

# Master Thesis

The influence of menopausal status on bone turnover and disease control in breast cancer patients with metastatic bone disease treated with chemotherapy plus zoledronic acid: an exploratory retrospective cohort study



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## **Abstract**

**Background:** A recent meta-analysis showed that the adjuvant use of zoledronic acid (ZA) in postmenopausal women with early breast cancer (BC) leads to a reduction in the risk of breast cancer death by 17%. We investigated the effect of the hormonal status (pre [PrM] vs late post menopause [PoM]) on bone turnover and disease control among women with BC and bone metastases (BM) treated with ZA and chemotherapy (CT).

**Methods:** In this retrospective cohort study, we collected clinicopathologic variables, urinary N-terminal telopeptide (NTX) and serum tumor marker levels from women with BC and BM treated with CT and ZA. Patients were divided in PrM (<45 years) and PoM (>60 years). Study endpoints were NTX, CA15.3 and CEA variation at 3, 6 and 9 months, and time to first-line CT failure and survival. We performed multilevel mixed-effects linear regression models to assess the variation of repeated measures and cox regression models for time to event outcomes.

**Results:** Forty patients were eligible for analysis (8 PrM and 32 PoM).

After introduction of ZA and CT, NTX and tumor markers declined in the overall cohort. Response profile was similar between menopausal groups at month 3 and at later time points (p-value for time-hormonal status interaction at month 3=0.957). Furthermore, tumor markers response profile was also equal between groups.

Median time to first-line CT failure in PrM and PoM women was 15.2 and 17.4 months, respectively. No significant difference between groups was found, either using a univariate analysis or after controlling for visceral disease involvement (p=0.399 and 0.469, respectively). Likewise, no differences in survival were found.

**Conclusions:** In this cohort, no differences were found in terms of NTX or tumor markers control according to menopausal status. Similarly, no difference in time to first-line CT failure or survival was found.

**Keywords:** breast cancer, bone metastases, zoledronic acid, bone remodeling markers, menopausal status.

## Resumo

**Introdução:** Uma meta-análise recente demonstrou que uso adjuvante de ácido zoledrónico (AZ) em mulheres pós-menopáusicas com cancro da mama precoce (CM) conduz a redução do risco de morte por CM em 17%. Investigámos o efeito do estado hormonal (pré [PrM] vs pós-menopausa tardia [PoM]) na remodelação óssea e controlo de doença em mulheres com CM e metástases ósseas (MO) tratadas com AZ e quimioterapia (QT).

**Métodos:** Neste estudo de coorte retrospectivo, colhemos variáveis clinico-patológicas e quantificámos o telopéptido N-terminal (NTX) urinário e marcadores tumorais (MT) séricos em mulheres com CM e MO tratadas com QT e AZ. As doentes foram divididas em PrM (<45 anos) e PoM (>60 anos). *Endpoints* do estudo: variação do NTX, CA15.3 e CEA nos meses 3, 6 e 9, tempo até falência de QT de primeira-linha e sobrevivência. Quando apropriado foram usados os testes de Wilcoxon rank-sum, modelo de efeitos lineares mistos, teste log-rank e modelo de Cox.

**Resultados:** Quarenta doentes foram elegíveis para análise (8 PrM e 32 PoM).

Depois da introdução de AZ e QT, os níveis de NTX e MT caíram no coorte global. O perfil de resposta não diferiu entre grupos no mês 3 ou em tempos posteriores (valor-p para interação tempo-estado hormonal no mês 3=0.957). Ademais, o perfil de resposta dos MT também não diferiu entre grupos.

O tempo mediano até falência de primeira-linha de QT em PrM e PoM foi de 15.2 e 17.4 meses, respetivamente. Não foi identificada diferença significativa entre grupos, quer em análise univariada quer após controlo para envolvimento visceral (p=0.399 e 0.469, respetivamente). Igualmente, não houve diferenças em termos de sobrevivência.

**Conclusões:** Neste coorte, não foram identificadas diferenças no controlo de NTX ou MT em função do estado menopausico. Igualmente, não foi identificada diferença no tempo até falência de primeira-linha de CT ou sobrevivência.

**Palavras-chave:** cancro da mama, metástases ósseas, ácido zoledrónico, marcadores de remodelação óssea, estado menopausico.

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## Introduction

This document constitutes the final thesis of the Double Master in Clinical Research from NOVA Medical School (NOVA University) and Northeastern University.

This document is an original scientific dissertation (*dissertação de natureza científica original*) as per *Regulamento n.º 680/2010*.

This project was presented at the “Spring Meetings 2014” (Évora, Portugal) in poster format and was awarded a prize for the second best poster presented in the meeting.

## Background and significance

### a) Breast cancer epidemiology

Breast cancer (BC) is the most frequently diagnosed and the most common cause of women cancer related death in Europe with an estimated incidence and death rate of 108.8 and 22.4 cases per 100 000 women per year, respectively.[1] More specifically, the estimated incidence and death rate in Portugal is of 85.6 and 18.4 cases per 100 000 women per year, respectively.[1] Furthermore, breast cancer is also the most common cause of cancer related death in women both in developed and developing regions.[2]

In Europe and in the USA, the majority of patients currently diagnosed with BC present with early or locally advanced disease in terms of stages of disease progression, allowing the use of therapies with curative intent.[3] However, 5-10% of breast cancers are diagnosed with metastatic disease. Furthermore, from those treated with curative intent, around 40% will develop disease recurrence.[4] While locoregional recurrence might be eligible for curative interventions, those with distant relapse are almost universally incurable.

### b) Bone metastases and skeletal related events

In patients with metastatic breast cancer bone is the most common involved site, either as *de novo* disease or after disease relapse. While the 5-years incidence of bone metastases in patients treated with curative intent is around 5%, 70% of those with metastatic disease have bone involvement at some time during disease evolution.[5] Liver, lung and less frequently the brain are also typical target organs of breast cancer metastatic spread.

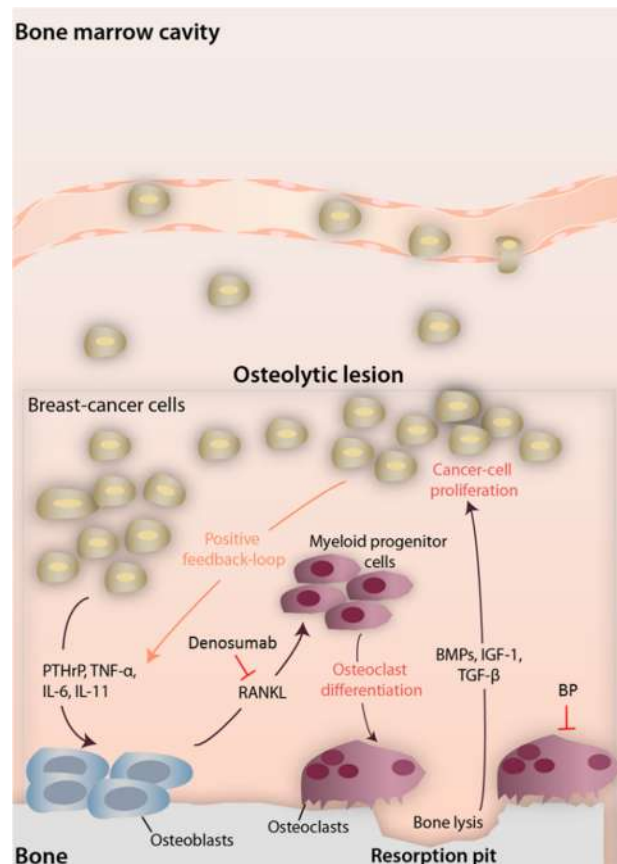
The exact mechanisms of breast cancer cells tropism to the bone is not fully understood. However, both anatomical characteristics, as blood drainage following the Batson plexus, and molecular features, as e.g. the chemoattractant action of bone marrow derived CXCL12 that induces tropism of tumor cells expressing CXCR4 and CXCR7, partially explain the homing process of breast cancer cells to the bone. After reaching the bone, Src activity seems to play a major role in supporting

cancer cell survival in the bone marrow microenvironment. This process appears to be accomplished by facilitating CXCL12-CXCR4-AKT signaling and by conferring tumor cells resistance to TNF-related apoptosis-inducing ligand (TRAIL).[6] Bone metastasis are also more common in patients whose tumors express the estrogen receptor (ER).[7] As a matter of fact, Src is active in most estrogen receptor positive and in a minority of estrogen receptor negative tumors.[6] Furthermore, Src and the ER seem to interact.[8] Despite this interaction, Src expression signature is more strongly associated with bone disease than the ER *per se*, an association that seems independent in nature from the ER. In line with this, even when restricting to patients with ER-negative tumors, Src expression is still highly associated with bone metastatic development, a fact that underlines the relevance of Src expression as, at least, a marker of activation of relevant pathways leading to a successful adaptation of cancer cells to bone environment.[6]

Breast cancer cells further elicit a cascade of events that lead to a profound change in bone metabolism. Bone is under continuous remodeling, a process accomplished by the coupled activity of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells). Cancer cells reshape this equilibrium to activate osteoblasts. This process is at least partially accomplished through the production of parathyroid hormone-related peptide (PTHrp), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 (IL-1), IL-6, IL-8 and IL-11 by cancer cells.[9] Activated osteoblasts release activator of nuclear factor  $\kappa$  B ligand (RANKL) that ultimately activates osteoclasts; hence, it induces bone resorption, which leads to the release of growth factors entrapped in the bone matrix (transforming growth factor- $\beta$  (TGF- $\beta$ ), bone morphogenetic proteins (BMPs), insulin like growth factor (IGF) and fibroblast growth factor).[9, 10] This last step is the closing gap in the loop, as cancer cells will benefit from these growth stimulation factors, therefore leading to more bone turnover activation: a loop referred as the vicious cycle of bone metastases.[11]

Bone remodeling modifications ultimately conduct to increased bone fragility[12] and development of skeletal related events (SRE).

**Figure 1** - Interactions between bone and cancer cells in predominantly osteolytic breast cancer lesions. Sites of action of denosumab and bisphosphonates are also noted. Bone metabolism with resorption and formation occurs. The depicted mediators emphasize the predominant pathways. See text for further details.



Skeletal related events include a series of adverse outcomes derived from the presence of bone metastases, namely excessive pain requiring intervention, as radiotherapy or surgery, bone fracture, myeloid compression and hypercalcemia. The interaction between cancer and bone cells can induce the development of several types of lesions: lytic (mostly bone degrading), blastic (mostly bone forming) or mixed (lesions presenting a mixture of lytic and blastic features). Either of these lesions represent a profound change in bone architecture and therefore of bone resistance to mechanical stress. In patients without therapy aiming to control bone metabolism, a rate of approximately 4 SREs per year is expected, significantly impairing patients' quality of life and ultimately, survival.[13, 14]

c) Markers of bone remodeling

Bone remodeling involves degradation of current bone matrix and formation of new bone matrix; in fact, both biochemical processes lead to the release of several biochemical by-products amenable of being quantified in, e.g., blood or urine.[15] NTX (amino-terminal crosslinked telopeptide of collagen type I) is a by-product of collagen degradation and one of the most commonly used markers of bone metabolism, in specific of the bone resorbing arm. On the other hand, ALP (alkaline phosphatase) is a by-product of osteoblast activity; hence, a marker of bone formation.[15] Other types of bone remodeling markers are listed in [table 1](#).

Bone markers are relatively non-invasive indicators of bone metabolism and several studies tested their role in the clinical setting. Urinary NTX has been characterized in patients with or without bone metastases, and receiving or not bone modifying agents.[16, 17] In fact, this and other bone remodeling markers (as CTX, carboxy-terminal crosslinked telopeptide of collagen type I) are being explored in several perspectives in the clinical setting, namely a) to evaluate the risk of developing bone metastases[18], b) to diagnose new bone metastases[19, 20], c) to evaluate response to therapy/patient outcomes (SRE, bone disease progression and overall survival)[21, 22], as well as d) as a tool to titrate the dose and frequency of administration of bone targeted agents[23]. The most relevant use of bone remodeling markers was however during the trials that led to the approval of new bone targeted agents as denosumab, when the variation of these markers was used as a central study endpoint.[24] Noteworthy, its role in the clinical setting is still exploratory and, therefore, not recommended outside clinical trials.[25]



**Table 1** – Types of bone remodeling markers.

<p><b>Bone formation markers</b></p>	<p>Bone specific alkaline phosphatase (BALP) Osteocalcin (OC) Propeptides of type I procollagen (P1NP and P1CP)</p>
<p><b>Bone resorption markers</b></p>	<p>Amino-terminal crosslinked telopeptide of collagen type I (NTX) Calcium Carboxy-terminal crosslinked telopeptide of type I collagen (ICTP or CTX-MMP) Carboxy-terminal crosslinked telopeptide of collagen type I (CTX) Deoxypyridoline (DPD) Hydroxyproline (Hyp), Hydroxylysine (Hyl) Non collagenous bone matrix proteins: bone sialoprotein (BSP) and osteopontin (OP) Osteoclast-derived enzymes: tartarate resistant acid phosphatase 5b (TRAP5b) and cathepsin k and L Pyridinoline (PYD) Parathyroid hormone related peptide (PTH-rp) Vitamin D</p>
<p><b>Regulators of bone turnover</b></p>	<p>Dickkopf-1 (DKK-1) Osteoprotegerin (OPG) Receptor activator of NFkB ligand (RANKL) Sclerostin</p>

d) Bone modifying agents

After the establishment of metastatic bone lesions two strategies are commonly implemented to control the impact of tumor cells in the bone: the use of anti-tumor treatments and/or bone directed treatments (osteoclast inhibitors). Anti-tumor treatments include targeted therapy (e.g., hormone therapy or anti-HER therapies), chemotherapy or radiation therapy. On the other hand, bone directed therapy is composed of bone modifying agents, namely bisphosphonates (BP) and denosumab ([table 2](#)).

**Table 2** – Bone modifying agents. NA – not applicable.

Pharmacologic Category		Agents	Relative potency
Bisphosphonates	Non nitrogenous-bisphosphonates	Etidronate	1
		Clodronate	10
		Tiludronate	10
	Nitrogenous-bisphosphonates	Pamidronate	100
		Alendronate	500
		Ibandronate	1000
		Risedronate	2000
	Zoledronate	10000	
Monoclonal antibodies		Denosumab	NA

BP are a group of pyrophosphate analogous with an intense tropism to the bone where, after incorporation into the matrix and subsequent absorption by osteoclasts (during the process of bone resorption), they act as potent osteoclast inhibitors ([figure 1](#)). There are two classes of BP, non-nitrogenous and nitrogenous. While the first act by substituting the terminal pyrophosphate moiety of adenosine triphosphate (ATP), resulting in a nonfunctional ATP molecule that competes for functional ATP in the osteoclast energy metabolism, the latter inhibit the osteoclast enzyme farnesyl diphosphate synthase (FDS). FDS is a critical isoprenyl diphosphate synthase or prenyl transferase in the mevalonate pathway that is fundamental for the synthesis of sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA and ubiquinone.[26] The inhibition of isoprenoid – farnesyl diphosphate and geranylgeranyl diphosphate synthesis by the inhibition of FDS prevents the post-translational prenylation of small GTPase signaling proteins such as Rho, Rac, Rab, Rap and Ras leading to disruption of osteoclast function and induction of apoptosis.[26]

On the other hand, the fully humanized monoclonal antibody denosumab acts by sequestering RANKL ([figure 1](#)). This process limits the natural action of RANKL on the osteoclast precursor surface receptor RANK, preventing osteoclast differentiation and ultimately bone resorption.

BP (in specific zoledronic acid, pamidronate and ibandronate), but also denosumab, are widely used in clinical practice as tools to reduce the morbidity of bone metastases. Despite subtle differences in relative efficacy of this agents[27, 28], major international guidelines support the use of either zoledronic acid (ZA) or denosumab to prevent and delay SREs.[25, 29]

e) Anti-tumoral action of bisphosphonates

BP are critical tools to prevent and delay SREs; however, pre-clinical and clinical studies are increasingly supporting a direct and indirect anti-tumoral action of these agents. Some of the anti-tumor mechanisms of action of BP include a direct cytotoxic action, but also other indirect mechanisms, such as anti-angiogenesis and immune system activation.[30] While the preclinical identification of these effects seems clear, the clinical translation of these findings is less clear, and, so far, seems to be restricted to hormone-sensitive patients under an estrogen depleted environment (either due to post-menopausal status or due to ovarian suppression). Furthermore, the effect is more clear in nitrogenous-bisphosphonates, in specific for ZA.[31] Of note, more than 80% of the women with BC are post-menopausal, which opens a wide window for eventual intervention/applicability.[3] Nevertheless, at present, available clinical practice guidelines do not recommend the use of BP as a therapeutic tool in the adjuvant treatment of breast cancer.[25, 29, 32]

In the last decade, a group of trials documented relevant pieces of information regarding the anti-cancer activity of BPs in the adjuvant treatment of breast cancer. The ABCSG-12 was a phase III trial that tested the adjuvant use of ZA (4 mg every 6 months for 3 years) in 1803 pre-menopausal women under ovarian suppression and tamoxifen or anastrozole.[33, 34] After a median follow-up of 8 years, those patients receiving ZA had a 33% decrease in the risk of recurrence (Hazard-ratio [HR] for disease-free survival [DFS] 0.77, 95% Confidence Interval [CI] 0.60 – 0.99;  $p=0.042$ ) and a trend towards a decrease in the risk of death (HR for overall survival [OS] 0.66, 95% CI 0.43-1.02;  $p=0.064$ ). Noteworthy, this effect was clearer in patients older than 40 years of age (HR for DFS 0.70, 95% CI 0.51 – 0.96 vs. 0.95, 95% CI 0.62 – 1.46 in women with less than 40 years of age; HR for OS 0.56, 95% CI 0.31 – 0.96 vs. 0.94, 95% CI 0.45 – 1.98 in women with less than 40 years of age).

The ZO-FAST and Z-FAST trials also tested the effect of BPs in terms of cancer related outcomes. Despite their design to test as central outcomes bone health related outcomes (as variation in bone mineral density and the occurrence of bone fracture), they also tested for cancer related outcomes as secondary outcomes (disease recurrence). In ZO-FAST (1065 women) and Z-FAST (602 women) postmenopausal women receiving adjuvant letrozole were randomized for upfront zoledronic acid (4 mg every 6 months) or zoledronic acid only after bone fracture or significant decline in bone mineral density.[35, 36] Despite the concordant results between trials in terms of improved bone mineral density for patients receiving upfront ZA, only those patients enrolled in the ZO-FAST derived a benefit in terms of disease recurrence (HR for DFS 0.66, 95% CI 0.44 – 0.97).[35, 36]

The AZURE trial was designed to have as a primary outcome the clinical impact of adjuvant ZA in terms of breast cancer control related outcomes.[37] In this phase III study, 3360 pre or post-menopausal women were randomized to standard adjuvant therapy with or without ZA (4 mg every 3-4 weeks for the first 6 months and then every 3-6 months for a total of five years). Primary outcome was disease-free survival. At a median follow-up of around 5 years, no significant difference in terms of DFS or OS was documented in the overall cohort. However, in a pre-planned subgroup analysis, late post-menopausal women (those > than 5 years post-menopause) derived a

significant benefit: 25% reduction in the risk of invasive disease or death (adjusted-HR 0.75, 95% CI 0.59-0.96) and 26% reduction in the risk of death (adjusted-HR 0.74, 95% CI 0.55-0.98).

As referred before, most of the available clinical evidence of the anti-tumor action of BPs is derived from trials that used zoledronic acid; however, other BPs were also tested. The NSABP B-34 tested the role of clodronate both as a strategy to test the action of this BP in terms of bone health related outcomes (as bone mineral density), but also in terms of disease recurrence.[38] In this study, 3323 women (pre and post-menopausal) were randomized to receive standard adjuvant therapy with or without oral clodronate (1600 mg/d for a total of 2 years). After a median follow-up of around 7 years, only those 50 years of age or older had a benefit in terms of DFS (bone and extra-bone). However, no clear improvement in terms of OS was documented for both age groups. Pamidronate was also tested as a strategy to reduce the risk of disease recurrence in breast cancer.[39, 40] In a small trial Kokufu and colleagues recruited 90 patients with node positive ( $\geq 4$  positive nodes) early breast cancer treated with curative intention to receive pamidronate (n=33; 45 mg pamidronate 4 times every 2 weeks) or standard follow-up (n=57) based on personal preference.[39] After a median follow-up of five years, a reduction in the incidence of bone metastases in the group receiving pamidronate was found (12.1% vs. 40.4% in the control group; p=0.005). A non-significant numerical difference favoring pamidronate was also found in terms of distant metastases (36.4 vs. 56.1%; p=0.071) and non-osseous metastases (33.3 vs. 52.6%, p=0.077). Nevertheless, no difference regarding overall survival or disease-free survival was found. Another study testing a similar hypothesis recruited 429 perimenopausal stage I-III breast cancer patients receiving therapy with curative intent.[40] From these, 258 received pamidronate (15mg iv every 4 weeks and oral pamidronate 100mg daily during adjuvant chemotherapy) while 171 didn't. Even though patients receiving pamidronate had a lower incidence of bone metastases (2.3% vs. 8.7% in the control group; time of endpoint evaluation not specified) no difference was found in terms of disease-free survival or overall survival.

Finally, a recent meta-analysis analyzed individual patient data from several randomized trials comparing the use of BP to no BP (either placebo-controlled or open control) in terms of the risk of recurrence and death.[31] In this study (preliminary data after analysis of 75% of 23,573 patients of interest), a statistically significant reduction of 17% in the risk of death and distant recurrence was found for post-menopausal women treated with BP only. Furthermore, a reduction of 35% in the risk of bone-specific recurrence was shown, an effect also restricted to the group of post-menopausal women. As a matter of fact, pre-menopausal women seem not to benefit from BPs in terms of the risk of disease recurrence and death.

When taken together, an increasing body of clinical evidence is pointing towards an effect of BPs, and more specifically ZA, as a tool to reduce the risk of recurrence and death when used in the adjuvant treatment of post-menopausal women (5 years after menopause establishment or older than 40 under ovarian suppression) with hormone receptor positive breast cancer. We should note however that no current international clinical guideline is recommending the use of BPs in this setting.[25, 29, 32]

f) Framing the research question

Metastatic breast cancer survival has improved over time, mostly after the introduction of most effective therapies, as taxanes, aromatase inhibitors and anti-HER agents. Other relevant gains were achieved with more rational use of already available drugs tailored to certain risk specific groups. BPs, and most specifically ZA, seem to have antitumor properties which depend on the systemic hormonal environment. In fact, only late post-menopausal women (or premenopausal under ovarian suppression therapy) seem to benefit from zoledronic acid in terms of disease recurrence and survival. However, the available data are only derived from patients in the early breast cancer setting, and there is no consistent evidence that BPs improve survival in metastatic breast cancer patients nor was the effect of the hormonal environment tested in this circumstance.

In this study we aim to test if the hormonal environment significantly impacts patient outcomes, as bone disease control, by means of the measurement of urinary NTX levels, serum levels of tumor markers, time to first-line chemotherapy failure and overall survival in patients with metastatic breast cancer affecting the bone and being treated with zoledronic acid and chemotherapy.

## Study Objectives

To test whether the hormonal environment (pre vs. late post-menopausal) significantly impacts bone disease control, as measured by urinary levels of NTX, serum levels of tumor markers, time to first-line of chemotherapy failure and overall survival in patients with breast cancer affecting the bone and being treated with zoledronic acid and chemotherapy.

- a) **Primary objective:** To determine the impact of the hormonal environment (pre vs. post-menopausal) on urinary levels of NTX at 3 months in patients receiving zoledronic acid and chemotherapy;

Hypothesis: Post-menopausal patients will present a more pronounced decline in the urinary NTX levels at 3 months when compared to pre-menopausal women.

- b) **Secondary objectives:** To determine the impact of the hormonal environment (pre vs. post-menopausal) in patients receiving zoledronic acid and chemotherapy on the following parameters:

- Urinary NTX levels at 6 and 9 months;

Hypothesis: Post-menopausal patients will present an increased decline in the urinary levels of NTX at 6 and 9 months when compared to pre-menopausal women.

- Serum CA15.3 and CEA levels (tumor markers);

Hypothesis: Post-menopausal patients will present an increased decline in the serum levels of CA15.3 and CEA when compared to pre-menopausal women.

- Time to first line chemotherapy failure;

Hypothesis: Post-menopausal patients will present a longer time to first line chemotherapy failure when compared to pre-menopausal women.

- Overall survival;

Hypothesis: Post-menopausal patients will present a longer overall survival when compared to pre-menopausal women.

## Methods

### a) Study population

Patients with the diagnosis of metastatic breast cancer involving the bone (*de novo* or post-disease recurrence), receiving zoledronic acid and chemotherapy, diagnosed and treated at the Department of Medical Oncology from Hospital de Santa Maria – CHLN from 2000 and 2009 and with quantifications of urinary NTX available.

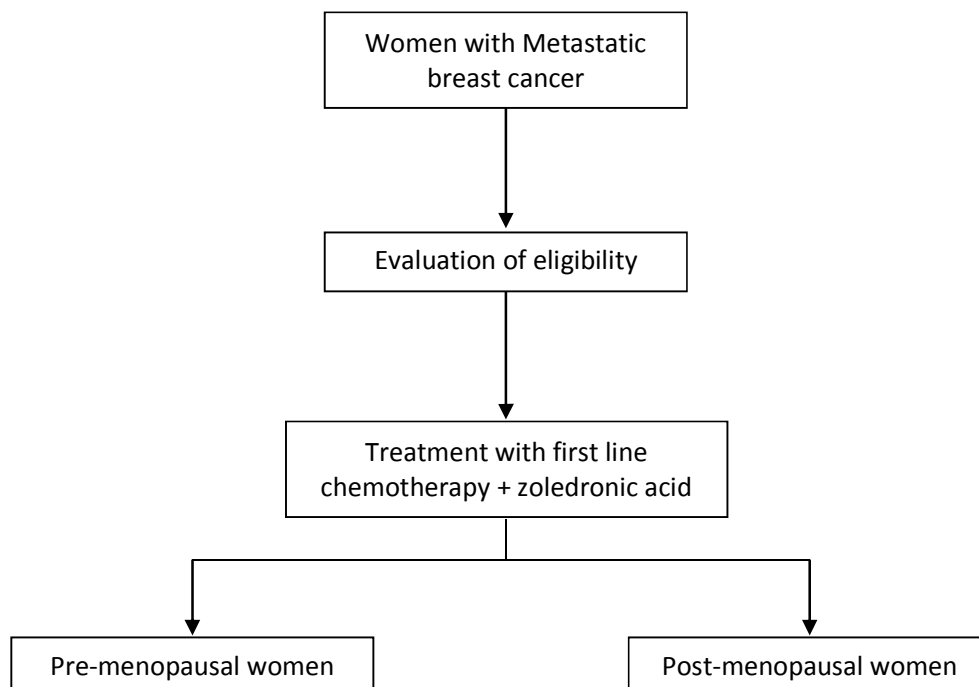
Evidence of breast cancer was obtained from the histopathology report, while evidence of bone involvement was obtained from imaging.

We will exclude patients with previous diagnosis of other oncologic disease, except contralateral breast cancer that is disease-free for more than 5 years, non-melanoma skin cancer or pre-invasive cervix cancer. We will also exclude patients with a follow-up of less than 3 months, with previous treatment for metastatic disease, previous treatment with BPs and pregnant women or women with psychiatric conditions.

### b) Study design and diagram

This study is a single center retrospective cohort study.

**Figure 2** – Study diagram.



### **c) Data collected and study procedures**

To collect the clinical variables of interest we directly extracted clinical information from medical records, both physical and electronic.

The collected variables were the following:

- Demographics: age at diagnosis, race and menopausal status;
- Disease and tumor characterization: date of diagnosis, WHO performance status, AJCC staging (T, N, number of involved lymph nodes and M), hormone receptor status, HER2 status, grade, histology, previous (neo)adjuvant chemotherapy regimen, (neo)adjuvant trastuzumab, (neo)adjuvant chemotherapy, date of disease relapse, metastatic disease characterization (date of metastatic diagnosis, number and localization of lesions);
- Biomarkers and imaging: NTX, CEA, CA15.3, CT (thorax/abdomen/pelvis), scintigraphy, simple radiography of the bone.
- Other endpoints: date of disease progression (visceral and bone), date of introduction of first and second line chemotherapy, date of SRE and date of death.

### **d) Definition of outcomes and other definitions**

Biomarkers response: variation of NTX and tumor markers (CEA and CA15.3) from baseline (before introduction of BPs) and time points of interest.

Time to first line chemotherapy regimen failure: time from introduction of BPs plus first line chemotherapy to the introduction of second line chemotherapy regimen.

Menopausal status: Menopausal status will be assigned by age group. Women will be assigned as pre-menopausal if less than 45 years of age; late post-menopausal women will be those with more than 60 years of age.

### **e) Evaluation of urinary NTX**

We used Osteomark NTx Urine ELISA (Inverness) to determine urinary NTX. The detection limit of the NTX assay is 20 nM BCE (bone collagen equivalents units – assay value, does not include creatinine excretion). The urinary levels of NTX in bone collagen equivalent units are expressed as the ratio of NTX to urine creatinine excretion. Urinary NTX was defined as low (NTX < 50 nmol BCE/mmol creatinine) or high (NTX ≥ 50 nmol BCE /mmol creatinine). The cutoff values for NTX were chosen to reflect the approximated levels of normal. However, the normal range for urinary NTX varies according to several factors, as age, gender and endocrine environment. While the upper level of normal (ULN) in young healthy adults is approximately 50 nmol/mmol creatinine, after menopause, the ULN level is approximately 100 nmol BCE/mmol creatinine.[22]



## f) Statistical considerations

### Descriptive statistics

Tabulation of baseline demographic, clinical, pathological and treatment characteristics were performed according to menopausal status using Pearson's  $\chi^2$ , Fisher's exact test, t-test or Wilcoxon rank-sum test when appropriate. Graphical representations of variation of NTX and tumor markers through time were performed.

### Analysis of outcomes

- 1) NTX and tumor markers (CEA and CA15.3) at baseline, 3, 6 and 9 months in the full cohort and by menopausal status
  - a. Wilcoxon rank-sum test was performed to test differences between menopausal groups at specific time points: baseline, 3, 6 and 9 months.
  - b. Linear mixed effects models for longitudinal data were used to model mean variation of NTX and tumor markers over time given the correlated nature of the outcomes (repeated measures). We used a model with random intercepts and slopes based on the hypothesis that individuals vary not only in their baseline level of response, but also in terms of their changes in the mean response over time. We further allowed the mean of intercepts and slopes to depend on the fixed effect menopausal status category (independent variable of primary interest). While the fixed effect parameter menopausal status informs us on how the sample means differ between pre and post-menopausal women, the random effect parameters represent the broad variability among subjects. We restricted analysis to 3 time intervals, from baseline to 3, 3 to 6 and 6 to 9 months.
  - c. NTX and tumor markers were analyzed in absolute and log transformed values as a strategy to focus on relative changes of biomarker at different time points and obtain a normal distribution of biomarker levels. Main results will be presented using the log transformed version of the biomarkers level.
  
- 2) Time to first line chemotherapy failure and overall survival
  - a. The time to first line chemotherapy failure was analyzed using survival analysis techniques. The events of interest were failure of first line chemotherapy and death. We used the Kaplan-Meier estimator to estimate the survival function and Kaplan-Meier plot according to menopausal status.
  - b. Log-rank test was used to test the univariate difference between survival curves of the group of pre and post-menopausal women.

- c. Cox proportional hazards model was used to test the multivariate difference between survival curves according to menopausal status. Outline of the model:
- Outcome/dependent variable: failure of first line chemotherapy and death;
  - Independent variable: menopausal status;
  - Covariates: estrogen receptor status, HER2 receptor status, histologic grade, histology and concomitant visceral involvement. Due to limitations of sample size/number of events only bivariate models were built.

#### Sample size calculation

This study is of exploratory in nature, so no prior knowledge is available in terms of primary outcome to perform a sample size calculation. Furthermore, we are constrained by a limited set of NTX evaluations. No further NTX evaluations were performed due to funding constrains. We will use the present results as the foundation for future assessment of feasibility of a larger study testing the same hypothesis.

#### Sources of confounding and strategies to avoid confounding

The following variables were selected as potential sources of confounding: estrogen receptor status, HER2 receptor status, histologic grade, histology and concomitant visceral involvement. Multivariate analysis used as a strategy to overcome confounding.

#### **g) Ethical considerations**

This study was approved by the ethics committee of the Lisbon Academic Medical Center. A waiver of the use of informed consent was granted based on the fact that urine and blood samples had already been collected for the purpose of the evaluation of NTX and tumor markers in the context of related studies and clinical practice, respectively. Moreover, the investigation *per se* included no more than minimal risk in the form of loss of confidentiality. To overcome this liability, several measures were taken. Some of these measures included the de-identification of patients and subsequent re-identification using a study specific numerical study code (p.e. 001); variables were also coded numerically (e.g., yes/no variables will be coded as 1/0, respectively) and a separated code of variables created. Moreover, electronic files were always kept inside CHLN network or in a very limited set of other encrypted computers that had continuous anti-virus protection. Furthermore, electronic files were always stored using an alphanumeric password protection (either the specific file or after placing the file inside a password protected .rar library).

## Results

### a) Cohort characteristics

A total of 40 women were included in this study, 8 (20.0%) of which pre-menopausal and 32 (80.0%) post-menopausal. Relevant patient's clinical and pathologic characteristics are summarized in [table 3](#). As a matter of fact, the groups were reasonably balanced regarding most of the selected variables. Noteworthy, median ages are compatible with corresponding menopausal status. Furthermore, most tumors were estrogen receptor positive, as expected from a cohort of patients with bone disease as first site of relapse, and had a fairly good performance status, which enabled the use of chemotherapy as a treatment option.

**Table 3** – Cohort baseline characteristics according to menopausal status.

	Pre-menopausal women	Late post-menopausal women	P-value
Number, %	8 (20.0)	32 (80.0)	-
Age at diagnosis, median (IQR)	39.8 (35.9 – 44.2)	65.6 (60.1 – 67.8)	-
Age at diagnosis of metastatic disease, median (IQR)	44.8 (40.6 – 46.5)	67.2 (62.9 – 72.9)	-
Disease-free interval (excludes stage IV patients), median (IQR)	39.7 (22.9 – 85.8)	42.6 (28.0 – 83.4)	0.366
Histology, n (%)			
Invasive Ductal carcinoma	7 (100)	19 (79.2)	0.737
Other	0 (0)	5 (20.8)	
Unknown	1 (12.5)	8 (25.0)	
Estrogen receptor status			
Positive	7 (100)	21 (80.8)	0.559
Negative	0 (0)	5 (19.2)	
Unknown	1 (12.5)	6 (18.8)	
HER2 status			
Positive	1 (20.0)	5 (27.8)	0.608
Negative	4 (80.0)	13 (72.2)	
Unknown	3 (37.5)	14 (43.8)	
Stage IV (at diagnosis), n (%)			
Unknown	1 (12.5)	9 (47.4)	0.345
	2 (25.0)	13 (40.6)	
Visceral involvement at diagnosis of metastatic disease, n (%)			
Unknown	3 (37.5)	4 (15.4)	0.315
	0 (0)	6 (18.8)	
Performance status, median (IQR)	0 (0 - 0)	0 (0 - 1)	0.132
Total follow-up, median (IQR) (months)	36.8 (30.0 – 53.0)	53.3 (24.9 – 62.7)	0.080

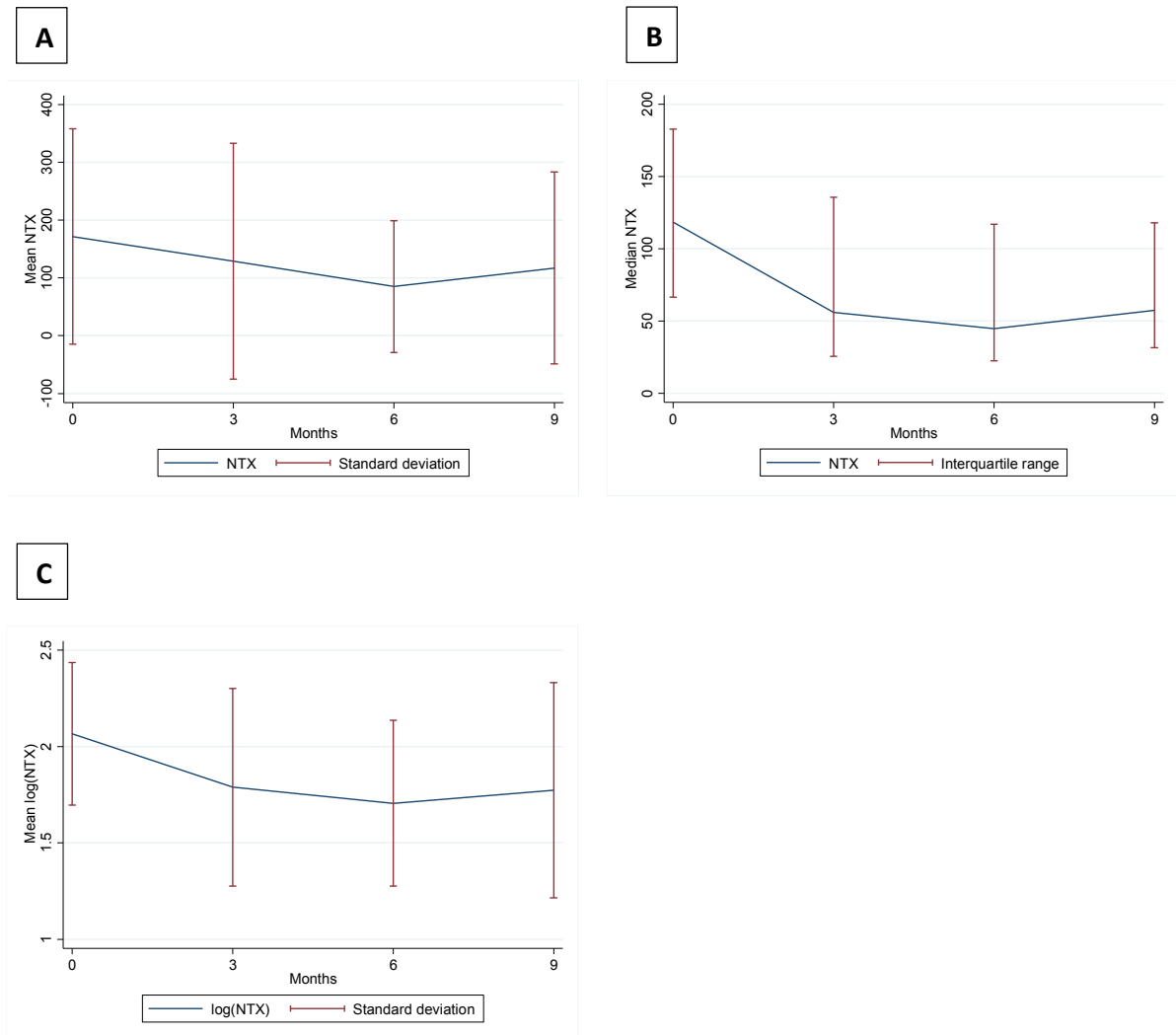
### a) Urinary NTX

The majority of patients had elevated levels of urinary NTX at baseline, which is compatible with active disease in the bone. Moreover, when evaluating the full cohort, after the introduction of zoledronic acid and chemotherapy (at diagnosis - baseline) a decrease of NTX is notable ([table 4](#) and [figure 2](#)), with 41.2% of the patients with elevated NTX at baseline reaching an NTX < 50 nmol/mmol creatinine at month 3. Furthermore, after log-transforming NTX values, a trend for an overall decline in mean  $\log_{10}(\text{NTX})$  in the interval 0 – 3 months ( $p=0.125$ ) and a statistically significant decline of  $\log_{10}(\text{NTX})$  in the interval 3 – 6 months (0.013) was found. Importantly, the proportion of patients that were lost to follow-up in terms of urinary NTX measurements was high: while at month 6 75% of the patients had a valid measurement of urinary NTX, at month 9 only 37.5% had. Of note, no clear differences in terms of age, disease progression or vital status were found between lost to follow-up and retained patients; therefore, we considered this missing information as missing completely at random.

**Table 4** – Urine NTX at several time points from diagnosis. NTX measured in nmol/mmol creatinine.

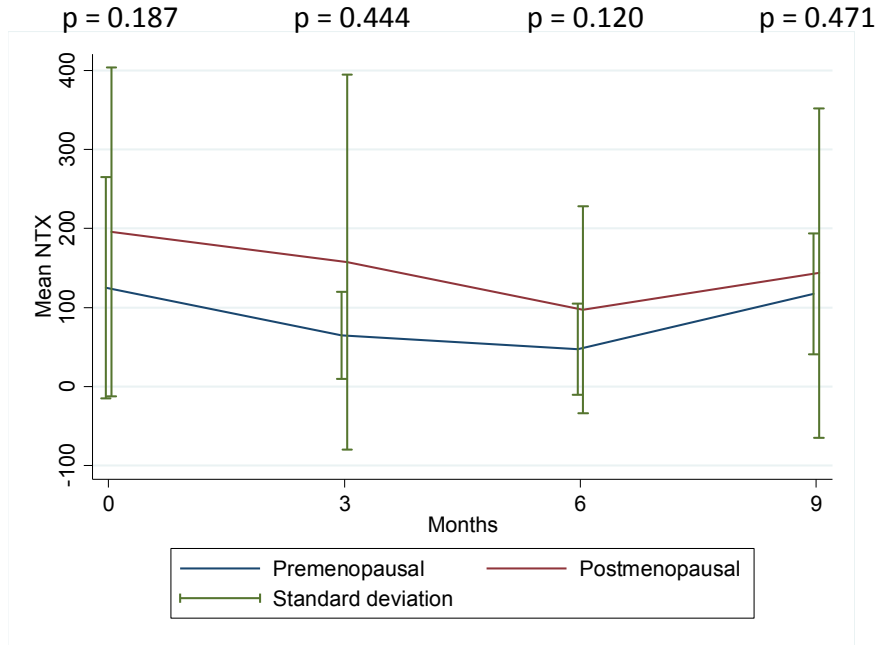
	<b>Baseline</b>	<b>3 months</b>	<b>6 months</b>	<b>9 months</b>
Number, %	40 (100)	39 (97.5)	30 (75.0)	15 (37.5)
Urine NTX, median (IQR)				
All	119.8 (66.5 – 211.1)	58.2 (25.6 – 135.9)	46.23 (20.7 – 123.9)	62.1 (32.8 – 162.9)
Pre-menopausal	70.9 (43.5 – 137.2)	49.7 (32.9 – 80.0)	26.2 (12.3 – 47.2)	100.3 (50.8 – 200.7)
Post-menopausal	121.3 (71.9 – 245.0)	61.0 (25.6 – 161.9)	55.8 (24.8 – 124.8)	57.4 (31.5 – 139.3)
Elevated urine NTX, n (%)				
All	34 (85.0)	23 (57.5)	24 (60)	35 (87.5)
Pre-menopausal	5 (62.5)	4 (50.0)	3 (37.5)	8 (100)
Post-menopausal	29 (90.6)	19 (59.4)	21 (65.6)	27 (84.4)
NTX normalization (if elevated at baseline), n (%)				
All	-	14 (41.2)	14 (41.2)	4 (11.8)
Pre-menopausal	-	4 (80.0)	5 (100)	0 (0)
Post-menopausal	-	10 (34.5)	9 (31.0)	4 (13.8)

**Figure 3** – Mean (A), median (B) and mean  $\log_{10}$  (C) variation of urine NTX at several time points after diagnosis in the full cohort.

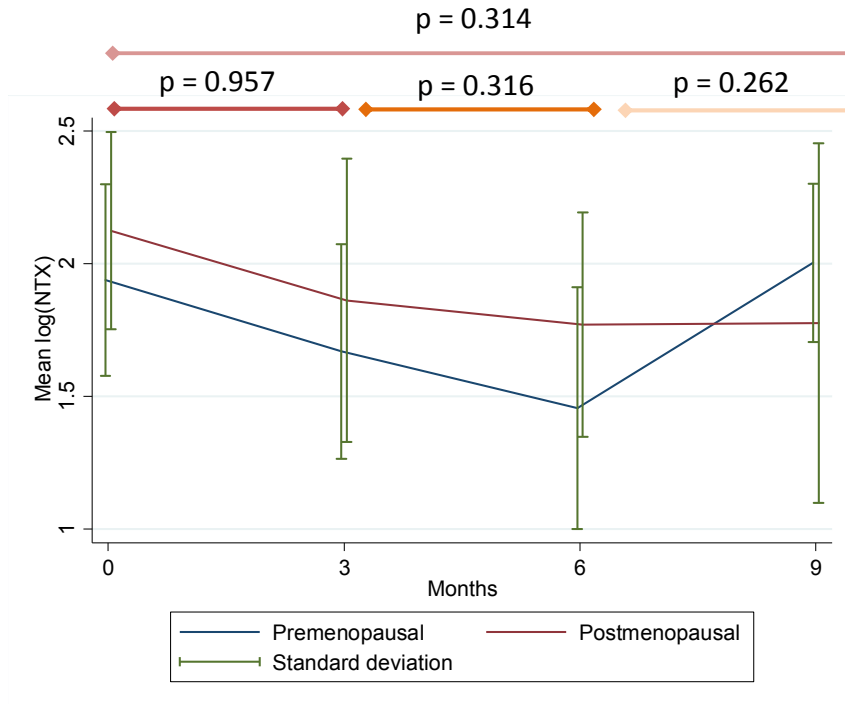


When stratified by menopausal status ([figure 3](#)), post-menopausal women present persistently absolute higher levels of mean NTX, namely at baseline and at 3 months; however, such differences do not reach statistical significance. In fact, such differences are non-significant through all the follow-up period. After log-transforming NTX values ([figure 4](#)), the early response (baseline to month 3) to zoledronic acid and chemotherapy is equal between groups ( $p=0.957$ ). Both groups seem to “escape” zoledronic acid plus chemotherapy control after month 6, as demonstrated by an increase of NTX in both groups. Of note, in absolute and relative terms, urinary NTX levels seems to increase more pronouncedly in the group of premenopausal women in this interval ([figure 3 and 4](#)); however, this difference is non-significant ( $p=0.262$ ). Moreover, when all the follow-up period is taken together, the response profile of urinary NTX levels to zoledronic acid and chemotherapy does not differ between pre and post-menopausal women ( $p=0.314$ ).

**Figure 4** – Mean NTX at several time points after diagnosis according to menopausal status. Test of differences in mean values is shown.



**Figure 5** – Mean response profile of  $\log_{10}(\text{NTX})$  at several time points after introduction of zoledronic acid plus chemotherapy according to menopausal status. Test of interaction time - menopausal group is shown.

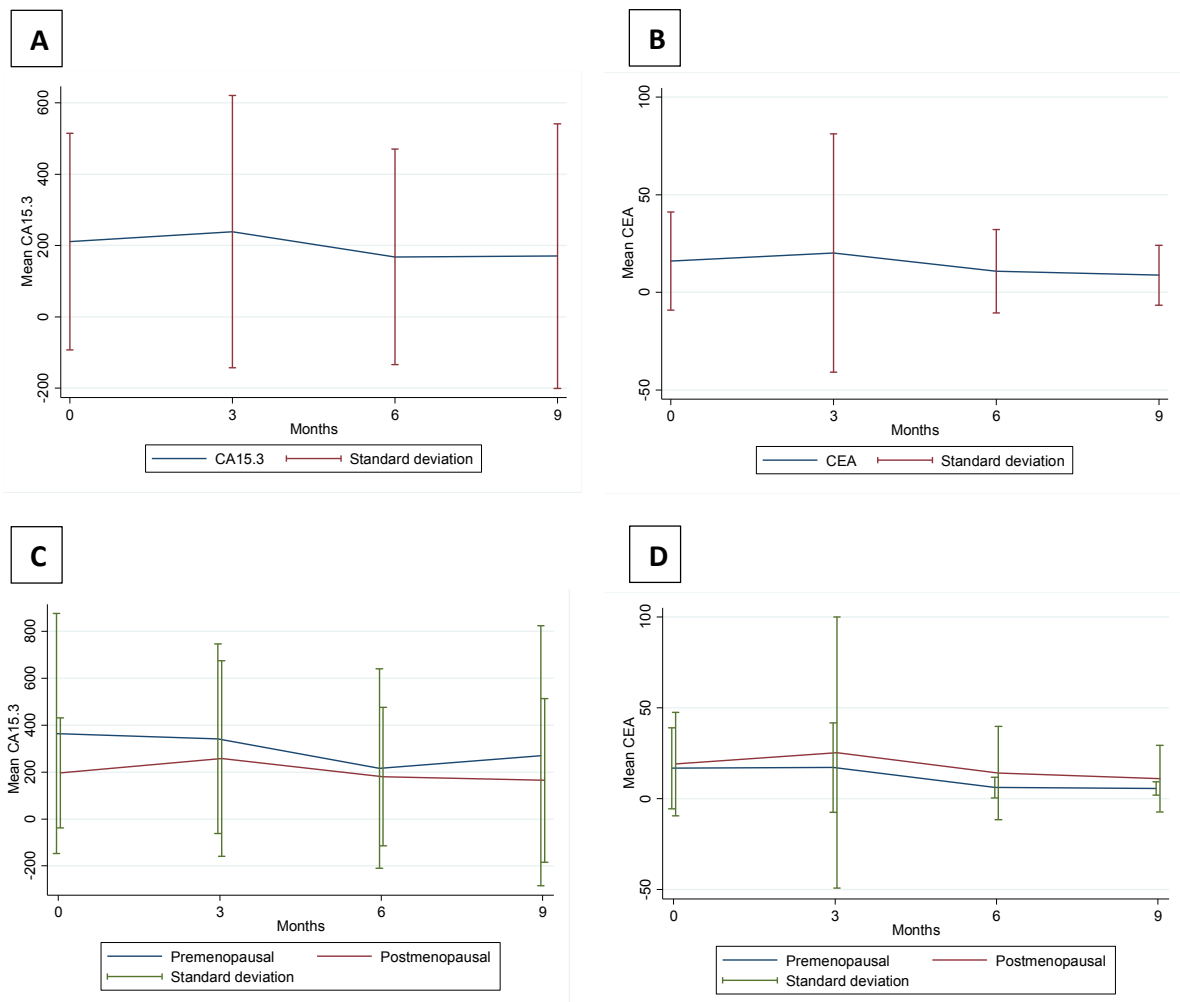


**b) Tumor markers (CA15.3 and CEA)**

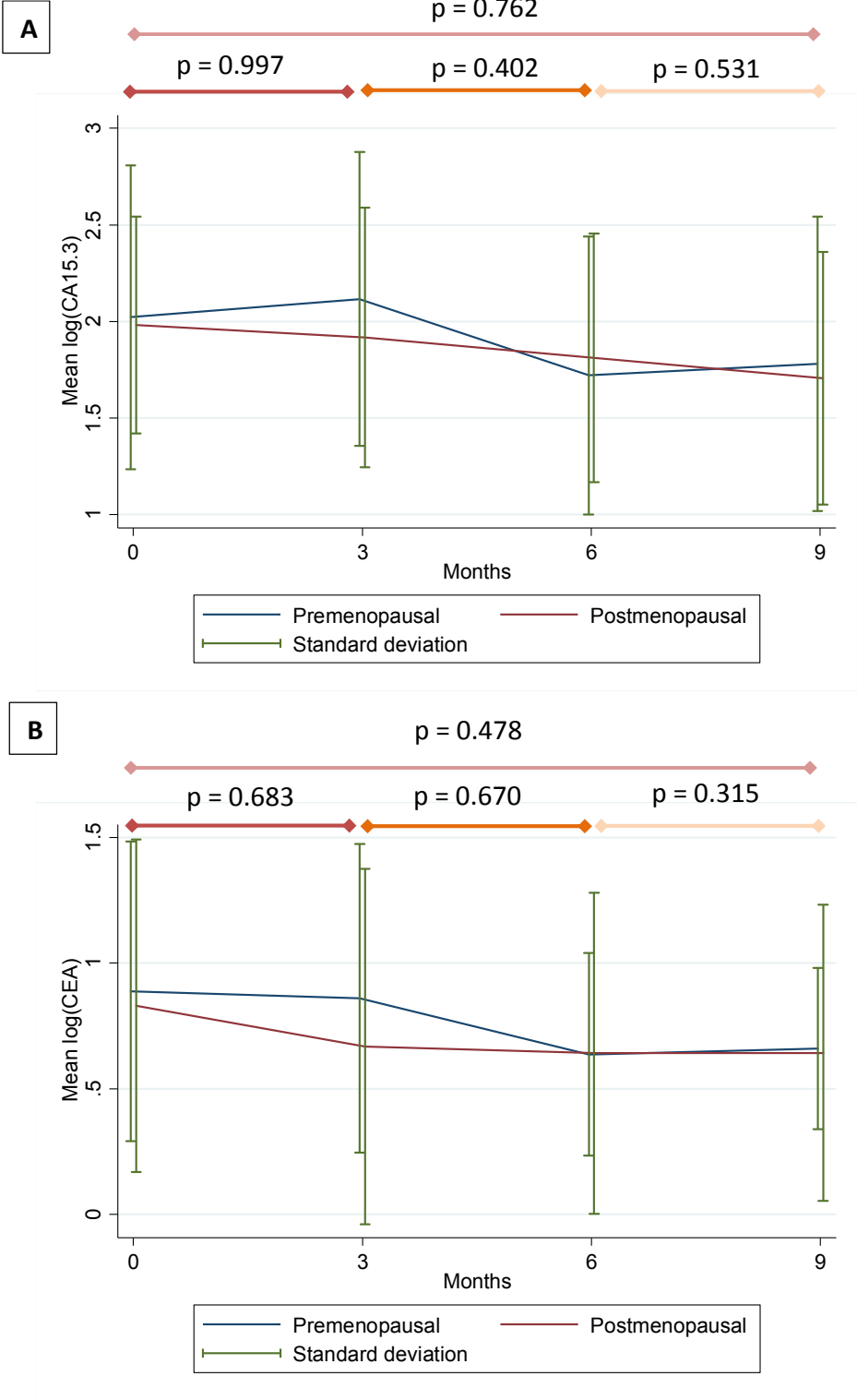
The majority of patients had elevated levels of serum CA15.3 and CEA at baseline, which is compatible with active breast cancer. After the introduction of zoledronic acid and chemotherapy (at diagnosis - baseline) a slight numerical decrease of both markers is identifiable (figure 5 and figure 6); however, only a trend for decreased values through the interval 0 – 9 months is found ( $p = 0.058$  and  $0.057$ , for CA15.3 and CEA respectively).

When stratified by menopausal status (figure 6), no clear differences are found between pre and post-menopausal women in the log transformed values, either at baseline or at the following time points, both for CA15.3 and CEA.

**Figure 6 – Mean response profile of CA15.3 and CEA at several time points after diagnosis in the full cohort (A and B, respectively) and according to menopausal status (C and D, respectively).**



**Figure 7** – Mean response profile of  $\log_{10}(\text{CA15.3})$  (A) and  $\log_{10}(\text{CEA})$  (B) at several time points after introduction of zoledronic acid plus chemotherapy according to menopausal status. Test of interaction time - menopausal group is shown.

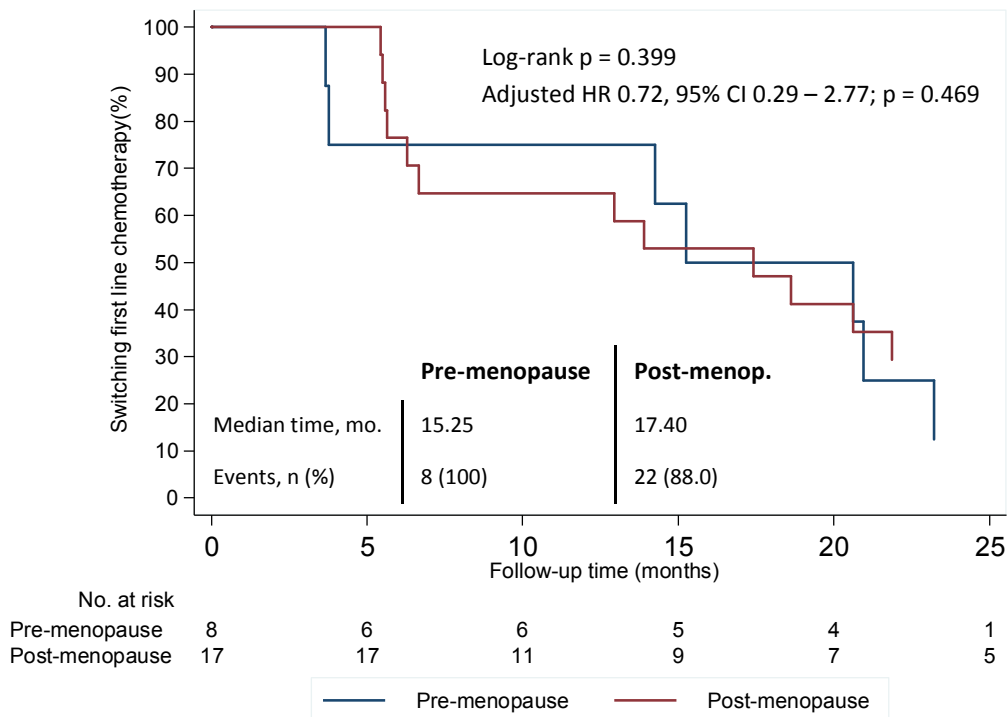




**c) Time to first-line chemotherapy failure**

When evaluating time to first-line chemotherapy failure, the full cohort presented a median follow-up time of approximately 17 months (IQR 6.3 – 23.2). A total of 30 events (90.9%) were documented, of which 8 (100%) in the premenopausal group and 22 (88.0%) in the post-menopausal group. The median time to chemotherapy failure was of 15.3 months for pre-menopausal women and 17.4 for post-menopausal women. In [figure 7](#) a representation of the survival function according to menopausal status is depicted. When evaluating the univariate association of menopausal status and other clinical and pathological variables with change of first line chemotherapy, only the presence of visceral involvement at diagnosis of metastatic disease was significantly associated with time to chemotherapy switch ([table 5](#)). Furthermore, when testing the role of menopausal status on time to first line chemotherapy failure controlling for visceral involvement a persistent non-significant association was noted ([figure 7](#)). Despite the intention to control for other variables, due to the lack of available observations and the fact that only concomitant visceral involvement was significantly associated with first-line chemotherapy failure, only this covariate was included in the model.

**Figure 8** – Time to first-line chemotherapy failure according to menopausal status. Adjusted HR controlling for visceral involvement is shown. HR – Hazard ratio; CI – Confidence interval; mo – months; n – number.



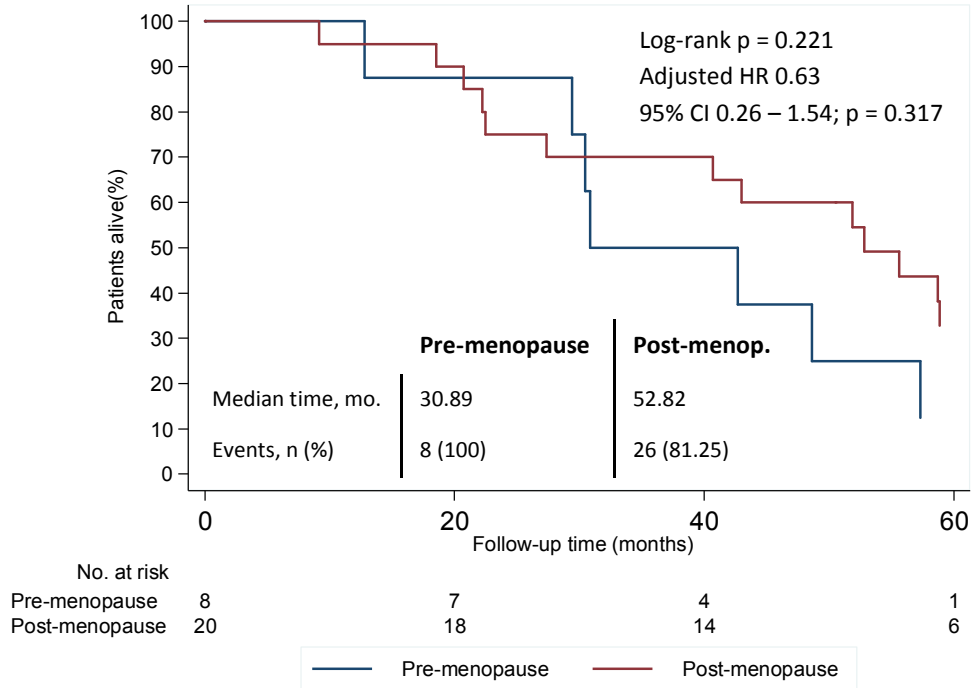
**Table 5** – Univariate association of baseline characteristics with time to first line chemotherapy failure.

	<b>P-value</b>
Menopausal status	0.399
Age at diagnosis of metastatic disease	0.673
Time from diagnosis to metastatic disease	0.272
Histology	0.882
Estrogen receptor status	0.867
HER2 status	0.915
Stage IV at diagnosis	0.418
Visceral involvement at diagnosis of metastatic disease	0.015
Performance status	0.240

**d) Overall survival**

When evaluating overall survival, the full cohort presented a median follow-up time of approximately 49.6 months (IQR 28.4 – 60.4). A total of 34 events (85.0%) were documented, of which 8 (100%) in the premenopausal group and 26 (81.3%) in the post-menopausal group. The median time to death was of 30.9 months for pre-menopausal women and 52.8 for post-menopausal women. In [figure 8](#) a representation of the survival function according to menopausal status is depicted. When evaluating the univariate association of menopausal status and other clinical and pathological variables with change of first line chemotherapy, no significant associations were found ([table 6](#)). However, HER status reached significance at 90% level of confidence, so we decided to adjust menopausal status to this variable as well in a bivariate model. In this bivariate model, no significant association was found as well ([figure 8](#)). As for the evaluation of time to first-line chemotherapy failure, despite the intention to control for other variables, due to the lack of available observations no other key variables were tested in the model.

**Figure 9** – Overall survival according to menopausal status. Adjusted HR controlling for HER2 status is shown. HR – Hazard ratio; CI – Confidence interval; mo – months; n – number.



**Table 6** – Univariate association of baseline characteristics with overall survival.

	P-value
Menopausal status	0.221
Age at diagnosis of metastatic disease	0.999
Time from diagnosis to metastatic disease	0.294
Histology	0.733
Estrogen receptor status	0.618
HER2 status	0.094
Stage IV at diagnosis	0.662
Visceral involvement at diagnosis of metastatic disease	0.186
Performance status	0.377

## Discussion

Pre-clinical[30] and more recently clinical[31] evidence is accumulating in favor of an anti-cancer activity of BPs, more specifically in the adjuvant treatment of post-menopausal women with breast cancer. In the present study we intended to explore the differential anti-cancer effect of BPs according to the hormonal environment in metastatic patients receiving ZA and chemotherapy. In this subgroup of patients, no evidence of a differential anti-cancer activity of ZA between pre and post-menopausal women was found when evaluating anti-cancer activity in terms of variation of NTX (as surrogate for bone disease activity), variation of CA15.3 and CEA (as surrogate for overall disease activity), time to first line chemotherapy failure (as surrogate for overall disease progression) and overall survival.

BPs were developed and introduced in clinical practice as a tool to decrease the morbidity of bone metastases given their properties as bone remodeling modulators, in specific as osteoclast inhibitors. In this context, the primary endpoints of pivot trials were essentially centered on the rate of SREs, time to first and subsequent SRE, improvement in pain control and general quality of life. As a matter of fact, compared to placebo BPs reduced the SRE risk by 15% (RR 0.85, 95% CI 0.77-0.94), with a median 28% reduction (range, 14–48%) of SREs.[41] However, no impact on overall survival was found.[41]

Other studies tested the efficacy of BPs (zoledronic acid, pamidronate and clodronate) in the metastatic setting with a focus on their anti-cancer activity with generally inconclusive findings. However, based on the data derived from adjuvant studies, ours was the first study to test the impact of menopausal status in the anticancer activity of BPs.

Two studies tested the anti-cancer activity of ZA in the metastatic setting. The MACS1295 study (NCT01129336), previously referred as Z-ACT, tested the anti-tumoral action of ZA in patients with HER2-negative metastatic breast cancer receiving standard first or second line therapy irrespective of the menopausal status. In this study, patients were allocated to two different arms according to the presence of bone metastases. Patients with bone metastases received standard therapy and ZA (4 mg IV monthly during months 1-18), while patients without bone metastases were randomized to receive standard therapy with or without ZA (same schedule). Primary outcome was the number of participants with progression free survival at month 18, and secondary outcomes included the percentage of patients with circulating tumor cells count above 5 cells per 7.5 mL of peripheral blood, at several time points. Unfortunately, from the 150 planned patients without bone metastases to be recruited, only 15 were included, making the interpretation of results not viable (results available only at [clinicaltrials.gov](http://clinicaltrials.gov)). Despite not stratifying according to the menopausal status, this study, if properly completed, could have contributed to the improved understanding of the clinical anti-tumor action of the zoledronic acid in the metastatic setting. Furthermore, the BISMARCK study (NCT01129336) indirectly tested the anti-tumor action of zoledronic acid.[42] In this phase III non-inferiority study, 289 patients with bone metastases from breast cancer were

randomized to receive zoledronic acid, either as a standard fixed schedule (4mg IV over 15 minutes once every 3-4 weeks for 24 months) or under a set of rules dependent on NTX variation. Despite the accrual limitations that led to premature study closure, the NTX-directed schedule group had a higher proportion of patients presenting with an SRE, thus failing to demonstrate non-inferiority of this approach. Of note, overall survival was selected as a secondary outcome in this study. Given that patients in the standard schedule arm had more than the double of ZA administrations, this study can help us gain some insight on the anti-tumor action of zoledronic acid in terms of overall survival. No stratification based on menopausal status is planned so far.

Clodronate and pamidronate (in its oral formulation) were also tested as anti-neoplastic agents, in specific, about their ability to prevent the development of bone metastases in patients with breast cancer without clinical evidence of bone involvement and irrespective of menopausal status.[43–45] Despite the fact that all studies were considerably underpowered to show a difference between groups, when compared to placebo, no difference was found in the incidence of new bone metastases. A recent meta-analysis summarized these findings, and found no overall effect of these agents on the incidence of bone metastases in patients with metastatic breast cancer without clinical evidence of bone metastases (HR 0.99, 95% CI 0.67 – 1.47).[41]

So far, while the studies performed in the early setting seem to show a protective action of BPs in terms of disease recurrence and survival in patients that are estrogen deprived (post-menopausal or under ovarian suppression), no such evidence is available in the metastatic setting. As a matter of fact, the role of menopausal status is still unexplored in the metastatic setting; however, fundamental differences between the early and advanced disease settings might limit the anti-tumoral action of BPs in latter stages. First, the burden of disease of metastatic disease is larger, than e.g., residual disease after treatment with curative intent. The intensity of BPs anti-tumor effect might only be clinically relevant for smaller volumes of disease, hence limiting the action in advanced cancer. Second, the central action of BPs might be mostly relevant before metastatic disease fully develops. In this regard, BPs seem to interfere with the vascularization of the premetastatic niche at several tentative distant sites of metastatic development.[30] Bone medulla is also frequently considered a sanctuary for disseminated tumor cells.[46] Similarly to the action at premetastatic niches, BPs might change bone medulla in such a way that hampers DTC survival, either through the anti-angiogenic action or through the action on bone cells.[30] Third, post-menopausal women present a more active bone metabolism, which some hypothesized that might contribute to the establishment of bone metastasis.[18] BPs block bone resorption and unbalance bone metabolism towards bone creation. This phenomena might impair the fixation of tumor cells, a mechanism not transposable to the metastatic setting. On the other hand, certain mechanisms may play similar roles both in the early and metastatic settings. BPs disrupt the vicious cycle between cancer cells and bone remodeling activation, which decreases the availability of growth factors entrapped in the bone matrix. Likewise, the immune activation, namely through  $\gamma\delta$  T cells, would reasonably act on both settings of disease evolution.

The present study presents important limitations which is compatible with its exploratory nature and budget constraints. The first main limitation refers to the small sample size. Despite the preplanned design as an exploratory study, the group of premenopausal women was considerably small, which might not be representative of a conventional cohort of pre-menopausal patients. Second, a relevant lost to follow-up was found at 9 months. Regardless of the intention to perform the analysis of primary outcomes at 3 months, secondary outcomes interpretation is limited due to this fact. Indeed, the late acceleration of urinary NTX in the pre-menopausal patients cannot be properly valued in this context. Third, time to first line chemotherapy failure after the diagnosis of metastatic disease was considerably long. In fact, several of these patients stopped first line chemotherapy after a good clinical response and had a switch to hormone therapy before switching to second line chemotherapy, as common practice in patients with hormone receptor positive disease. The present analysis does not account for the type of hormone therapy received in the transition to second line chemotherapy and this fact might have been a source of heterogeneity. As a matter of fact, hormone therapy is known to interfere in bone remodeling[47, 48], and different options of hormone therapy might change bone remodeling differently. Finally, the same threshold of NTX normality was used both for pre and post-menopausal women for comparability reasons. It is long known that several factors interfere with bone remodeling markers levels, such as age, hormonal environment and gender. The persistently higher levels of NTX in post-menopausal women at baseline and after the introduction of zoledronic acid and chemotherapy (despite all of which non-significant) might be exclusively associated with this fact.

Noteworthy, to our best knowledge, none of the published studies so far tested the menopausal status as a modulator of BPs action as an anti-tumor agent. In light of our findings and study limitations this question remains unanswered.

## **Conclusions**

In a cohort of women with breast cancer and bone metastases receiving first line chemotherapy and zoledronic acid, no difference seems to exist in terms of urinary NTX variation at 3 months according to menopausal status. Furthermore, no differences was found in terms of CA15.3 and CEA variation according to menopausal status. Finally, time to first line chemotherapy failure or overall survival seems to be similar when evaluated according to menopausal status.

Taken together, these results do not support a differential action of zoledronic acid in the control of bone metabolism (as a surrogate of cancer cells activity) or other cancer control related outcomes according to menopausal status. However, these findings need to be considered side with the discussed study limitations. As a matter of fact, this body of data can only be used as preliminary findings for future studies intending to test the anti-cancer activity of BPs according to the hormonal environment.

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## List of abbreviations

ALP – Alkaline phosphatase  
BALP – Bone specific alkaline phosphatase  
BC/CM – Breast cancer/Cancro da mama  
BP – Bisphosphonates  
BSP – Non collagenous bone matrix proteins: bone sialoprotein  
CA15.3 – Cancer antigen 15.3  
CEA – Carcinoembrionic antigen  
CI – Confidence interval  
CT/QT – Chemotherapy/Quimioterapia  
CTX – Carboxy-terminal crosslinked telopeptide of collagen type I  
CXCL12 – chemokine (C-X-C motif) ligand 12  
CXCR4 – chemokine (C-X-C motif) receptor 4  
CXCR7 – chemokine (C-X-C motif) receptor 7  
DKK-1 – Dickkopf-1  
DTC – Disseminated tumor cells  
DPD – Deoxyproline  
ER – Estrogen receptor  
HER – human epidermal growth factor receptor 2  
HR – Hazard ratio  
Hyl – Hydroxylysine  
Hyp – Hydroxyproline  
ICTP – Carboxy-terminal crosslinked telopeptide of type I collagen  
MT – Marcador tumoral  
NTX – Amino-terminal crosslinked telopeptide of collagen type I  
OC – Osteocalcin  
OP – Osteopontin  
OPG – Osteoprotegerin  
P1NP – Propeptides of type I procollagen  
PTH-rp – Parathyroid hormone related peptide  
PoM – Post-menopausal  
PrM – Pre-menopausal  
PYD – Pyridinoline  
RANK – Receptor activator of NFκB  
RANKL – Receptor activator of NFκB ligand  
SRE – Skeletal related event  
TRAP5b – Tartarate resistant acid phosphatase 5b  
TRAIL – TNF-related apoptosis-inducing ligand  
USA – United States of America  
ZA/AZ – Zoledronic acid/Ácido zoledrónico

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