pathologic fracture, hypercalcaemia, surgery on bone and spinal cord compression [141].

1.2.8 Bone targeted therapy

While bone metastases contribute significantly to the morbidity associated with breast cancer, they are rarely the cause of disease related deaths. However, as aready reported, serious complications are associated with them, including chronic bone pain, hypercalcemia, SREs, which can lead to a dramatic decrease in the quality of life for breast cancer patients [142]. The current standard of care for the treatment of bone metastases includes systemic therapy, such as chemotherapy and bisphosphonates, as well as local treatments, such as surgery or radiation to bone. Treatment with intravenous bisphosphonates (IV-BPs) has been the current standard of care for maintaining skeletal

integrity and preventing skeletal complications. Recently Denosumab (Xgeva ®, Amgen), a monoclonal antibody against RANKL, has been introduced in the clinical standare of care [142].

1.2.8.1 Zoledronic Acid

Bisphosphonates are potent antiresorptive drugs in widespread use that are well suited to the treatment of metabolic bone disease. These drugs bind avidly to hydroxyapatite crystals at sites of active bone metabolism, achieving therapeutic concentrations. Bisphosphonates are released during bone resorption and are internalized by osteoclasts, leading to inhibition of bone resorption itself and induction of osteoclast apoptosis [143].

The use of drug treatments has a positive impact on the quality of life, inducing a reduction of skeletal related events (SRE) and death risk in patients with bone metastases from breast cancer [144-146]. Based on the results of large randomised controlled trials conducted 10-15 years ago, the bisphosphonates have become the standard of care for the treatment and prevention of skeletal complications associated with bone metastases in patients with breast cancer. In particular, Zoledronic acid (Zometa ®, Novartis) (ZA) is a potent third-generation nitrogen-containing bisphosphonate, and, in recent years, it has had widespread clinical use in patients with breast cancer [147]. Furthermore, many preclinical studies have demonstrated that ZA has both direct and indirect tumor activity, reducing proliferation and viability of tumor cell lines *in vitro* [148]. The direct action occurs in a dose and time dependent manner to inhibit proliferation and induce apoptosis in breast cancer cell lines. The indirect action depends on the modification of bone microenvironment that is less hospitable for cancer cells growth. Furthermore, ZA is known to inhibit tumor cell adhesion and invasion and its potential antiangiogenic activity has recently been discovered. In animal models, a reduction in skeletal tumor burden and slower progression of bone lesions was observed after ZA treatment [149-151]**.**

Zoledronic acid molecular mechanism of action depend on the inhibition of the mevalonate pathway and in particular the farnesyl diphosphate synthase (FPP synthase) [152]**.**. The mevalonate pathway is involved in the production of cholesterol and isoprenoid lipids such as isopentilinil adenosine diphosphate (IPP), the farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) [153-154]**.** The loss of FPP and GGPP as a result of the activity of BPs prevents post- translational lipid modification (prenylation) of small GTPases such as Ras, Rho and Rac. The inhibition of the enzyme farnesyl diphosphate

synthase is possible because the NBPs act as an analogue of the transition state of isoprenoidi [155].

The prenylation is important because the lipid groups that are linked to proteins serve to anchor these on cell membranes where they participate in protein – protein interaction. The GTPase fail to translocate to the plasma membrane and this leads to the inhibition of the antiapoptotic regulatory Ras/Raf-1/MEK/ERK1-2 pκB/Akt leading to activation of caspase-3 leading all'apoptosi. As a result of inhibition of FPP synthase we have the production and accumulation of Apppl an intracellular ATP analogous is able to induce in vitro osteoclasts apoptosis by inhibiting the translocase mitocondrial ADP / ATP. It was recently demonstrated the presence of ApppI in vivo [157-159].

The modified proteins control many cellular functions of osteoclasts , such as traffic endosomal control, the signaling of integrins, the rippling of the membrane , the control of cell morphology and the apoptosis [160-163].

Recent clinical data in the adjuvant setting of breast cancer has also shown that ZA also increases disease free survival [164-165]. However, one of the most important limitations of this drug, which makes the direct anticancer effects difficult to demonstrate in vivo, is its pharmacokinetics profile. In fact, after an infusion of 4-mg dose of ZA, the drug remains in the plasma 1-2 h before localization to bone, with a plasmatic peak of 1μM. Studies on rats and dogs showed that ZA levels rapidly decreased in plasma and non calcified tissue, but higher levels persisted in bone and slowly diminished with a half-life of about 240 days. The results seemed to indicate a portion of ZA is reversibly taken up by the skeleton, and the disposition in blood and non calcified tissue is governed by extensive uptake into and slow release from bone; so efforts are required to allow the clinical translational of in vitro results to reach an increase of anticancer activity of this drugs. A method to reach this goal is to increase the availability of this drugs in extra-bone tissues and improve their plasma half life encapsulating them in liposome vehicles. Other strategies could be change schedule treating patients with low dose protracted administration of ZA or use synergistic combinations of drugs.

It has been demonstrated that ZA also has direct anti-tumor activity carried out by the induction of apoptosis and the activation of the immune system through the response of lymphocytes T [166].

ZA acid also induces the reduction of the expression of the gene COX-2 and then of prostaglandins in tumor cells , leading to the inhibition of chemioattrattive effect of stromal cell-derived factor- 1 (SDF-1) and the downregolation of the CXCR-4 receptor for this factor.

The recruitment of T cell population Λδ occurs through identification by these cells of the nitrogenous bisphosphonate that is exposed on cancer cells surface [153]. T cells Λδ then induce the lysis of neoplastic cells by inhibiting tumor-induced osteolysis. ZA acid shows anti -tumor activity even outside of the bone microenvironment, particularly when administered in combination with other anticancer drugs such as taxanes, doxorubicin and platinum -derived compounds ; showing synergism or addition or in the anti- neoplastic activity. In particular, it has been shown in some studies that the administration of chemotherapy and then of ZA acid sensitizes tumor cells to the action of ZA acid thus inhibiting cancer progression [167].

Several ZA dosing schedules have been proposed for the treatment of osteoporosis and bone metastases [168]. However, these schedules need to be optimized to maximize its antitumor effects. The metronomic approach has already been studied, and, in particular, daily or repeated therapies with bisphosphonates have been reported to inhibit skeletal tumor growth in mouse models [169]. In cancer patients with bone metastases, repeated intermittent low-dose therapy with ZA has been shown to induce a decrease in VEGF levels in cancer patients.

Zoledronic acid reduces the risk of skeletal complications of 30-50% and not only for breast cancer but for an extensive range of solid cancers [167].

Indeed, it can reduce the production of numerous growth factors and cytokines at the level of the bone microenvironment (IGF-1 and IGF -2 , FGFs) , making it less attractive as a site of migration, colonization, adhesion and invasion, proliferation and survival for cancer cells [170].

1.2.8.2 **Denosumab**

The Denosumab is a monoclonal antibody directed against RANKL that mimics the effect of endogenous OPG. It binds with high affinity to RANKL , preventing binding to its receptor RANK, and this leads to inhibition of the processes of recruitment, maturation and activation of osteoclasts resulting in a reduction of bone resorption [170]. In the United States and Europe Denosumab use was initially permitted only for the treatment of patients with postmenopausal osteoporosis, while recently it has been allowed its use for the prevention of SREs in patients with bone metastases from solid tumors. Denosumab is administered by subcutaneous injection, eliminating the requirement of ZA for intravenous infusion.

Phase III clinical trials that compared treatment with denosumab and ZA acid have been conducted on patients with bone metastases from breast cancer and prostate cancer.

Denosumab treatment appears to be superior to treatment with ZA acid in terms of the risk of developing SREs . The time for the appearance of the first and subsequent SRE is higher after treatment with denosumab compared to treatment with ZA acid . Furthermore there is also a decrease of bone turnover markers (uNTx / Cr) significantly higher in the treatment with denosumab (uNTx/Cr -80 % versus -68 % with denosumab with ZA acid) [171]. Overall survival, disease progression, and rates of severe and serious adverse events were similar between both study arms.

In separate analyses evaluating the respective effects of ZA acid and denosumab on pain and health-related quality of life (HRQoL) in all patients included in the study, a similar time to pain improvement was observed in both treatment arms. However, patients with a baseline score of no/mild pain significantly had longer median time to develop moderate/severe pain when treated with denosumab (295 days) compared with ZA acid (176 days; HR: 0.78; 95% CI: 0.67e 0.92). Moreover, a greater percentage of patients treated with denosumab than with ZA acid had a clinically meaningful improvement in HRQoL, regardless of their pain level at baseline ($p < 0.05$). These results are in agreement with those obtained in other phase III trials performed in patients with advanced cancer such as prostate, other solid tumors or multiple myeloma [172].

This superiority suggests that a greater inhibition of osteoclast-induced bone resorption of Denosumab compared with ZA acid, as evident by increased suppression of bone turnover markers, translates into improved clinical outcomes, such as the prevention of SRE.

Safety profile of Denosumab has been generally well tolerated in several clinical trials conducted in advanced cancer patients. RANKL has been identified as a costimulatory cytokine for T-cell activation, and this is the reason for expecting a higher risk for infectious diseases. However, preclinical studies revealed no increased risk of bacterial infections. Also, in a phase III study comparing denosumab with ZA acid in metastatic breast cancer, there was no increase in the number of infectious adverse events (48.8% with ZA acid vs. 46.4% with denosumab) or infectious serious adverse events (8.2% ZA acid vs. 7.0% denosumab) [173]. In fact, in that trial only hypocalcemia were more frequently observed with denosumab. In contrast, acute-phase reactions (including pyrexia, fatigue, bone pain, chills, arthralgia and headache) were 2.7 times more common with ZA acid than with denosumab (27.3% vs. 10.4%, respectively) as well as adverse events potentially associated with renal toxicity (8.5% vs. 4.9%, respectively). Renal toxicity might include increased blood creatinine and blood urea, oliguria, renal impairment, proteinuria, decreased creatinine clearance, acute renal failure and chronic renal failure. Thus, denosumab represents a valid therapeutic option for patients with bone metastases

suffering from chronic renal failure. Lastly, a low incidence of osteonecrosis of the jaw was anticipated in metastatic cancer patients.

Cancer induced bone loss

Patients with breast cancer often develop bone loss secondary to cancer treatment itself. Several mechanisms of bone loss due to cancer treatment have been identified [174]. Firstly,there is bone loss as a result of estrogen deprivation. In premenopausal women bone density loss averages 8% in the first year of treatment with premature ovarian suppression due to chemotherapy induced amenorrhoea [175]. Secondly, there is bone loss due to endocrine anticancer therapies. The effects of tamoxifen, a selective estrogen receptor modulator,on bone are dependent on the actual physiologic estrogen concentration. Tamoxifen causes bone loss in premenopausal women, but is bone protective in post menopausal women [176]. Aromatase inhibitors (Ais) in post menopausal women lower the estrogen level. As a consequence of the estrogen deprivation, on average a 2.6% loss of bone density in the first year of breast cancer treatment has been found [177]. In contrast, bone loss during natural menopause is typically 1% per year. Finally, chemotherapies and adjuvant drugs, such as steroids, affect bone density directly or indirectly. Chemotherapy treatment causes bone loss by directly damaging bone architecture or inducing early menopause in premenopausal women. The role of denosumab in preventing aromatase-inhibitor induced bone loss has been studied in the Hormone Ablation Bone Loss Trial in Breast Cancer (HALT-BC) study. This trial examined the efficacy of denosumab (60mgs every 6 months for 2 years) vs. placebo for preventing bone loss among 252 postmenopausal women with early-stage breast cancer who were receiving anaromatase inhibitor. After 24 months of follow- up, a significant difference of 7.6% in lumbar spine bone density of patients treated with denosumab compared to placebo was found. Similarly, a significant difference of 4.7% was detected in total hip bone density in advantage of the denosumab treated group.

1.3 Tumor markers

In the susequent paragraph a number of possible innovative markers for bone metastases prediction will be presented.

1.3.1 β2 -microglobulin (B2M)

The beta 2 -microglobulin is a plasma protein of the family of betaglobuline present mainly on the surface of immune system cells such as lymphocytes and macrophages. An identified role of β2 -microglobulin as a growth factor and signaling molecule in cells 186

-189. The expression of β2- microglobulin increases during the progression of several types of cancer including also breast cancer [178]. B2M has multiple roles in tumor development and metastasis because it mediates tumorigenesis, angiogenesis and osteomimicry. It is also capable of activating bone stromal cells as mesenchymal stem cells, osteoclasts and osteoblasts [179-180]. The B2M therefore promote the development of bone metastases in several ways:

- Increases the expression of matrix proteins such as osteocalcin,and bone sialoprotein - mimicking the bone microenvironment and promoting the growth and survival of tumor cells ;

- Promotes the growth of osteoclasts, osteoblasts and mesenchymal stem cells in the bone microenvironment promoting primary and metastatic cancer cells growth ;

- Promotes bone homeostasis and the induction of HIF- 1α in tumor cells promoting the growth in the skeleton ;

- It acts as a coupling factor between osteoclasts and osteoblasts by increasing the interactions between the tumor and the bone marrow stroma, triggering a vicious cycle of metastatic progression to bone ;

- Finally it seems to induce EMT and determines the acquisition of stem-like properties of the tumor cells .

1.3.2 Connective tissue growth factor (CTGF)

The connective tissue growth factor is a secreted protein, rich in cysteine, which belongs to the CCN family. This family of proteins interacts with a variety of extracellular molecules such as adhesion molecules, proteoglycans and growth factors including TGF-β [181].

CTGF also modulates various cellular functions such as chemotaxis, differentiation and apoptosis.

CTGF is highly expressed in cell lines of breast carcinoma (MDA-MB-231) and, in combination with other genes such as IL-11, CXCR4 and OPN converts cancer cells with low metastatic potential in tumor cells with high metastatic potential [182].

1.3.3 Heparanase (HPSE)

Heparanase (HPSE) is an enzyme whose active form cleaves the glycosidic bonds of the heparan sulphates glycosidic produce fragments of 10-20 residues that interact with growth factors but without binding to the extracellular matrix or the cell surface. The cutting of heparan sulfates promotes the erosion of the basement membrane by facilitating the invasion and the formation of metastasis. It seems to play an important role in breast cancer, where its expression is correlated with tumors of large size and high metastatic power, and it is also implicated in the induction of angiogenesis. The heparanase leads to the release of osteolytic agents, such as syndecan- 1 [183], which binds and regulates the activity of effector molecules such as IL- 8, and FGF.

It also increases osteoclastogenesis through synergistic interaction of heparin with IL- 11 , and this leads to the activation of STAT3 that promotes the formation of osteoclasts. Although the IL-8 expressed by tumor cells binds to residues of heparan sulfate by heparanase produced by the cutting, and this leads to an increase of osteoclastogenesis and bone resorption and activation of the vicious cycle .

1.3.4 osteonectin (SPARC)

The osteonectin, also called SPARC, is a glycoprotein of 32-46 kDa originally discovered in bone for its ability to bind collagen type I . Sparc appears to mediate an intermediate state of adhesion that promotes cell motility. Initially it was thought that the osteonectin produced in bone serve as a chemo-attractant for cancer cells cancer in prostate cancer, but the lack of reliable identification of a specific receptor for this protein has modified the hypothesis of the role of SPARC in bone metastatic process .Later it was demonstrated that neoplastic cells of breast cancer produce a high levels of osteonectin compared to healthy breast tissue. It is possible that the cells of breast cancer, overexpressing osteonectin into the bone microenvironment and overexpressing osteonectin , can promote the process of invasiveness following the proteolytic cleavage of SPARC by certain proteases such as cathepsin K. The peptides that are produced have high affinity for collagen and regulate various growth factors such as VEGF, PDGF and FGF2 promoting tumor associated angiogenesis. The expression of SPARC is also related to an increased production of metalol proteinases as 1, 2 , 3 and 9 that regulate the shaping of the matrix and induce a inflammatory response [184] .

1.3.5 trefoil factor 1 (TFF1)

TFF1 (formerly pS2) is a small secreted protein rich in cistein 223 , 224. It is constitutively expressed in the stomach where it has a key role in the normal differentiation of the gastric glands. In addition, interacting with mucins , TFF1 participates in the proper organization of gastric mucosa 228. TFF1 is produced and secreted in an autocrine and /or paracrine in response to inflammation and damage to the gastrointestinal tract. High expression of TFF1 maintains the integrity of the epithelial cells by inducing the migration of cells and inhibiting the anoikisi during the migration process. High levels of TFF1 were observed in a variety of cancers such as prostate and breast [185-187] .

1.3.6 RANK

RANK is a receptor that is expressed primarily on the surface of osteoclasts and is implicated in the activation of NF -kB . The binding with RANK-L expressed by osteoblasts and bone marrow stromal cells and secreted by T cells active promotes the differentiation and maturation of osteoclasts, inhibits apoptosis and leads to a consequent increase of boneresorption. As already described above, this process can be controlled by the production of OPG by osteoblasts, and other cell types,as discussed above. RANKL and OPG are important regulators produced by the bone marrow microenvironment , involved in controlling the formation and activation of osteoclasts. The cancer cells that reach the bone microenvironment secrete factors that influence the process of bone resorption leading to the establishment of a vicious cycle [110]. It has been demonstrated that the cells of breast cancer (and also the melanoma cells) express RANK [188-189] and are attracted by RANKL expressed at the level of the bone microenvironment . It was also demonstrated in a retrospective study that the combination CXCR4/RANK is a predictive marker of bone release by increasing the risk of bone metastases by 9.3 -fold in patients with bone metastases compared to disease-free patients and patients who relapsed to viscera [190].

1.3.7 Chemokine receptor type 4 (CXCR4)

It is known that cytokines and chemokines and their receptors play an important role in the regulation of the tropism of the organs in metastasis. Among the most important receptors which are expressed by the cells of breast cancer are the CXCR4 receptor. CXCR4 binds to its ligand cxcl12/sdf1 constitutively expressed by endothelial cells, osteoblasts and other stromal cells of the bone marrow. The activation of the SDF-1/CXCR4 pathway not only regulates the homing and migration of tumor cells in the bone but also the adhesion,

invasion, and the rearrangement of the cytoskeleton of cancer cells. Confirmation of the relevance of this pathway in bone metastases were obtained in the murine model [191].

1.3.8 Bone sialoprotein (IBSP)

The bone-sialoprotein is synthesized by bone cells including osteoclasts, osteoblasts and osteocytes. It facilitates the attachment of cancer cells to the bone and increases the metastatic power .

The IBSP could act as an adhesion molecule for tumor cells that express it , because its expression on the surface of tumor cells allows homing and retention of these cells at the level of the bone microenvironment interacting with integrins that are expressed by specialized cells bone and hematopoietic stem cells [192].

1.4 Multidisciplinary approach and translational research

Bone Metastases, as already reported, are responsible for high morbidity and reduced quality of life due to clinical complications defined as SREs. Such complications reduce functional independence and quality of life, decrease survival rates and increase healthcare costs.

The current treatment for metastatic breast cancer aims to obtain meaningful clinical responses, improved quality of life, long-term remissions, prolonged survival and goes so far as to hope for a complete cure in a small percentage of cases. The treatment of this malignancy has become progressively complex and currently includes either well-known anti-tumour agents or bone-targeted molecules aimed at preventing bone complications and improving patients' quality of life and the treatment outcome in a multidisciplinary programme. The importance of a multidisciplinary approach in the management of BMs is also widely accepted to reduce the frequency bone metastases complications, and to improve patients' quality of life and prognosis reducing the high rate of hospitalisation, with the ensuing social and economic consequences.

Translational research

Translational research is the definition for a type of research in which laboratory scientists and clinicians work closely so that the waiting time between a laboratory discovery and its application in clinical practice are as short as possible. The ongoing dialogue between researchers with different expertise enables the identification of clinical problems for which you are trying to study a solution in the lab. For example we are in the era of the tailored therapy in which patients are treated according to their genomic features [193].

1.5 Aims

Cancer patients mainly do not die for the primary tumor, but rather for the formation of metastases.Many of the most common cancers such as breast, prostate and lung commonly metastasize to the bone, indeed more than 50% of patients with prostate cancer or advanced breast show bone metastases. Bone metastases are responsible for different clinical complications defined as skeletal-related events (SREs) such as pathologic fractures, spinal cord compression, hypercalcaemia, bone marrow infiltration and severe bone pain requiring palliative radiotherapy [137]. Such complications are often devastating for patients and substantially reduce their functional independence and quality of life, decrease survival rates and increase healthcare costs [138]. The general aim of these three years research period was to improve the management of patients with bone metastases through two different approaches of translational research. Firstly in vitro preclinical tests were conducted on breast cancer cells and on indirect co-colture of cancer cells and osteoclasts to evaluate bone targeted therapy singly and in combination with conventional chemotherapy. The activity of ZA, and Denosumab as bone targeted therapy was evaluated with the purpose of finding the rationale for improving the available therapeutic strategies. In order to reach this goal the molecular mechanisms of action of the different drugs were studied in breast cancer cell lines with different molecular pattern to highlight the difference in terms of sensitivity to the drugs and in terms of molecular mechanisms of action. The results obtained could serve as preclinical rationale of possible new clinical studies on cancer patients with bone metastases.Another important criticism of the treatment of breast cancer patients, is the selection of patients who will benefit of bone targeted therapy in the adjuvant setting. In recent years there are a number of studies that showed a benefit in the use of bishposphonates and Denosumab before the diagnoses of bone metastases, as a preventive aim only in some subsets of patients. Validated markers for the prediction of bone metastases has not been found yet, so to fill the gap of uderstanding who could benefit to be treated in the adjuvant setting, a retrospective case control study was secondly planned to evaluate the predictive role of bone metastases in the primary tumors of breast cancer patients. The case series included patients with bone relapse (cases), patients with visceral metastases (first group of control) and patients with no evidence of disease (second group of controls), i.e patients operated for a breast cancer, but without any diagnosed relapse. The markers were chosen according to the literature about microarray studies aimed at discriminating between patients at higher risk of relapse.

2 Materials and methods

2 A Preclinical study

Cell culture: cancer cells

The evaluations were performed on four breast cancer cell lines, MCF-7, SKBr3, MDA-MB-231, obtained from the American Type Culture Collection (Rockville, MD), and BRC-230, established in our laboratory [194]. Hormone receptor and HER2 status are listed in Figure 1. The cell lines were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of 45% HAM F12 and 45% DMEM supplemented with 10% fetal calf serum, 1% insulin and 1% glutamine (Mascia Brunelli s.p.a., Milan, Italy). Cells in the exponential growth phase were used for all experiments.

Collection of conditioned media

Cells were cultured until they reach 90-100% confluency and then supplied with fresh media that was collected 24 hours later, aliquoted and stored at – 20°C.

Osteoclasts

Human osteoclasts were obtained from the differentiation of peripheral blood monocytes (PBMC) of a healthy donor who gave written informed consent to take part in the study. Monocytes were isolated from whole blood by Ficoll density gradient. Briefly, heparinized whole blood (30 mL) was diluted 1:1 with phosphate-buffered saline, layered on 15 ml of lymphocyte separation media (Lymphosep, Biowest, Nuaillé, France) and centrifuged without brakes at 1,000g for 30 minutes. The PBMC layer was collected and washed twice with phosphate-buffered saline and resuspended in αMEM (LONZA, Basel, Switzerland) containing 10% fetal bovine serum and 1% glutamine. Cells were counted and plated at a concentration of 250,000 cells per 0.32 cm^2 well. After 24 hours the media was removed and differentiation toward osteoclasts was induced by αMEM supplemented with 25 ng/ml of MCSF and 30 ng/ml RANKL (Peprotech, Rocky Hill, NJ, USA), or by αMEM with 10% MDA-MB-231 conditioned media. The media was changed every 2-3 days and mature osteoclasts were observed after 7, 14 and 21 days of differentiation.

Drugs

Cisplatin (Bristol-Myers Squibb S.p.A) was stored at room temperature and diluted in medium before use.

Zoledronic acid (Zometa®) (ZA), kindly provided by Novartis (Milan, Italy), was solubilized and stored at a concentration of 50 mM in sterile water at -20°C and diluted in medium before use.

Denosumab 120 mg/ml (XGEVA®) (Thousand Oaks, CA, USA) was stored at 4°C.

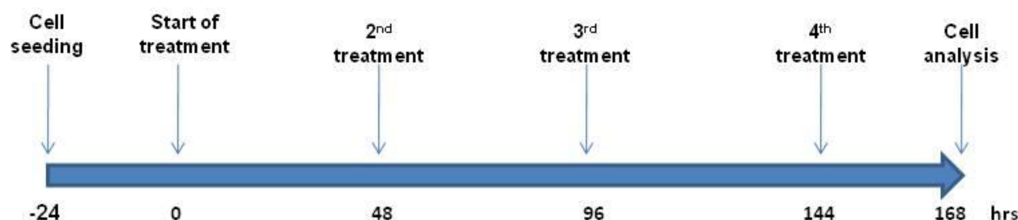
Cancer cell exposure to drugs

Single drug exposure: ZA Acid treatment

Cells were exposed to 12.5, 25 and 50 µM of ZA in chemosensitivity assay, and to 50 µM of ZA for apoptosis and western blot analysis.

In the chemosensitivity assay, cells were exposed to Repeated (RS) and Non-repeated schedules (NRS). In NRS experiments, cells were exposed for 168 hrs, while in the RS experiments, cells were exposed every 48 hrs to the same ZA concentration (Figure 1). All experiments were done in triplicate and results were reported as the mean inhibition of 50% cell growth (IC50) [195].

Fig.1



Cell line	ER	PgR	HER2
MCF-7	+	+	-
SKBr3	-	-	+
MDA-MB-231	-	-	-
BRC-230	-	-	-

Combination drugs exposure: ZA-Cis

The four cell lines were exposed to ZA or platins for 72 h singly and in combination. ZA was tested at 50 µM for 72 h in continuous, meanwhile Cisplatin and For combination assays cell lines were exposed first to different concentrations of Cisplatin or Carboplatin in combination with ZA (50 µM) for 6 hours. After a wash out cells were than exposed to ZA (50 µM) for 72 hours [196].

Osteoclasts exposure to drugs

Drug Exposure

After 7 days of differentiation, osteoclasts were exposed to Denosumab (0.5, 1 and 5 µg/ml), ZA (0.1, 1 and 10 µM) and anti-MCSF (25, 75 and 225 ng/ml) for a further 7 days. The effect of the drugs was evaluated in terms of inhibition of osteoclastogenesis and osteoclast survival. The same concentrations were tested in MDA-MB-231 for 7 days and the effect of the drugs was assessed in terms of inhibition of proliferation.

Chemosensitivity assay

Sulforhodamine B (SRB) assay was used according to the method of Skehan et al. [195]. Briefly, cancer cells were trypsinized, counted and plated at a density of 3,000 cells/well (in 5000/well in the experiments of the association ZA-CIS) in 96-well flat-bottomed microtiter plates. Experiments were run in octuplicate, and each experiment had 3 biological replicates. The optical density (OD) of cells was determined at a wavelength of 540 nm by a colorimetric plate reader. Growth inhibition and cytocidal effect of drugs were calculated according to the formula reported by Monks et al. [195]: $[(OD_{treated} - OD_{zero})/(OD_{control} - OD_{zero})] \times 100\%$, when $OD_{treated}$ is $\geq OD_{zero}$. If $OD_{treated}$ is above OD_{zero}, treatment has induced a cytostatic effect, whereas if $OD_{treated}$ is below OD_{zero}, cell killing has occurred. The OD_{zero} depicts the cell number at the moment of drug addition, the $OD_{control}$ reflects the cell number in untreated wells and the $OD_{treated}$ reflects the cell number in treated wells on the day of the assay.

Treatment of cells for apoptosis, western blot assay and Pull down assays

Cells were plated at a density of 10^6 cells in a flask (75 cm^2) and were treated after 24 hrs from the seeding with 50 µM of ZA according to the two different schedules described above. For apoptosis analysis, cells were detached from the flasks by trypsin treatment at the end of treatment, washed twice with PBS and stained according to the different methods specified below. For western-blot analysis, cells were detached from the flasks; cells were then lysed by shaking for 5 minutes in B-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). For pull down assays cells after treatment were stimulated by EGF 100 ng/ml for 10 minutes at 37°C (Miltenyi, Bologna Italy) for Ras activity evaluation, and by Rho activator 1 (Cytoskeleton., Denver CO) 1 U/ml for 30 minutes at 37°C for Rho activity evaluation. Then cells were washed once with PBS, lysed by the cell lysis buffer by Cytoskeleton and detached by scraper. Protein concentration was assessed using BCA Protein Assay kit (Pierce).

Wound scratch

Wound scratch assay was used to determine the migration capability of the four cell lines after ZA and ZA cis treatment. Cells were grown and treated according to the different chosen schedules. A uniform cell-free area was produced by scratching a confluent monolayer with a scraper 24 hours befoe the end of the experiment, and then wound closure was observed to determine if cells could migrate or not [195]. The obtained datum is qualitative.

Western blot

An equal quantity of proteins was denatured and separated on Criterion-HCL gel 12.5% Tris (Bio-Rad, Hemel Hempstead, UK) and electroblotted onto Immobilon-P Transfer Membrane (Millipore). The membrane was stained with Ponceau S (Sigma Aldrich, Milan, Italy) to verify equal amounts of sample loading and then incubated for 2 hrs at room temperature with T-PBS 5% non fat dry milk (Bio-Rad). The membrane was probed overnight at 4°C with the specific primary antibody, after which horseradish peroxidise-conjugated secondary antibody diluted 1:5,000 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was added. Bound antibodies were detected by Immun-Star Western C kit (Bio-Rad), using Chemidoc XRS Molecular Imager (Bio-Rad). The following primary antibodies were used: anti-p21 (monoclonal, 1:100) (BioOptica, Milan, Italy), anti-caspase 3 (polyclonal, 1:500), anti-caspase 9 (polyclonal, dilution 1:500), anti-bax (polyclonal, 1:1000), anti-pMAPK (polyclonal, 1:1000) (Cell Signalling Technology, Inc., Beverly, MA, USA), anti-caspase 8 (monoclonal, 1:500) (Alexis Biochemicals, San Diego, CA), anti-RAS (polyclonal, 1:1000) (Stressgen, Exeter,UK), anti-Bcl-2 (monoclonal,1:100) (Dako Corporation, Glostrup, Denmark), anti MCL-1 (monoclonal 1:100) (BD Pharmingen, San Jose, CA), anti rap1 (monoclonal 1:1000) (Abcam, Cambridge, UK) and anti-actin (polyclonal, 1:5000) (Sigma Aldrich), anti p-27 (monoclonal 1:2500) (BD Pharmingen, San Jose), anti-MAPK (polyclonal 1:1000) (Cell Signaling Technology).

Ras and Rho activity evaluation

For the evaluation of Rho and Ras activity the “Ras/Rho Activation Assay Biochem kit” by Cytoskeleton was used according to manufacturer’s instructions. Briefly, a pull down of the RAF-RBD/GTP-Ras complex and GTP-RHO Rhotekin-RBD, respectively, was performed. Then the amount of activated Ras is determined by a quantitative western blot using a Ras and Rho pan specific antibody, respectively. The band density was evaluated by the Quantity one software.

Apoptosis

Fragmented DNA generated in response to apoptotic signals was detected by terminal deoxynucleotidyl transferase (TdT)-mediated binding of 3′-OH ends. For TUNEL assay, cells were fixed for 15 minutes in 1% paraformaldehyde in PBS on ice, suspended in ice cold ethanol (70%) and stored overnight at -20°C. Cells were then washed twice in PBS and resuspended for 5 minutes at 4°C in PBS with 0.1% Triton X-100. Then , samples were incubated in 50 µl of solution containing FITC-conjugated dUTP deoxynucleotides and TdT 1:1 (Roche Diagnostics GmbH, Mannheim, Germany) in a humidified atmosphere in the dark at 37°C for 1 hour and 30 minutes, washed in PBS, counterstained with propidium iodide (2.5 µg/ml, MP Biomedicals, Verona, Italy) and RNAse (10 Kunits/ml, Sigma Aldrich) at 4°C for 30 minutes in the dark and then evaluated by flow cytometry.

Flow cytometric analysis was performed using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA). Data acquisition and analysis were performed using FACSDiva software (Becton Dickinson). Samples were run in triplicate and 10,000 events were collected for each replica. Data were the average of three experiments, with errors under 5% [195].

Cell cycle

After treatment of the evaluated drugs, cells were fixed in ethanol 70%, stained in a solution containing propidium iodide (10 mg/ml, MP Biomedicals, Verona, Italy), RNAse (10 kunits/ml, Sigma Aldrich) and NP40 (0.01%, Sigma Aldrich) overnight at 4°C in the dark, and analyzed by flow cytometry. Data were expressed as fractions of cells in the different cell cycle phases.

It was performed a pulse and chase experiment to evaluate S Phase. We performed the pulse and chase experiment on MDA-MB-231 with RT treatment. We decided to perform analysis on MDA-MB-231, because it is the cell line more sensitive to ZA.Samples were taken at baseline, after 72h of treatment, at 168 h (end of treatment) and after a 48 h washout [195].

Bi-parametric BrdU-DNA content determination.

BrdU (20mM, Sigma Aldrich, Milan, Italy) was added to cell medium 15 minutes before the start of scheduled treatments. Cells were incubated with the reported regimen or in medium without drugs. At the end of every selected exposure time, cells were fixed in ice-cold ethanol (70%), stored overnight (O.N.) at -20°C, washed twice in PBS and incubated in HCl 2N for 25 min at room temperature. Samples were then washed with 4 ml of Na2B4O7 (0.1M, pH8.5, Sigma Aldrich, Milan, Italy), incubated for 15 min at room temperature in PBS containing 0.5% Tween 20 (Biorad) and BSA 1% (Sigma Aldrich) and

subsequently incubated with a anti-BrdU mouse antibody (NeoMarkers) (1/50 dilution in 0.5% Tween 20 and BSA 1%) for 1 h at room temperature in the dark. Cells were washed in PBS and incubated with a FITC-conjugated anti-mouse immunoglobulin antibody (Dako Cytomation) (1/50 dilution in 0.5% Tween 20 and BSA 1%) for 1 h at room temperature in the dark. Before the cytofluorimetric analysis, samples were finally washed with PBS and stained with propidium iodide 5 mg/ml (MP Biomedicals) and RNAse (MP Biomedicals) 1mg/ml in PBS O.N. at 4°C in the dark.

Osteoclast Quantification

Mature osteoclasts were fixed at the different time points by incubation in 3.7% PBS buffered formaldehyde (Polyscience, Niles, IL, USA) for 20 minutes at room temperature and then stained for tartrate resistant acid phosphatase (TRAP kit, Sigma-Aldrich, Steinheim, Germany). Nuclei were counterstained with hematoxylin (TRAP kit). Osteoclasts were counted as multinucleated (more that 3 nuclei) TRAP-positive cells. Images of mature osteoclasts were acquired at different magnifications with Axiovision software. Each experiment was performed in quadruplicate and repeated at least 3 times**.**

MSCF and IL-6 Secretion

MCSF and IL-6 secretion were evaluated in MDA-MB-231 and osteoclast media by ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analyses

Differences between dose response, apoptosis and schedules of treatments were determined using the Student's t test for unpaired observations. Statistical analyses were performed using the Statistical Package for Social Science (SPSS, version 17.0) and statistical significance was defined as $p < 0.05$. All p values were two-sided.

2 B Clinical study

This was a retrospective observational case-control study conducted at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (I.R.S.T.), in Meldola, Italy. Our primary objective was to evaluate the predictive role of several gene expression markers, determined by Real Time PCR in fresh frozen primary tissues, in the development of bone metastases in breast cancer patients.

The study was designed to focus on 3 groups of patients operated on for breast cancer: the first was composed of 30 patients with radiologically confirmed bone metastases (BM)

which developed within 5 years of surgery; the second (30 patients) had radiologically confirmed visceral metastases (VM) which developed within 5 years of surgery; both groups formed the relapsed patient subgroup. The third group (30 patients) comprised patients with no evidence of disease (NED) at a minimum follow-up of 5 years. The protocol was reviewed and approved by the local ethics committee and performed in accordance with Good Clinical Practice guidelines.

Choice of markers

With regard to choice of marker, we have used microarray results [182, 187] and all studies found in literature that deal with gene profiling, sites of metastatization, and key molecular pathways involved in the metastatic process of breast cancer to bone. They were chosen moreover, on the consideration of metastatic process as a sequential multi-step program. Tumor cells at the primary site acquire properties allowing them A) to invade surrounding stroma and supplying vasculature and gain access to the bloodstream: in this phase epithelial and mesenchymal transition takes place, i.e. the acquisition of a more invasive phenotype B) to survive in the blood circulation (Beta 2-microglobin, CTGF, TFF1 ;C) to home to a specific secondary site(s) (as CXCR4 and RANK);D) to survive and colonize this secondary site (as Beta 2-microglobin, CTGF, CXCR4, RANK, SPP1, SPARC, HPSE, IBSP) [198-200].

Biomolecular determinations

Surgical tumor specimens were fresh frozen in nitrogen liquid; then tissues were homogenized and total RNA were extracted by Trizol (Invitrogen, Paisley, UK) following the manufacturer’s instructions. RNA was purified by a silica–cartridge extraction system and it was treated with DNAse I (Qiagen).

Five hundred ng of RNA were reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA). The final mixture was incubated at 25°C for 5 min, at 42°C for 20 min, at 47°C for 20 min, at 50°C for 15 min and 5 min at 85°C (mercatali et al 2011).

Real-Time PCR was performed using the 7500 Real-Time PCR System (Applied Biosystem) using the TaqMan assay custom plate system (Applied Biosystem). After retrotranscription reactions, amplification was performed in a final volume of 20 µl containing 2x Gene expression master Mix (Apllied biosystem), 2 µl of CDNA in a total volume of 20 µl. The reaction mixtures were all subjected to 2 min at 50°C, 10 min at 95°C followed by 40 PCR cycles at at 95°C for 15 sec and 60°C for 1 min for overall markers.

The stably expressed endogenous β-actin and HPRT genes were amplified and used as reference genes. Twelve markers were analyzed: Trefoil factor 1 (TFF1), bone sialoprotein (ibsp), heparanase (hpse), osteopontin (spp1), agr2, SPARC, CTGF, COMP, delta2-microglobulin and RANK.

All RT-PCR experiments were run in duplicate. The amount of transcripts was normalized to the endogenous reference genes and expressed as n-fold mRNA levels relative to a calibrator using Applied biosystem Software using a comparative threshold cycle (Ct) value method (delta delta Ct). The calibrator used was a Standard RNA sample extracted from a normal breast tissue (Ambion).

Statistical analysis

Descriptive statistics were reported as proportions and median values. The relationship between patient status and markers was analyzed using non-parametric ranking statistics (Median test). In the absence of internationally accepted cut-off values for overall markers, the cut-off maximally discriminating between control groups and BM patients was identified using receiver operating characteristic (ROC) curve analysis. Ninety-five percent confidence intervals (CI) were calculated for sensitivity and specificity values. Statistical analyses were carried out with SAS Statistical software (version 9.1, SAS Institute, Cary, NC, USA).

The diagnostic relevance of the dichotomized markers were evaluated using an univariate logistic regression model. The significant marker upon univariate analysis were entered into a multivariate logistic regression model considering marker as continuous variables . All the analyses were performed using SAS System version 9.3 (SAS Institute, Cary, NC, USA).

3 Results

3 A Preclinical study

Single drug exposure: ZA acid

Cytotoxic activity

ZA cytotoxicity was assessed and IC50 value was calculated (Figure 2). The IC50 vaslues for Repeated (RS) were lower than those for Non-repeated ones (NRS) in all cell lines analyzed.

Triple negative cell lines: The NRS treatment produced, in MDA-MB-231 cells, a IC50 mean value of 29 µM compared to 23 µM for RS, with a drop of 26% compared to standard treatment, (p =0.042) (Figure 2). BRC-230 cells were more sensitive to ZA for both treatment, and more specifically, the IC50 decrease was 14% higher with RS compared to NRS (p =0.003). Moreover, a cytocidal effect was observed with RS, inducing a LC50 of 49 µM and 40 µM in MDA-MB-231 and BRC-230, respectively.

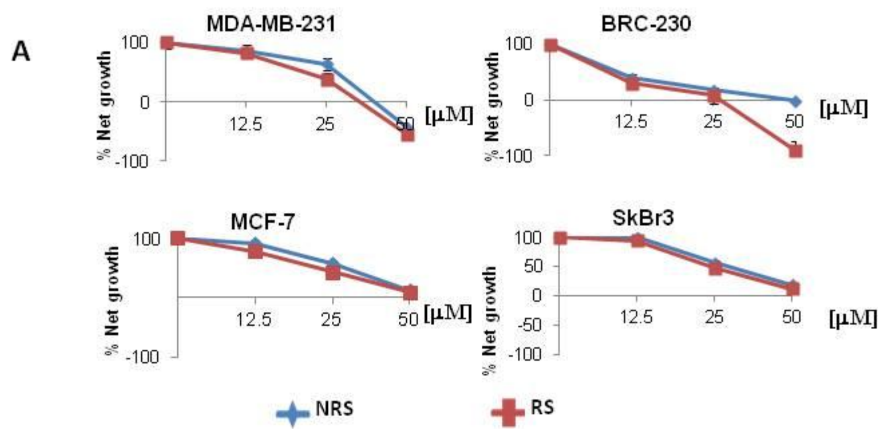
MCF-7 and SkBr3 cell lines: NRS treatment induced IC50 values of 23.6 µM and 25.2 µM in MCF-7 and SKBr3, respectively, while the RS schedule resulted in IC50 values of 29.0 µM (MCF-7) and 26.4 µM (SKBr3) (Figure 2). Neither of the two treatment schedules induced a cytocidal effect. As the highest concentration produced the strongest effect in all cell lines, this was chosen for all subsequent experiments.

Effect of ZA acid on the mevalonate pathway and proliferation markers

Triple negative cell lines: Both treatments induced a strong decrease in RAS expression in MDA-MB-231 and BRC-230 cells. There were no changes in MAPK levels after treatment in BRC-230 cell lines, meanwhile it was observed a strong decrease after both treatment in MDA-MB-231. Furthermore, a strong reduction of (Figure 3) pMAPK was reported in BRC-230 and, only slightly, in MDA-MB-231. Although both dosages inhibited the migration power of both cell lines, this reduction was more evident in BRC-230 (Figure 4). This result was confirmed by WB analysis of RHO, which decreased after treatment.

Ras activity in MDA-MB-231 was evaluated and a decrease of about a half of its activity was observed in both schedules, Ras expression levels in the RT decreased of about 10 times in RT and about twice in NRT (Figure 3). Considering the datum as ras expression/ras activity only with RT treatment a difference pre-post treatment was observed. About Rho, a difference between Rho activity pre-after treatment (data not shown) was not observed.

Fig. 2



B

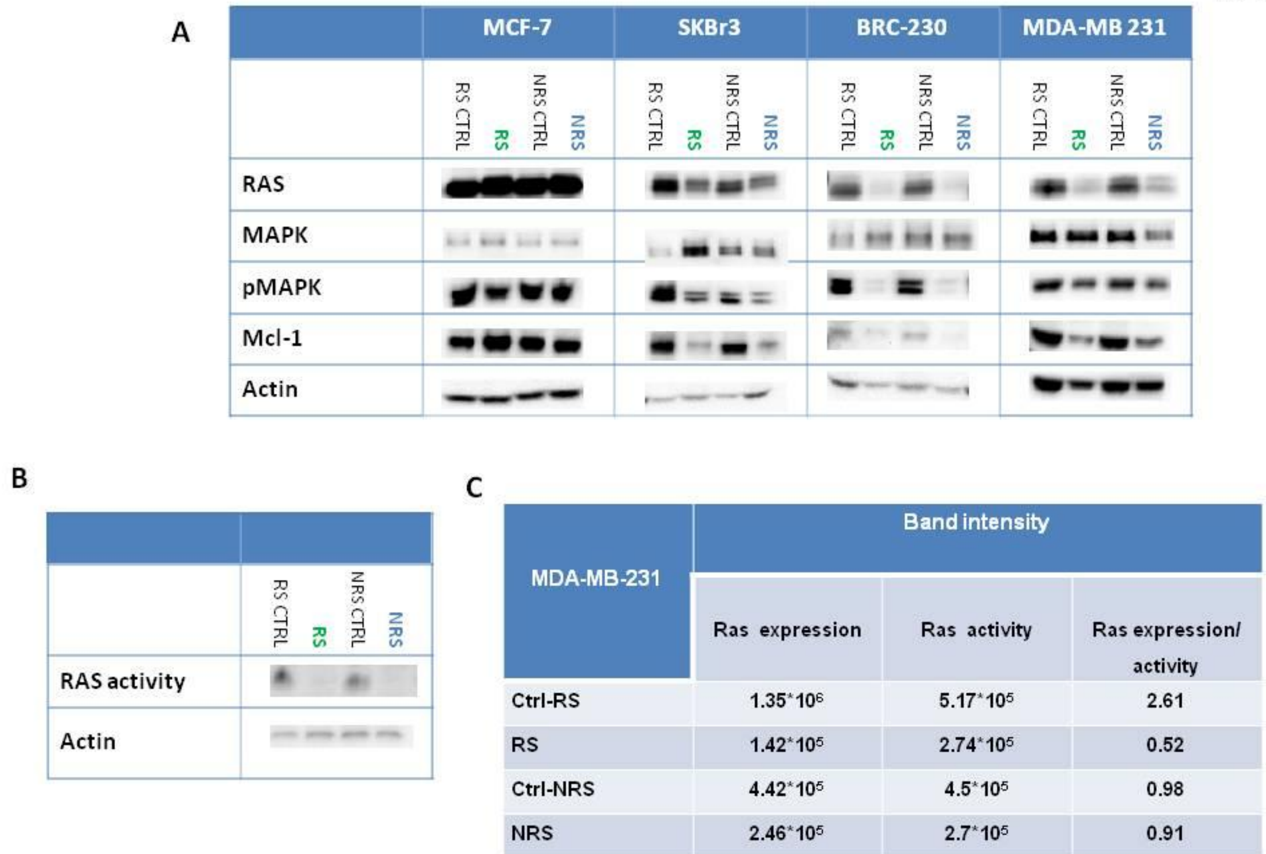
	MDA-MB-231 [μM]		BRC-230 [μM]		MCF-7 [μM]		SkBr-3 [μM]	
	NRS	RS	NRS	RS	NRS	RS	NRS	RS
IC50	29	23*	10.5	9*	29	23.6*	26.4	25.2
LC50	n.r.	48.5	n.r.	40	n.r.	n.r.	n.r.	n.r.

MCF-7 and SkBr3 cell lines: In these two cell lines, the reduction in RAS and pMAPK was lower compared to that observed in triple-negative cells, and was more evident in SKBr3 cells (Figure 3). MAPK levels were not different pre-after treatment. In MCF-7 cell lines and there was a slight increase of protein after RT treatment in SKBr3. Both treatment schedules did not modify the migration power of either cell line. This result was also confirmed by the absence of modulation of RHO expression by WB (Figure 4).

The differences observed in the cytotoxicity data and in the modulation of the mevalonate pathways cannot be attributed to a different uptake of ZA of the cell lines. In fact, no difference was detected in the accumulation of unprenylated Rap1A, a surrogate marker of ZA uptake (Figure 5).

Apoptosis

Triple negative cell lines: ZA induced apoptosis in both the triple negative cell lines used as experimental models (Figure 6). Both treatment schedules induced a significant percentage of apoptotic cells compared to the untreated control.

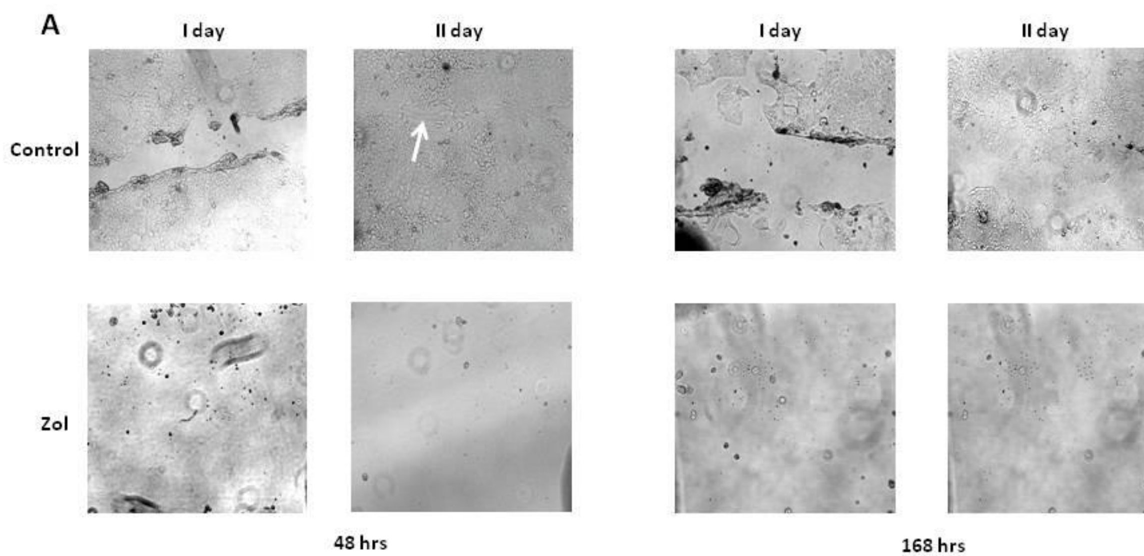


MDA-MB-231	Band intensity		
	Ras expression	Ras activity	Ras expression/ activity
Ctrl-RS	1.35*10^6	5.17*10^5	2.61
RS	1.42*10^5	2.74*10^5	0.52
Ctrl-NRS	4.42*10^5	4.5*10^5	0.98
NRS	2.46*10^5	2.7*10^5	0.91

However, MDA-MB-231 showed a higher percentage of apoptotic cells following RS compared to NRS treatment, without reaching the statistical significance (44% compared to 30.6%). Conversely, BRC-230, showed a higher percentage of apoptotic cells after NSR treatment (48%) compared to RS (40%), without reaching the statistical significance. Apoptosis was confirmed by western blot by a decrease in the levels of pro-caspase 3, 8 and 9 in both cell lines, without detection of the active forms. In MDA-MB-231, the levels of Bcl2 expression decreased after both treatments, whereas in BRC-230 the protein was not appreciably expressed (Figure 6). Furthermore, a decrease of mcl-1 expression was detected in both cell lines.

MCF-7 and SkBr3 cell lines: No apoptosis was observed in MCF-7, even if the presence of debris was detected. An almost complete disappearance of Bcl2 expression was also observed in MCF7 cells treated with RS. In SKBr3, the percentage of apoptotic cells was higher in treated cells following both treatment schedules compared to untreated control (not significant) (Figure 6). In addition, a strong reduction of MCL-1 was observed only in the SKBr3 cell line for both treatments. However, NRS treatment induced a higher percentage of apoptotic cells (31%) in this cell line compared to the RS treatment (14%).

Fig. 4



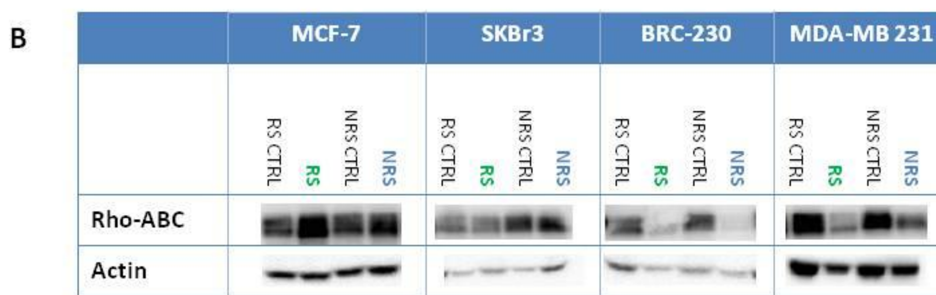


Fig. 5

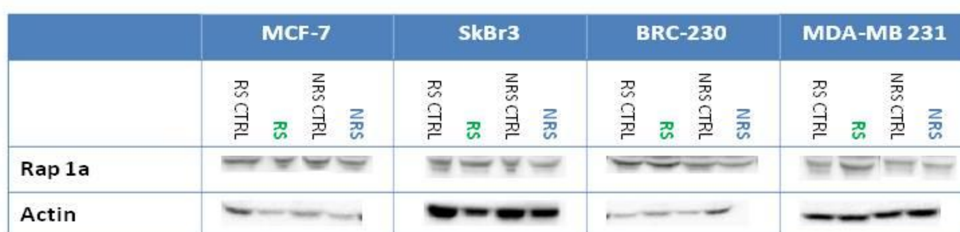
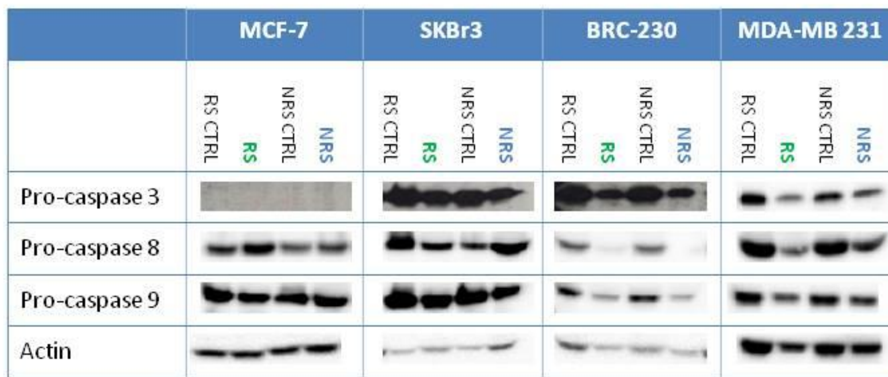
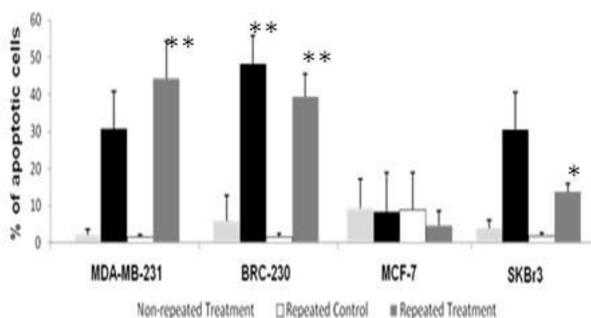


Fig. 6



A



B

Cell cycle analysis

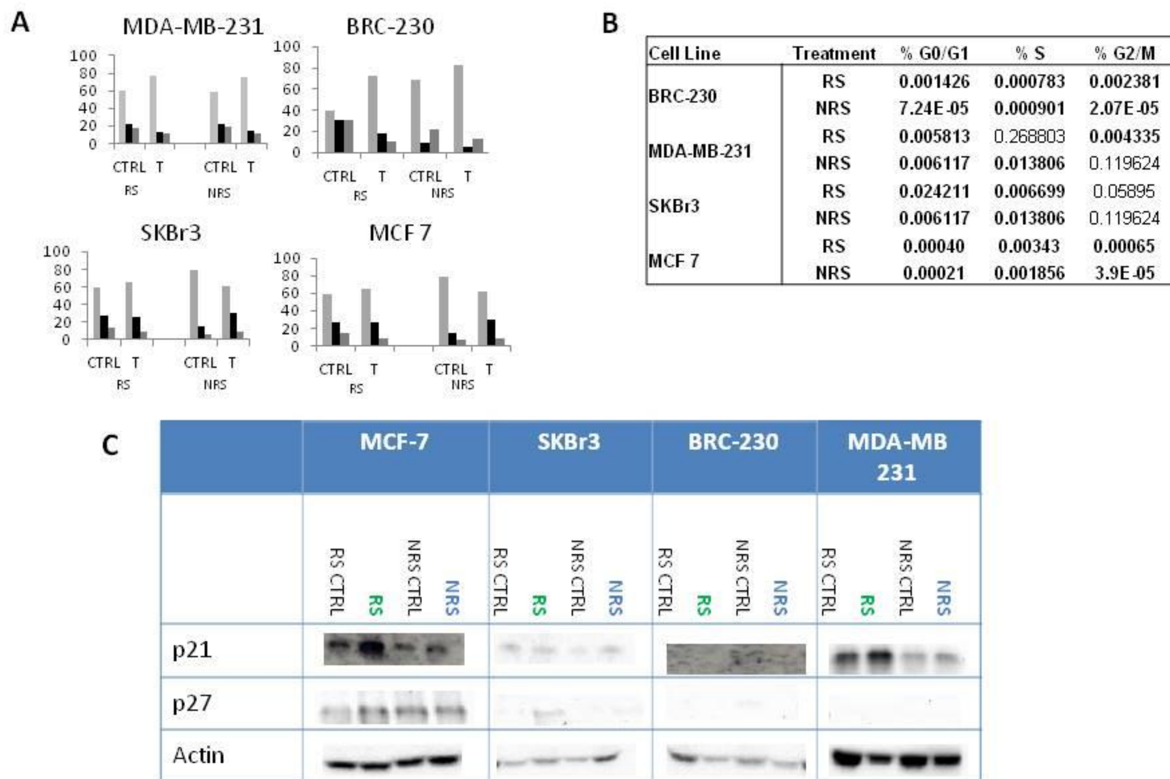
Triple negative cell lines Both treatment schedules induced a significant increase of the percentage of cells in G0/G1 in all cell lines used (Figure 7) compared to untreated controls. The percentage of cells accumulated in G0/G1 was 45.5 % higher after RS with respect to control compared to NRS treatment (16.9%) in the BRC-230 cell line. The accumulation in G0/G1 was also confirmed by the increase in p21 expression in RS in MDA-MB-231cells, whereas in BRC-230, the protein was not appreciably expressed. p27 expression can not be evaluated in any of the two lines.

SKBr3 and MCF-7: In SKBr3 cells, RS treatment induced an accumulation of cells in G0/G1 resulting in an increase of about 9% compared to untreated cells (p =0.005). Instead, NRS induced a cell accumulation in the S phase with a 50% increase of blocked cells compared to controls (p =0.01). Cell cycle perturbation was confirmed by an in increased of p27 in both cell line after ZA treatment.

We performed the pulse and chase experiment on MDA-MB-231 with RT treatment. After 72 and 168 h, all untreated cells were BrdU positive, indicating that every cell had entered S phase at least once, demonstrating again a regular cell proliferation. On the contrary, after 72 and 168 h, treated cells showed a fraction of BrdU negative cells, i.e. not proliferating, confirming ZA has arrested at least a fraction of cells in G0/1 phase. Moreover, it has to be noted that after 72 h, very few treated cells, BrdU positive or

negative, entered or leaved the S phase, as showed by the absence of clearly visible/detectable S and G2/M phase, even from the P.I. fluorescence axis only. 48 h after the end of treatment almost all cells were dead, in a late apoptosis stage, and therefore no more analyzable to correctly study the S phase Therefore, these last data were not reported in fig.

Fig. 7



Cell Line	Treatment	% G0/G1	% S	% G2/M
BRC-230	RS	0.001426	0.000783	0.002381
	NRS	7.24E-05	0.000901	2.07E-05
MDA-MB-231	RS	0.005813	0.268803	0.004335
	NRS	0.006117	0.013806	0.119624
SKBr3	RS	0.024211	0.006699	0.05895
	NRS	0.006117	0.013806	0.119624
MCF 7	RS	0.00040	0.00343	0.00065
	NRS	0.00021	0.001856	3.9E-05

Drug combinations: ZA-cisplatin

Based on the previous results of ZA activity drug combinations were performed using one dose of ZA -50 µM- for 72 hours. Five doses of Cisplatin -0,001, 0,01, 0,1, 1 and 10 µM were used. Interesting results were reached with Cisplatin and ZA combination on triple negative cell lines BRC-230 and MDA-MB-231. MDA-MB-231 cells were very sensitive to the combined treatment with Cisplatin and ZA. Indeed the IG_{50} obtained combining the two drugs in this cell line was lower than 0,001 µM whereas Cisplatin alone did not reach the IG_{50}, neither when administrated at the highest concentration (Fig 8A). BRC-230 were more sensitive to Cisplatin alone respect to MDA-MB-231 with an IG_{50} of 4.6 µM. But the decrease in survival percentage obtained with drug combination was lower respect to MDA-MB-231 with an IG_{50} of 0,005 µM (Fig 8B).

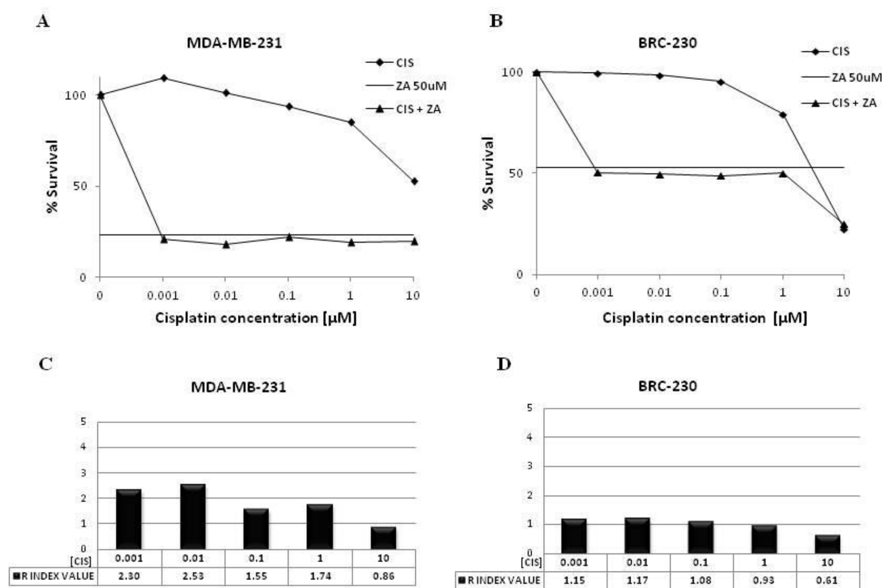


Fig 8

In MDA-MB-231 the combination of ZA and Cisplatin produced an important synergistic effect which yielded an R index higher than 1,5 for all Cisplatin concentrations except 10 µM. The synergism was particularly evident at lower concentrations of Cisplatin -0,001 and 0,01 µM- (Fig 8 c). An additive effect was reached combining Cisplatin and ZA in BRC-230 for all Cisplatin concentrations. Even in this cell line the addictive effect was higher at lower concentrations of Cisplatin (Fig 8d). No synergistic nor additive effect was observed combining Cisplatin on the ormonal receptor positive line MCF7 and on the HER-2 expressing line SKBr3. Based on these results the subsequent experiments were performed on Cisplatin and ZA combination in triple negative cell lines BRC-230 and MDA-MB-231.

Effect on proliferation pathways

A strong reduction of p-MAPK level was observed in BRC-230 after combination of Cisplatin and ZA, respect to untreated control, and especially at the lowest dose of Cisplatin -0,001 µm-. This reduction was absent in single treatments. Furthermore in MDA-MB-231 cell line the expression of MCL-1 was down-regulated after combined treatment but not in single drug exposure. Finally pM-TOR was found dramatically down regulated in MDA-MB-231 cell line after ZA alone and especially after combination with all Cisplatin concentrations (Fig 9).

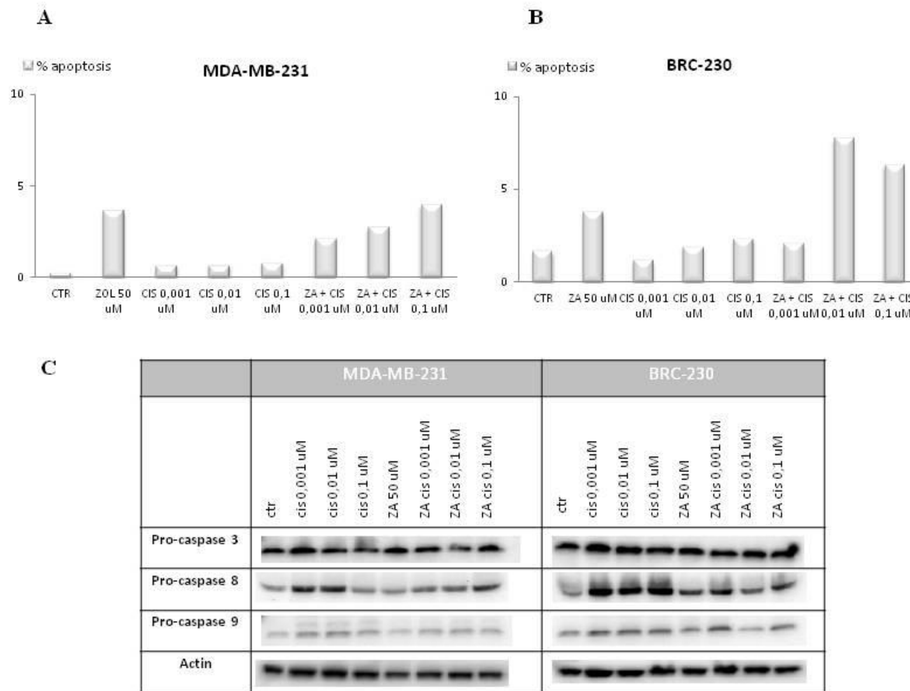


Fig 9

Figure **9:** protein expression levels of p-MAPK and MCL-1 detected by western blot analysis pre and after treatment with Cisplatin (0,001 µM; 0,01 µM and 0,1 µM) and/or ZA 50 µM.

Apoptosis induction

Assessment of apoptosis by TUNEL assay showed that either single drug exposure either combination of ZA with Cisplatin induced a small, not statistically relevant, increase in apoptotic cells percentage respect to controls for both cell lines. In MDA-MB-231 the apoptotic cell percentage did not exceed 5% in all the tested concentrations of Cisplatin alone and in combination with ZA (Fig 10A). In BRC-230 the apoptosis percentage reached 7,7 % and 6,3% after combination of ZA and Cisplatin 0,01 uM and 0,1 uM, respectively (Fig 10B). These data are in agreement with western blot analysis of caspase 3, 8 and 9. A substantial increase on the clived form of the three caspases neither a decrease on the pro-caspases levels were observed after all treatments (Fig 10C).

Fig

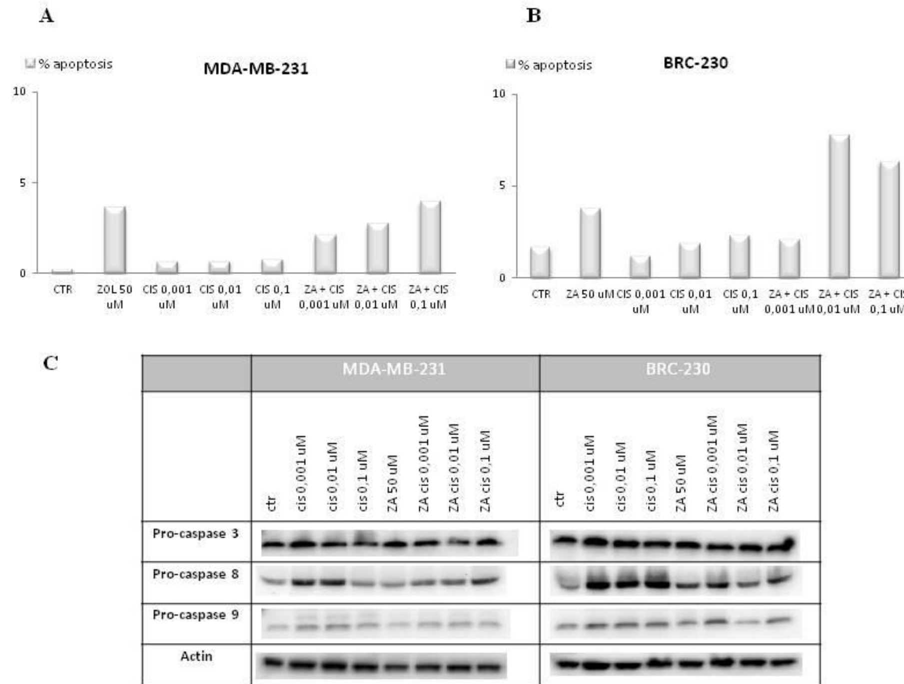


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Figure **10:** protein expression levels of pro-caspase 3, pro-caspase 8 and pro-caspase 9 detected by wester blot analysis pre and after single or combined treatments.

3.4 Cell cycle perturbation

Combination of ZA and Cisplatin did not produce a significant block of the cell cycle in G0-G1 or G2 phases in both triple negative cell lines. We observed a slight increment, respect to control, on the percentage of cells in G0-G1 after treatment with ZA alone and in combination with all Cisplatin concentrations in MDA-MB-231(Fig 11A) and with Cisplatin 0,001 and 0,01 µm in BRC-230 (Fig 11B). This data were in agreement with western blot analysis of p-21: p-21 levels were found to be up-regulated, respect to control, after combination of ZA and Cisplatin at all the tested doses in MDA-MB-231, but only at the lowest doses of Cisplatin for BRC-230 (Fig 11C).

Effect on migration ability

Treatment of cells with combination of ZA and Cisplatin resulted in a decreased migration rate respect to untreated control cells. The reduction of migration rate was found using the scratch assay in both triple negative cell lines: untreated cells and cells treated with Cisplatin alone were able to close the wound scratch by migration, whereas cells treated

with ZA alone and in combination with Cisplatin at all concentrations did not migrate and were unable to close the wound scratch.

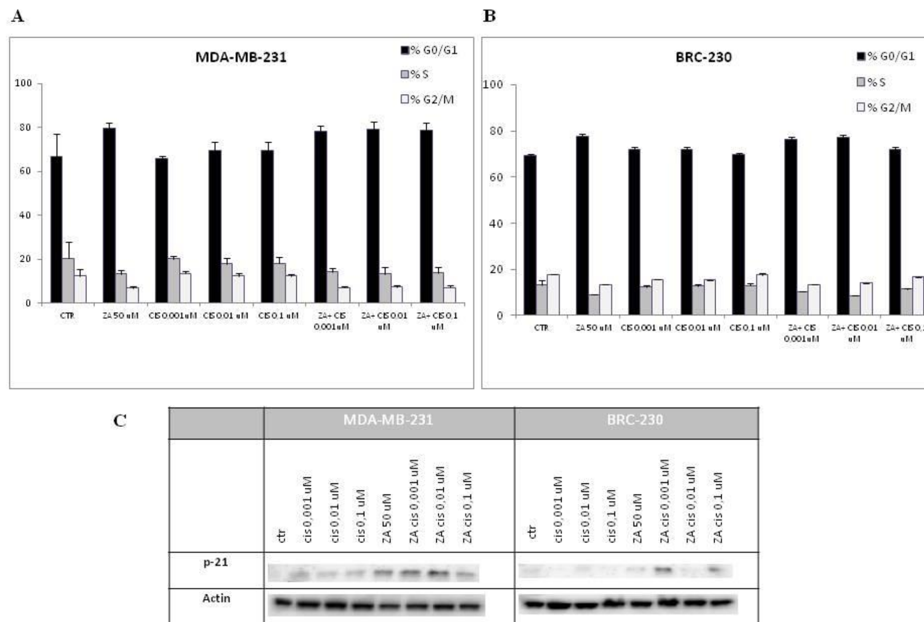
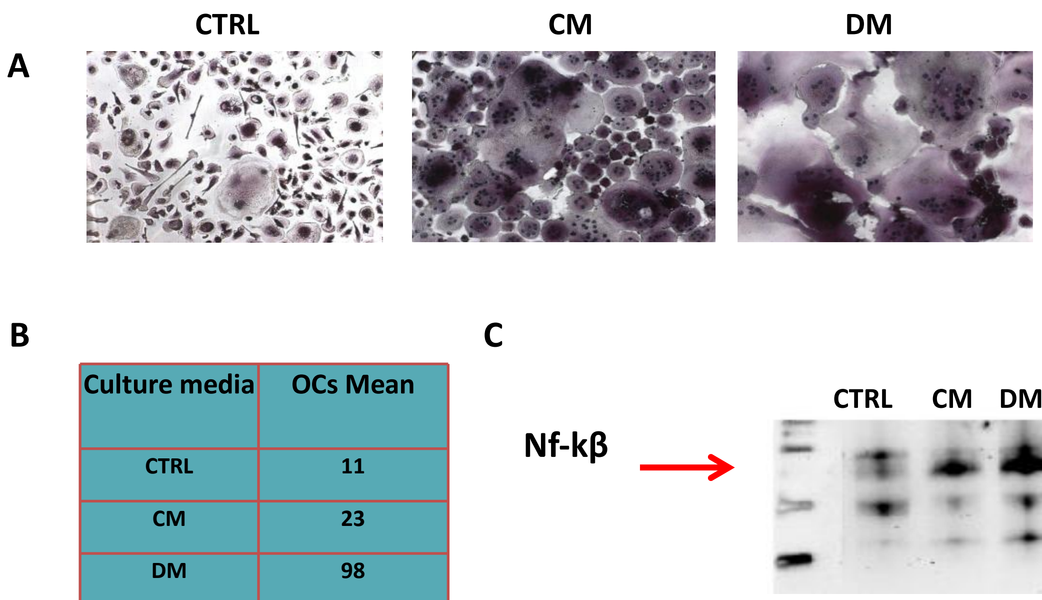


Fig 11



Culture media	OCs Mean
CTRL	11
CM	23
DM	98

Fig 12

Co-colture experiments

Model Validation

The effect of the soluble mediators produced by MDA-MB-231 on the process of the differentiation of peripheral blood monocytes into osteoclasts were studied. Conditioned media from MDA-MB-231 (CM) and RANKL-MCSF-supplemented media (differentiation media, DM) induced *in vitro* osteoclastogenesis after 14 days of culture. The number of differentiated osteoclasts, counted as TRAP- positive multinucleated cells, doubled with breast cancer-conditioned media compared to control media. The nuclear factor kappa-B was upregulated in CM- and DM- stimulated osteoclasts with respect to undifferentiated monocytes (Fig. 12*A*, *B*, *C*).

MDA-MB-231 cells were found to secrete high levels of MCSF, a growth factor required for osteoclast differentiation. MCSF levels in MDA-MB-231 significantly increased when cells were cultured under confluent conditions ($p = 0.009$) .

MCSF and IL-6 Profile

The presence of MCSF and IL-6, were evaluated in different samples (CTRL, CM and DM) during osteoclast culture. Baseline levels of MCSF varied among the 3 culture media: DM (supplemented with 25 ng/ml of MCSF), showed the highest concentration of this factor, CTRL showed no concentration, and 10% MDA-MB-231 CM showed a significantly ($p = 0.0003$) higher concentration than that of CTRL. Starting IL-6 levels were not different among the 3 culture media. During culture cytokines leveles changed with a MCSF decrease after 14 days in DM-cultured osteoclasts but increased in osteoclasts cultured in CTRL media and CM. Conversely, IL-6 decreased during time with any of the three different media (Fig. 13*A*, *B*).

Effects of drugs on Osteoclastogenesis

It was evaluated if the two different bone-targeted molecules produced an effect Osteoclasts induced by CM and DM. Treatment with Denosumab blocked osteoclast differentiation and proliferation: Osteoclasts were numerically and dimensionally reduced with respect to untreated cells. Treatment with ZA at all of the tested doses induced apoptosis on osteoclasts. ZA-treated osteoclasts showed a condensed and vacuolated cytoplasm, respect to undifferentiated control cells (Fig. 14).

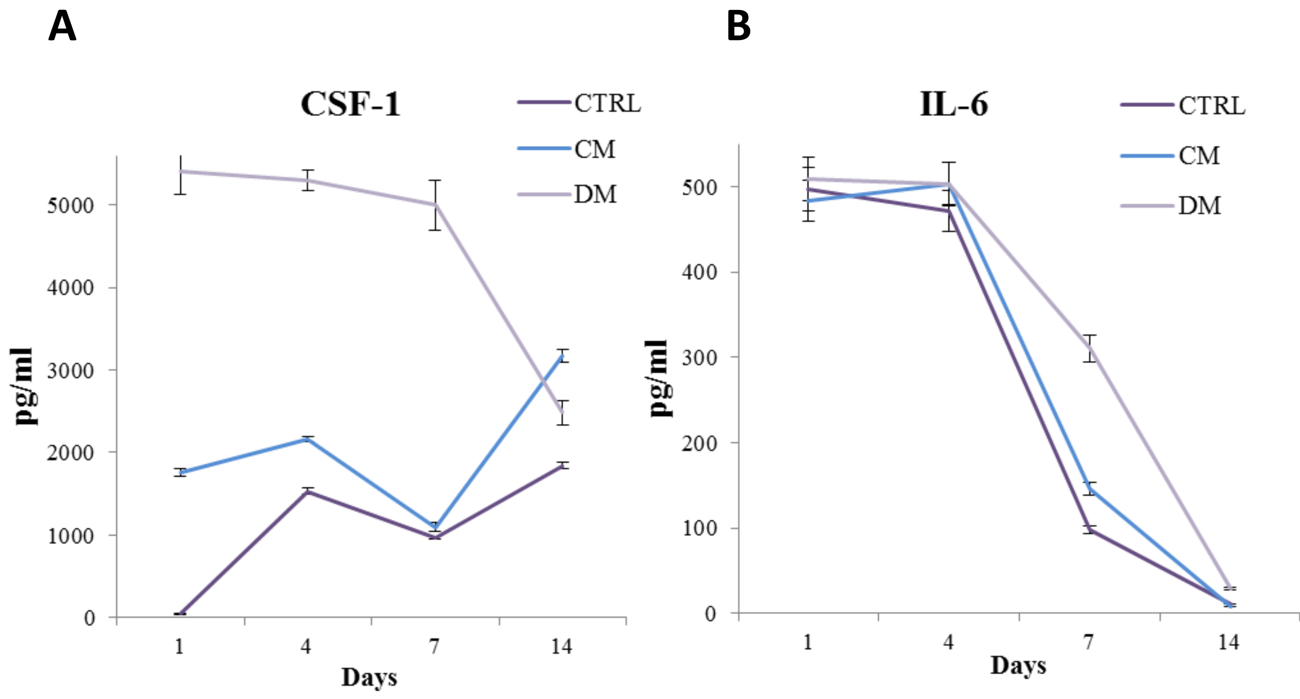


Fig 13

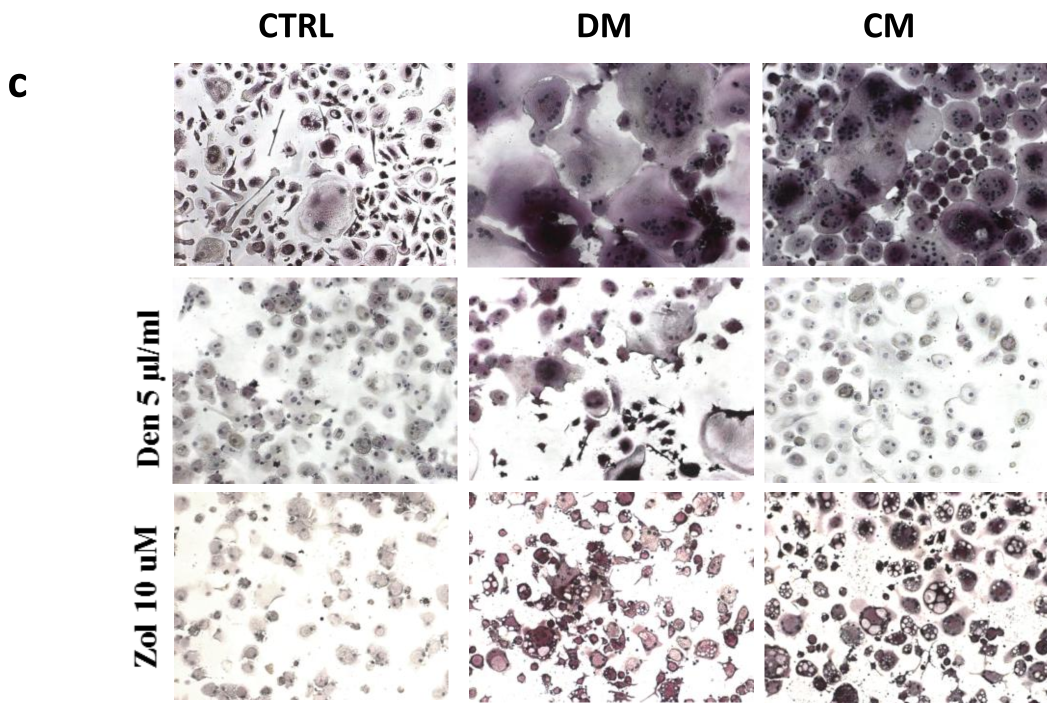


Fig 14

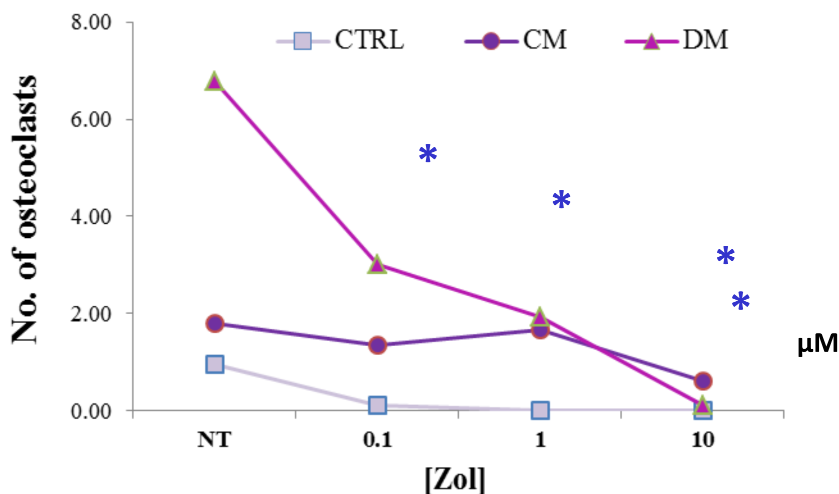
The sensitivity of osteoclasts to the 2 drugs differed on the basis of the osteoclastogenesis conditions. Breast cancer-induced osteoclasts were less sensitive to ZA than DM-induced osteoclasts. Only the highest concentration of ZA produced a significant decrease in the number of osteoclasts when differentiation was stimulated by MDA-MB-231 culture media (p = 0.0319), whereas all concentrations had a significant effect on DM-induced osteoclasts (p = 0.004 for ZA 0.1 µM, p = 0.0009 for ZA 1 µM and p = 0.0005 for ZA 10 µM) (Fig. 15*A*, *B*).

Breast cancer-induced osteoclasts showed similar sensitivity to Denosumab with respect to DM-induced osteoclasts. Only the highest concentration of Denosumab induced a significant decrease in osteoclast numbers when DM was used (p = 0.0031 for Denosumab 1 µg/ml and p = 0.0029 for Den 5 µg/ml), and only the two highest concentrations had a significant effect on for osteoclasts induced by MDA-MB-231 culture media (p = 0.029) (Fig.16 *A*, *B*).

Drug Effects on Cancer Proliferation

The effect of the 2 drugs on cancer cell proliferation was tested in the MDA-MB-231 cell line. None of the 2 drugs showed a significant anti-proliferative effect at any of the tested concentrations. Survival percentages were comparable with those of control cells.

A

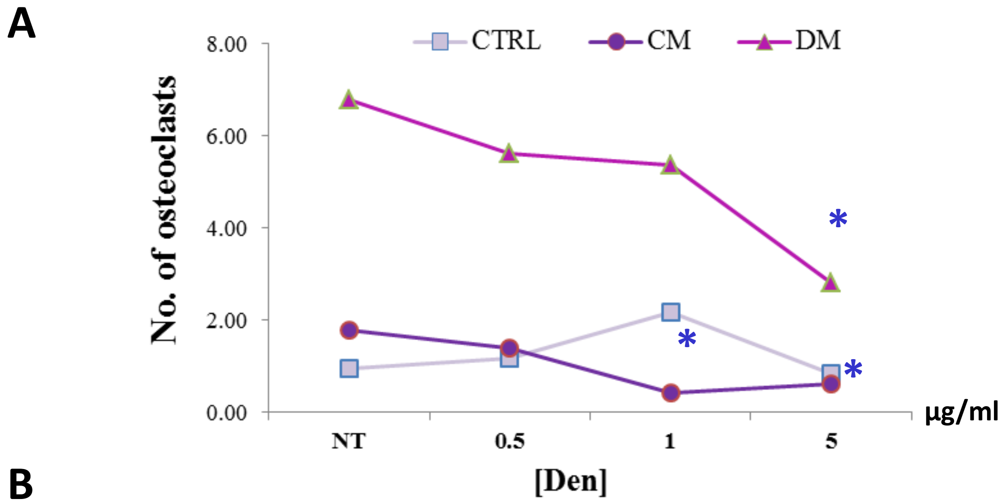


B

	CTRL			DM			CM		
Dose	**Mean**	**Std Dev**	***p* value**	**Mean**	**Std Dev**	***p* value**	**Mean**	**Std Dev**	***p* value**
NT	1.19	1.91		5.13	0.42		1.73	0.14	
Zol 0.1	0.10	0.14	**0.5004**	1.38	0.65	**0.0040**	0.85	0.76	**0.1215**
Zol 1	0	0	**0.3927**	0.54	0.25	**0.0009**	1.00	1.34	**0.5797**
Zol 10	0	0	**0.3927**	0.03	0.04	**0.0005**	0.44	0.62	**0.0319**

Fig 16.

A

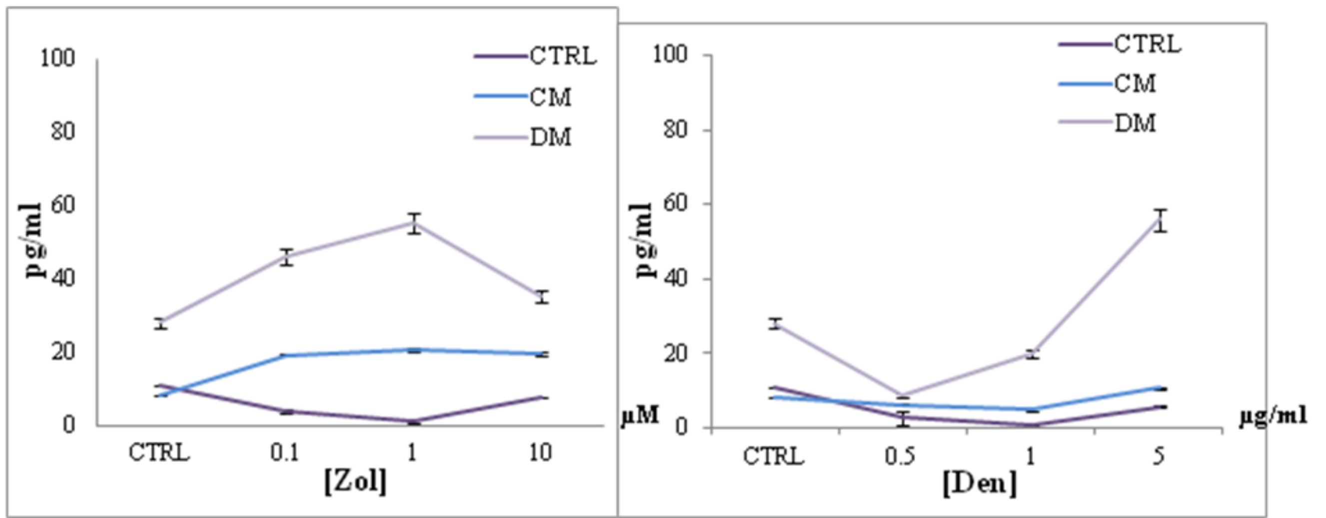


B

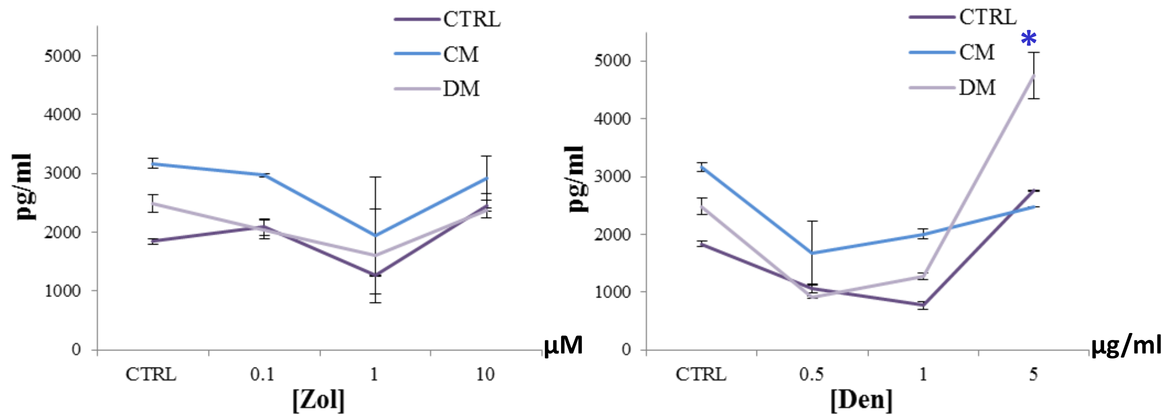
	CTRL			DM			CM		
Dose	**Mean**	**Std Dev**	***p* value**	**Mean**	**Std Dev**	***p* value**	**Mean**	**Std Dev**	***p* value**
NT	1.19	1.91		5.13	0.42		1.73	0.14	
Den 0.5	0.79	1.11	**0.7689**	4.17	3.54	**0.6842**	0.92	1.50	**0.4452**
Den 1	1.41	2.40	**0.9069**	4.17	3.54	**0.8422**	0.30	0.36	**0.0031**
Den 5	0.63	0.57	**0.6511**	2.30	1.41	**0.0294**	0.48	0.30	**0.0029**

The effect of the 2 drugs on MCSF and IL-6 levels in the different culture media (CTRL, CM and DM) was evaluated after a 14-day culture. IL-6 was only modulated in DM: a slight increase in the levels of this factor was induced by Denosumab at the highest concentration and by ZA at the intermediate concentration (Fig. 17*A*, *B*).

The highest concentration of Denosumab induced a significant increase in levels of this factor in DM. No significant changes were detected in CTRL media or CM at any of the concentrations of drug tested. Finally, ZA did not affect MCSF levels under any of different media conditions.



A



B

Fig 17:**Drug Effects on MCSF (a)and IL-6 *P*rofile (b)**

4 B Clinical study

Case series

Thirty patients (NED) were disease-free, while 60 had relapsed, 30 to viscera (VM) and 30 to bone (BM) tissue.

Markers analyses as continous variables

The median values of gene expression levels of each marker was evaluated in the 3 subgroups of patients NED, BM and VM: (Tab.1).

Different comparisons were performed between median levels of patients divided to understand if markers can discriminate between NED and metastatic patients first, and secondly if they have different trend also between patients with bone or visceral metastases.

Tab1 Median levels

Pazients	b2m	ctgf	Hpse	sparc	tff1	tnfrsf11a	cxcr4	ibsp
BM	0.40	0.71	3.11	4.52	430.64	0.66	2.76	0.69
VM	0.19	0.41	1.76	1.74	99.51	0.28	2.02	0.87
NED	0.22	0.49	2.35	2.41	32.79	0.56	0.78	0.42

In particular we compared

- **BM+VM vs NED:** to evaluate if the trend of markers are different in metastatic patients respect those with no relapse
- **BM vs NED:** to evaluate if the trend of markers are different in BM patients vs NED patients
- **BM vs VM:** to evaluate if the trend of markers are different in patients with different site of relapse

The Wilcoxon test highlighted TFF1 median levels were significantly higher in BM group in every comparison. Furthermore also CXCR4 expression was significantly different in metastic patients vs NED patients (P=0.0017) and in BM patients respect to NED patients (p=0.0177). (Tab 2)

Considering the comparison by Kruskal-Wallis test (comparison of medians of 3 groups) releaved a signifcant difference between TFF1 and CXCR4 (p=0.0043 and p=0.0039, respectively).In order to reduce variability each level oF gene expression was transformed on logaritmic scale (log_2) and univariate analyses was performed for continuous variables. Again Tff1 and CXCr4 (Tab 3) showed different levels in the comparisons metastatic patients vs NED and BM vs NED. In particular TFF1 OR was 1.274 (p di 0.0034) and CXCR4 OR was 1.636 (p= 0.0221).

Tab.2 Wilcoxon test for the comaprison of median levels of the different markers in comparison between two groups. (ns:not significant)

Markers	**BM+VM vs NED**	**BM vs NED**	**BM vs VM**
B2M	ns	0.0261	ns
CTGF	ns	ns	Ns
HPSE	ns	ns	ns
SPARC	ns	ns	ns
TFF1	0.0076	0.0024	0.0546
RANK	ns	ns	ns
CXCR4	0.0017	0.0177	ns
IBSP	ns	ns	ns

Tab 3 Univariate Analyses

Markers		BM vs NED		p		BM vs VM		p		BM+VM vs NED		p
	OR	IC 95%			OR	IC 95%			OR	IC 95%		
B2M	1.345	0.953	1.899	0.0916	1.210	0.863	1.696	0.2683	1.244	0.918	1.687	0.1591
CTGF	1.252	0.992	1.579	0.0583	1.077	0.857	1.354	0.5224	1.218	0.997	1.487	0.0531
HPSE	1.170	0.910	1.504	0.2198	1.050	0.831	1.327	0.6835	1.147	0.919	1.433	0.2256
SPARC	1.206	0.948	1.533	0.1267	1.010	0.795	1.283	0.9365	1.205	0.974	1.491	0.0854
TFF1	1.274	1.084	1.499	**0.0034**	1.176	0.994	1.391	0.0592	1.172	1.038	1.322	**0.0103**
RANK	1.158	0.888	1.509	0.2790	0.967	0.764	1.226	0.7838	1.178	0.938	1.480	0.1589
CXCR4	1.636	1.073	2.493	**0.0221**	1.096	0.787	1.526	0.5865	1.635	1.119	2.389	**0.0111**
IBSP	1.103	0.971	1.253	0.1316	1.047	0.945	1.160	0.3809	1.110	0.961	1.281	0.1553

Analyses of markers as dicotomic variables

Cut off values for this markers did not exist, so we chose for each marker the value that better discriminate between cases and controls according to receiver operating characteristic (ROC) curves(Fig.18 e Fig.19). Sensitivity and specificity of the prediction of bone metastases relapse were calculated. TFF1 was the most accurate markers witha sensitivity of 63% and a specifcity of 79% considering as control group NED patients and 77% considering as control group VM patients. L'Area Under Curve (AUC) was 0.74 considering the comparison BM vs NED and 0.65 considering BM vs VM (Tab.4 e Tab.5). The association of TFF1, B2M, CTGF e RANK bring to an increase of sensitivity of 79% without specificity decrease respect to markers considered singly.

Tab.4 BM vs NED: sensititvity and specificity

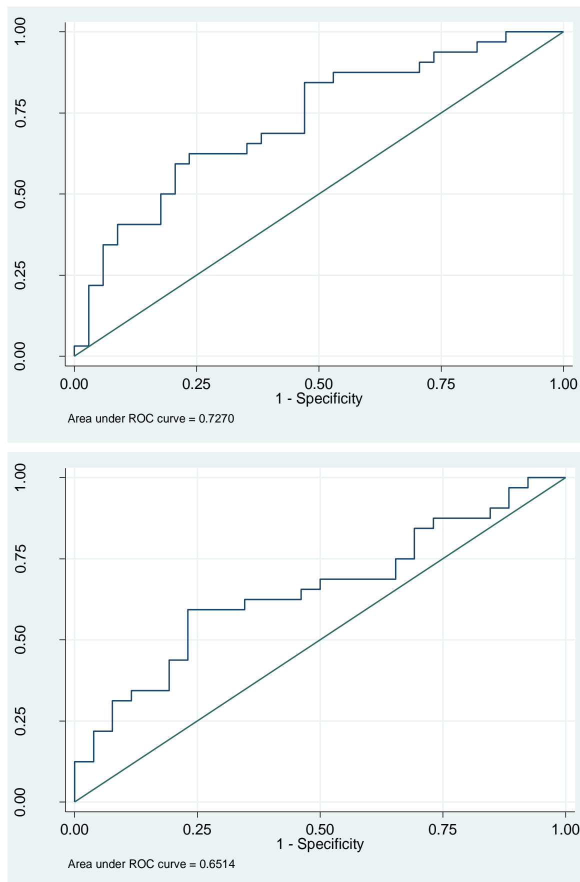
Markers	**Cut-off**	**Sensitivity IC 95% (%)**	**Specificity IC 95% (%)**	**AUC (range)**
B2M	1.1	27	94	0.6622 (0.52741 – 0.79701)
CTGF	3	30	97	0.6417 (0.50762 – 0.77580)
HPSE	17	18	97	0.6051 (0.46213 – 0.74804)
SPARC	38	9	100	0.6309 (0.49416 – 0.76755)
TFF1	350	63	79	0.7270 (0.60415 – 0.84990)
RANK	3.2	18	100	0.5788 (0.42643 – 0.73121)
CXCR4	4.5	35	100	0.7484 (0.57911 – 0.91762)
IBSP	10	20	100	0.5533 (0.37823 – 0.72844)

Tab.5 BM vs VM: sensititvity and specificity

Marcatori	Cut-off	Sensitivity IC 95% (%)	Specificity IC 95% (%)	AUC (range)
B2M	1.1	27	100	0.6085 (0.46236 – 0.75461)
CTGF	3	30	88	0.5501 (0.40070 – 0.69954)
HPSE	17	18	96	0.5148 (0.36032 – 0.66935)
SPARC	38	9	100	0.5344 (0.37551 – 0.69336)
TFF1	350	63	77	0.6514 (0.50871 – 0.79417)
RANK	3.2	18	96	0.4786 (0.31662 – 0.64052)
CXCR4	4.5	35	87	0.5221 (0.30575 – 0.73836)
IBSP	10	20	100	0.4925 (0.30486 – 0.68014)

Fig.18 TFF1 ROC curves

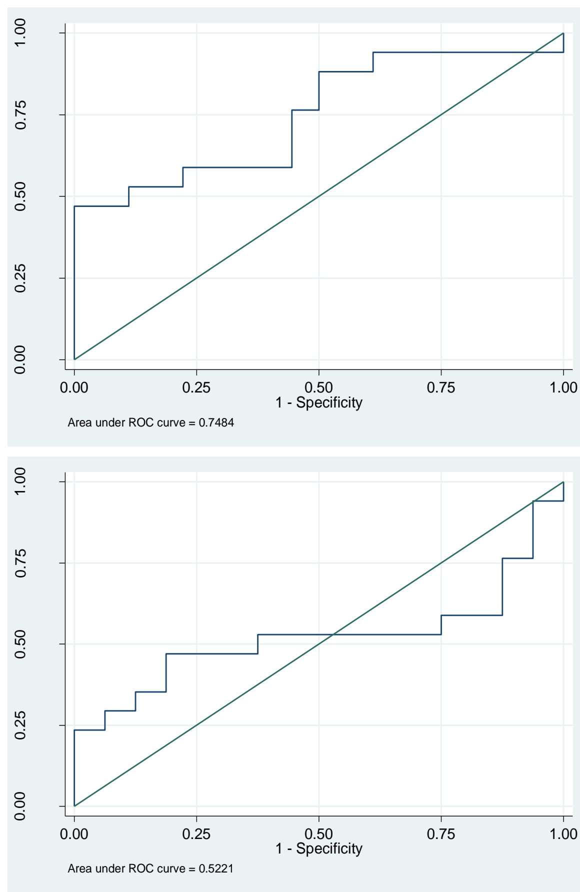
(BM vs NED e BM vs VM)



A significant datum also in this analyses was observed for CXCR4 in the comparison BM + VM patients vs NEDpatients obtaining an OR of 8.425 (p=0.0454) Tab..

Interesting results were also observed in the comparison between BM and NED patients; the increase of other markers, a part of TFF1 and CXCR4 was associated to a higher risk of bone relase; significant data were obtained for B2M,CTGF, TFF1 and CXCR4. Only TFF1, had a significant OR in the comparison BM vs VM.

Fig.19 CXCR4 ROC curves (BM vs NED e BM vs VM)



Tab. Univariate analyses of ditotomic variables

Markers		**BM vs NED**		**p**		**BM vs VM**		**p**		**BM+VM vs NED**		**p**
	OR	IC 95%			OR	IC 95%			OR	IC 95%		
B2M	6.000	1.186	30.346	**0.0303**	Non stimabile				2.880	0.584	14.195	0.1937
CTGF	14.348	1.716	119.942	**0.0140**	3.333	0.811	13.704	0.0951	9.325	1.162	74.829	**0.0356**
HPSE	7.329	0.831	64.633	0.0729	5.553	0.624	49.392	0.1242	4.440	0.522	37.724	0.1722
SPARC	Not calculable											
TFF1	5.235	1.777	15.423	**0.0027**	4.524	1.441	14.203	**0.0097**	2.836	1.066	7.546	**0.0368**
RANK	7.329	0.831	64.633	0.0729	1.072	0.287	3.998	0.9180	7.562	0.931	61.419	0.0583
CXCR4	10.560	1.239	90.008	**0.0311**	0.568	0.150	2.148	0.4048	8.425	1.044	67.979	**0.0454**
IBSP	4.552	0.481	43.071	0.1863	0.290	0.030	2.767	0.2821	3.055	0.342	27.307	0.3176

With multivariate analyses any association among markers was found; thus, TFF1 was the most accurate marker for the prediciton of bone metastases in cancer patients.

4 Discussion

4 A Preclinical study

In the present study, ZA induced cytostatic and cytocidal effects on breast cancer cell lines, in agreement with results from previous papers [201]. To mimic the bone microenvironment, concentrations of ZA used in the first sets of experiments (12.5, 25, 50 μM) were higher than the transient circulatory levels detected in patients. However, the concentrations used were in agreement with previously reported *in vitro* and *in vivo* data [202]. Moreover, it is well known that the pharmacokinetics and pharmakodinamic properties of ZA result in a rapid drug elimination by renal excretion and rapid uptake and accumulation within bone. This accumulation has also been supported by a xenograft study which showed a high bisphosphonate concentration in bone compared to plasma [203-206]. For the reasons described above, a higher concentration compared to that utilized in the clinical setting.

As expected, ZA induced dose-dependent effects on cell proliferation in all cell lines following both treatment exposures. However, the repeated treatment induced a statistically significant modulation of cell proliferation and cytotoxic effect only in triple negative breast cancer cell lines. These data support results obtained in a preclinical model of bone metastasis induced in a triple negative cell line, showing that the antitumor effect of bisphosphonates increases when the drug is administered at low dose with a daily or weekly schedule, inducing a reduction of osteolyisis and growth of tumor in the bone.

ZA is known to block enzymes of the mevalonate pathway such as farnesyl pyrophosphate synthase, and/or geranylgeranyl pyrophosphate synthase [207]. This block causes a deficiency in isoprenoids which are essential for the post-translation lipid modification of signalling GTPases such as RHO and RAS [208]. This study on ZA treatment on triple-negative lines to observe a modulation of RAS and RHO pathways; indeed, the decrease in RAS and pMAPK expression could explain the observed inhibition of cell proliferation. Furthermore we have demonstrated also the decrease of RAS activity after treatment.

There are conflicting literature data on breast cancer sensitivity to ZA, possibly due to the different HER2 and hormone receptors' patterns of breast cancers. A study reported that MCF-7 and MDA-MB-231 cell lines were similarly sensitive to bisphosphonates. Conversely, another study reported that clodronate reduced cell survival of MDA-MB-231, but not MCF-7 cells. Hu et al. have characterized genetic alterations and oncogenic pathway in different breast cancers subtypes, both in tissue and in cell lines, and found that

all mutations in *BRAF, KRAS* and *HRAS* were significantly associated with the triple negative subtype [209-211]. It has been hypothesized that triple-negative cell lines are more sensitive to ZA because the mevalonate pathway is blocked and the KRAS pathway is constitutively active. This *hypothesis fits in with the MDA-MB-231 cell line profile, which harbors mutated KRAS and BRAF*, while BRC-230 did not present any *BRAF*, *KRAS* and *HRAS* alterations (data not shown). However, BRC-230, presented a genetic amplification of EGFR and concomitant overexpression of the protein as observed in triple-negative breast cancers. The hormone receptor (MCF-7) and HER2-positive (SKBr3) cell lines, not presenting any alterations in *BRAF, KRAS, NRAS, HRAS* or *EGFR*, appear to be less sensitive to both ZA schedules. A possible explanation could be the lack of caspase 3 in MCF-7 and the overexpression of HER2 in SKBr3, which are involved in overcoming inhibition of the RAS pathway.

To evaluate the possible synergic effect of ZA and chemotherapeutic agents, cisplatin was chosen because conventional chemotherapy for breast cancer often employs DNA damaging drugs to prevent proliferation and stimulate apoptosis of cancer cells, especially in triple negatie breast cancer [212].

Cisplatin produced a synergist effect with ZA on the triple negative cell line MDA-MB-231, whereas an additive effect was reached on BRC-230. No effect was observed on the other two lines probably due to low drugs sensitivity of these lines. From one side this finding confirms previous results that highlighted the greater sensitivity of triple negative cells to ZA. As reported before, this result can be explained by genetic alterations on oncogenic pathways.

From the other side it has been demonstrated that the two triple negative lines have different sensitivity to ZA and Cisplatin. Results in the present study are quite interesting because ZA seems to sensitize MDA-MB-231 to Cisplatin whereas Cisplatin alone did not produce any effect on proliferation and survival. We do not have information about BRC-230 subtype because is a cell line isolated in our laboratory. Further molecular characterization are undergoing.

It is important to highlight that an high inhibition of cell proliferation for MDA-MB-231 was observed at low Cisplatin concentrations in association with ZA. Based on these results we decided to further evaluate other two lower concentrations -0,001 and 0,01 uM- of Cisplatin: as a result a greater synergistic effect was obtained..

Finally, the molecular mechanisms involved on the synergistic/additive effects was investigated: surprisingly, assessment of apoptosis showed that combination of ZA with Cisplatin induced a small, not statistically relevant, increase in apoptotic cells percentage

for both cell lines. The main molecular mechanism involved seems to be proliferation control. Even if we detected only a slight increment of cells percentage in G1 phase, we observed a relevant decrease of p-MAPK, Mcl-1 and p-mTOR expression levels and an increased p21. P-MAPK is part of the mevalonate pathway and this result is in agreement with previous demonstration that ZA produced his effect by modulating this pathway. Mcl-1, besides his anti apoptotic effect, was found to be involved in cell cycle and proliferation regulation and it was found modulated by ZA also in prostate cancer cell lines [213]. MTOR is critically involved in the mediation of cell survival and proliferation and some clinical trials with Everolimus- a new mTOR inhibitor- have already been done in metastatic breast cancer. In addition the PI3K/Akt/mTOR pathway is involved in chemotherapeutic drug resistance and response to radiation in breast cancer cells [214]. A previous study highlighted that inhibitors of mTOR have the potential to overcome drug resistance from topoisomerase II in solid tumors and it was demonstrated that ZA has the potential to enhance mTOR inhibition in osteosarcoma cells . Finally we know that MDA-MB-231 is a mesenchymal stem like subtype cell line that is responsive to mTOR inhibitors but resistant to Cisplatin [214]. Taken together, our findings can lead to the hypothesis that inhibition of mTOR proliferation pathway plays an important role in ZA anticancer activity and ability to overcome MDA-MB-231 resistance to Cisplatin.
All these remarks are essential to identify new molecular targets for the design of new preclinical and clinical trial investigations, especially in Triple negative breast cancer that lacks of molecular targeted therapies.

The effects of drugs on osteoclasts were also considered. It is known that cancer in any steps, from cancerogenis to metastases formation depend on mutations and changes in cancer cells, but also on the crosstalk between cancer and stromal cells. This deep communication is even more evident in bone metastases microenvironment, where the arrival of cancer cells determine the end of bone homeostasis with the develop of a vicious cycles in which cancer and bone cells help each other. With this in mind an in vitro model of indirect cocoltures has been developed to reproduce the effect exerted by breast cancer on human bone cells differentiation and results indicate that it could be used to improve the efficiency of preclinical trials. In particular, we confirmed that breast cancer cells have the potential to enhance osteoclastogenesis and to produce one of the soluble mediators needed for osteoclast differentiation: MCSF. The concentration of this cytokine significantly increased in breast cancer culture media when cells were cultured to 90%-100% confluence. After assessing breast cancer-induced osteoclastogenesis, we tested the effect of two conventional bone-targeted drugs in terms of their ability to interfere with this

cross-talk. We confirmed the different mechanisms of action of two molecules: ZA induced osteoclast apoptosis, while Denosumab blocked osteoclast differentiation and survival.

The efficacy of the two drugs differed in relation to the media used for differentiation induction. Breast cancer-induced osteoclasts proved less sensitive to ZA than osteoclasts induced by differentiation media containing only RANKL and MCSF.

This observation is in agreement with previous studies that demonstrated that breast cancer not only induces osteoclastogenesis, but also protects osteoclasts from undergoing apoptosis [215]. In contrast, sensitivity to Denosumab was similar among the differently induced osteoclasts, and the most important response was observed in osteoclasts induced by breast cancer. The superior ability of Denosumab to prevent skeletal-related events could be the result of these different mechanisms of action, which also resulted in a different efficacy of the inhibition of breast cancer-induced osteoclastogenesis [216].

Denosumab and ZA did not induce an antiproliferative effect at any of the tested concentrations, which seems in contrast to results from our previous results on the antitumor activity of ZA in breast cancer cells. However, the concentrations tested in the older studies that proved effective in inhibiting cancer cell proliferation were much higher than those used in the present work. Cytokine modulation in response to osteoclast differentiation and treatment was an interesting finding. IL-6, a putative cytokine involved in the direct stimulation of osteoclast maturation by breast cancer, was not produced by MDA-MB-231cells. IL-6 production by monocytes was not associated with osteoclastogenesis and progressively decreased during cell culture, independently of osteoclast stimulation. Furthermore, the modulation of the cytokine was not significantly involved in drug response, indicating that it was not essential for *in vitro* osteoclastogenesis. Conversely, a marked increase in MCSF levels was observed in osteoclast culture media after administration of the highest dose of Den, which may have been the result of the monocytes' response to the RANK ligand blockade. Monocytes, which produce MCSF in a variety of situations, may overexpress this cytokine when they are unable to complete differentiation. Our observations highlight a potential indication for using anti-MCSF antibodies in combination with Den, and further research into this area is ongoing.

4B Clinical study

The process leading to the development of bone metastases in patients with breast cancer is complex, and multi-step and requires the expression of specific genes that act together.

The processes that lead to the development of metastasis are still not fully understood.The problem of development of bone metastases has been addressed in recent studies, also considering the gene profile of primary tumors. Several panels of markers appear to provide important predictive information on distant recurrence of disease using assays with 70 or 21 geni although the results are inconsistent and not reproducible [217-218].One of the reasons for the lack of homogeneity of the results depends on the type of case series used, indeed some of these studies have been conducted in experimental animal models; only a few studies have evaluated the genetic profile of metastasis in biological samples collected from patients .The data obtained from the microarray analysis are only semi-quantitative and require confirmation by real -time PCR and studies conducted on samples of patients have provided for the evaluation of only a few markers. So there are no validated tumor markers which can predict the development of bone metastases. The availability of such a tool would provide clinicians with an important aid in the selection of the most appropriate therapy for each patient. To address this issue, we conducted a retrospective study of patients with breast cancer to evaluate the predictive role of markers selected in the development of bone metastasis. Ninety patients operated on for breast cancer, were selected and divided into three groups (each consisting of 30 patients): NED (disease-free patients), BM (patients who developed bone metastases) and VM (patients who developed visceral metastases). The markers were chosen to be evaluated by considering the results reported in the scientific literature on the most current gene profiling , to the sites of metastatization, and the key molecular pathways involved in the metastasis of breast cancer to bone, using RNA extracted from fresh tissue frozen.Although current technologies allow to use as starting material paraffin-embedded tissue, the possibility of exploiting fresh tissue or frozen allows to obtain RNA of better quality (less degraded) .In our study, the expression of TFF1 also appears to be significantly higher in the BM group compared to groups NED and VM individually .In univariate analysis with continuous variables transformed into a logarithmic scale (log2) for TFF1 we observe an OR of 1.214 with a p of 0.0034 in the comparison between the BM group and the group NED .

This result emphasizes the role of TFF1 in predicting an increased risk of development of bone metastases compared to the group of NED patients. The role of TFF1 as a predictor of bone metastasis was confirmed also considering categorized variables using a cut-off. As mentioned before the cut-off was chosen considering the ROC curves . The choice of a cut-off of 350 for TFF1 was made in such a way that the marker can efficiently discriminate the risk of development of bone metastasis is between groups BM and NED both between

groups BM and VM maintaining a high specificity and a good feeling. TFF1,indeed, was the marker showing the highest accuracy with a sensitivity of 63% and a specificity of 79% whereas the NED as a control group and 77 % considering patients as a control group VM. In this case, we preferred to select a cut-off which maintains a high specificity, since we are interested in identifying patients who are most likely to relapse to bone with a minimum of false positive results. The risk of bone relaspe for TFF1 levels higher repsect to cut off was 5235 (P = 0.0027) timefold than the other.

Taken together, the present data confirm and enhance the results reported by Smid highlights the important role that TFF1 has as predictive marker for the development of bone metastases.

Also are the results obtained for the marker CXCR4 . The study we conducted previously show that , high expression of CXCR4 , allows to identify patients at high risk of relapse to bone 242. In the current study we have seen, by univariate analysis with continuous variables transformed into a logarithmic scale (log2) , an OR of 1.636 with a p of 0.0221 in the comparison between the BM group and the group NED for CXCR4 . This indicates a significantly higher risk, associated with the presence of high levels of CXCR4, of BM development compared to the group of NED disease free patients

5 Conclusions

The present work dealt on improve the knowledge of mechanisms of action and potential of the bone targeted therapies for bone metastases patients. Two types of approaches were followed. Firstly bone targeted therapy singly or in combination with other drugs in different in vitro models to highlight new possible unknown mechanisms that could help clinicians to find new therapeutic strategies for this setting of patients. Secondly, the research of new markers to introduce in clinical practice to help clinicians to correctly select patients who could benefit of bone targeted therapy in adjuvant setting as a prevention for BM relapse.
Preclinical data confirmed the direct antitumor activity of ZA in human cell lines, as previously reported in *in vitro* and mouse models. Furthermore, an increase in the efficacy of ZA with repeated doses was highlighted. In addition, the two triple-negative breast cancer cell lines were more sensitive to ZA than the other cell lines. These results indicate that it would be interesting to carry out further trials on animal models and, after successful completion, on patients. Furthermore, we observed that ZA has a synergistic/additive effect with Cisplatin on triple negative cell lines; investigating the molecular mechanisms involved we found that control of proliferation pathways is probably the key of action of the drug combination. P21, pMAPK and mTOR pathways were find evidently regulated especially at lower doses of Cisplatin. Even if further evaluations are needed to elucidate the molecular mechanisms, a lot of new possible targets to be investigated have came out. Finally it would be very interesting to test these schedules on xenograft models and, moreover, on patients for several reasons. First of all considering the limited options for triple-negative breast cancer patients and second because, seeing that the synergist effect find on MDA-MB-231 was higher at lower doses of Cisplatin, this schedule could consent to reduce Cisplatin dosage, minimizing side effects of this chemotherapeutic agent. Further we developed a valid system to test the activity of bone-targeted molecules from a preclinical standpoint. The experimental model enabled us to investigate the molecular mechanisms governing the cross-talk between breast cancer and bone cells, and to understand how these are influenced by bone-targeted treatments.

We considered the results obtained by the translational retrospective study a possible useful support to clinicians in planning the therapeutic choice given the sometimes conflicting results reported in the clinic. In fact, some trials have evaluated the potential role in the prevention of bone metastases of ZA and Denosumab. In clinical practice, the action of

bisphosphonates on bone resorption mediated by osteoclasts seems to be a useful strategy to improve the results on adjuvant setting for breast cancer, and also prevents the development of SREs in patients with bone metastases. Recently it has been used in the treatment of bone metastases a monoclonal antibody directed against RANKL, the Densumab, which interferes with the axis RANK/RANKL/OPG. It is clear that the possibility of use of markers that allow to predict metastasis may help clinicians select the proper therapy for each patient, delaying the development of bone metastasis. This could provide a major change in the management of patients and in particular whose with bone metastases, with the hope of obtaining a reduction in the number and frequency of SREs with an increase in terms of clinical efficiency and cost.

In addition, patients considered at high risk of relapse in bone might be followed by bone radiological exam which is not included in the current guidelines for the clinical follow-up of disease free patients. Instrumental examinations, indeed, are required only when patients are symptomatic (eg Bone scintigraphy is required when patients have bone pain). Finally, the present study has identified new molecular players, as TFF1, involved in the natural history of metastatic process from the primary tumor to secondary anatomical sites, that, in the future , may be tested as a target for new biological drugs .

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