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Role of OPG-RANK-RANKL pathway in the pathophysiology, diagnosis and therapeutic follow-up of metastatic bone disease.

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Abstract

Background
Studies on animal models showed that the metabolic activity bones may promote formation and progression of bone metastases.
It was therefore suggested that drugs inhibiting bone metabolism may locally protect bone in metastatic disease and inhibit growth of tumour .

Aims
The aim of this study is to assess the role of RANK-RANKL-OPG pathway either as predictive and diagnostic marker or as therapeutic follow-up of metastatic bone disease.

Materials and methods
A group of 100 patients affected by breast or prostate cancer with bone metastases (43 females and 57 male) was recruited a: 43 females breast cancer patients were compared with 45 healthy women; 57 male prostate cancer patients were compared with 55 healthy subjects.
In order to evaluate calcium and phosphorus metabolism, bone metabolic markers, osteoprotegerin (OPG) and soluble receptor activator of nuclear factor-Kb ligand (sRANKL), all of the enrolled underwent a medical examination, bone densitometry and blood sampling.
All patients with metastatic disease were treated with zoledronic acid (ZA) 4 mg i.v. every 4 weeks and revalued after 18 months (T1).

Results
Comparing patients with bone metastases from breast cancer to controls, RANKL/OPG ratio resulted increased in patients (23,68±13,69 vs 16,18±2,75 $p<0,05$), as well as serum levels in patients with prostate cancer OPG (4.0 ± 0.46 vs 4,2 ± 0.65 pmol/L; $p < 0,05$), while sRANKL/OPG was reduced (12,94 ± 3.65 vs 18.8 ± 2.75%; $p < 0,05$).
Densitometric follow-up of patients with breast cancer showed a significant increase of the T-score at lumbar site (Ts:-1,59±1,16DS vs 0,89 ± 0,35 DS; $p=0.04$).
Furthermore was not showed any significant variations of bone density in both lumbar (Ts: -1,4 ±0,3 vs -1,33±0,8 DS) and femoral site (Ts: -0,9±0,75 vs -0,89±1,45 DS).

Conclusions
Results show that metastasis either from prostate or breast cancer is finely tuned by RANK-RANKL -OPG pathway and that ZA can play protective role against bone metastases.
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Introduction

The process of metastasization takes place thanks to the release of growth factors which support metastatic lesions in growing within bones.

Metastatic lesions persisting in several organs and tissues of patients affected by cancer, even after removal of primary tumour, do represent the pathophysiological base for the disease's resurgence. The survival of such cells and the development of metastases depend on tissue microenvironment and on neoplastic cells ability to adapt their selves to that microenvironment (1-3).

Particularly the bone tissue offers a favourable microenvironment both to survival and growth of cells from metastatic breast cancer, so causing significant morbidity and, consequentially, deteriorating life quality for patients (4-6).

Studies on animal models have shown that bone metabolic activity may promote bone metastases formation and progression (7-9). Supporting these, clinical studies have shown a significant association between bone turn-over, tested by biochemical marker, and progression of metastatic bone disease (10,11). It has been therefore suggested that drugs inhibiting bone metabolism may locally protect bone in metastatic disease and inhibit growth of tumour .

In fact, further studies on animal models with metastases from breast cancer have shown that reducing bone turn-over, before the colonisation by tumour cells takes place, can appreciably reduce metastatic lesions in number and in their progression (12-14).

Nonetheless it has also been observed that, on animals with pre-existing injury, the antiresorptive treatment by bisphosphonates does not affect the metastatic growth potential of cancer cells in the bone micro-environment, even if it does have an antiosteolytic effect (12,13).

In order to explain such a phenomenon, it has to be considered that growth of tumour is related to the degree of bone resorption suppression and thus to the dose of administered bisphosphonates; it has also to be considered that initial phase of growth of tumour strictly depends on the interaction with the bone microenvironment, still being able to become independent afterwards and develop autonomously (13,15).

In fact prostate and breast cancer frequently metastasize at bone level, where neoplastic cells induce formation of osteoclasts which leads to resorption of the bone itself.

In 84% of patients, prostate cancer, the most frequent in males, causes bone metastases which determine alterations of both bone formation and resorption (16-19).

It has also been shown that prostate cancer cells produce and secrete OPG (17) and produce the sRANKL that promote osteoclastogenesis. Further observations have revealed that particularly OPG may inhibit osteoclastogenesis by linking sRANKL, so preventing bone metastasis from developing (18, 20).

In vivo studies have highlighted that over-expression of OPG inhibits growth of tumour at bone level (21), and indirectly protects cancer cells from TRAIL mediated apoptosis (TNF-Related Apoptosis-Inducing Ligand), thus helping their survival (17).
Several studies, though conducted on small samples in order to reach definitive conclusions (17, 22), have found that serum levels of OPG were higher in patients with bone metastases from prostate cancer than in patients suffering just from prostate tumours (23).
No correlation has been showed with regard to PSA levels; while in some case it has also been shown that the over-expression of OPG and increased OPG/RANKL ratio correlate with markers of disease, such as Gleason score, TNM, and PSA levels.
All of the data so far reported suggest the opportunity of further studies in order to confirm the role of OPG as prognostic marker for prostate cancer.
Breast cancer metastasis frequently cause osteolytic activity which is not directly mediated by cancer cells, but by osteoclasts (24, 25, 26).
It has been shown that breast cancer cells do not express mRNA for RANKL, but, through interaction with bone marrow stromal cells and synthesis of PTHrP (parathyroid hormone-related protein), they can induce synthesis of RANKL as well as inhibit synthesis of OPG, so promoting the formation of osteoclasts and the growth of metastatic lesions (26, 27).
In metastatic breast cancer lesions PTHrP is the main mediator of osteoclastic activation (28).
After incretion of PTHrP, RANKL, which is produced by osteoblasts and stromal cells, links to RANK on osteoblastic precursors and induces the differentiation.
The bioavailability of RANKL is controlled by OPG and OPG/RANKL ratio regulates the osteoclastogenesis process (29,30).
It has also been found that, in metastatic lesions from breast cancer, OPG promote cancer cells survival by inhibiting activity of TRAIL (TNF-related Apoptosis Inducing Ligand) (26, 31, 32).

Aims

The aim of this study is to assess the role of RANK-RANKL-OPG pathway either as predictive and diagnostic marker or as therapeutic follow-up of metastatic bone disease.

Materials and methods

We have included in the study 100 patients suffering from bone metastasis from breast or prostate cancer, on average aged 57.6 ± 10.4:
Group of patients was compound of 43 women suffering from breast cancer and 57 male subjects affected by prostate cancer. All of them were compared with controls, which was on his turn compound of 45

healthy women and 55 healthy subjects not suffering either from any neoplastic pathology nor bone metabolic alterations.
All of the enrolled subjects (T0) underwent medical examination, lumbar and femoral bone densitometry and collection of blood sample for evaluation of serum bone metabolic markers: bone alkaline phosphatase (BALP), c-telopepide of type 1 collagen (CTX) Osteocalcin (OC) for osteoprotegerina (OPG) and soluble receptor activator of nuclear factor-Kb ligand (sRANKL).
All the serum assays have been processed by ELISA after storage at -20℃.
All of the patients affected by metastatic neoplastic pathology were treated, every 4 weeks, with zoledronic acid 4 mg for intravenous infusion lasting 15 minutes, and revalued after 18 months (T1).
They underwent new medical examination, lumbar e femoral densitometry, and blood sampling for the determination of parameters already valued at T0.
The statistical analysis has been implemented by multifactor analysis of variance (ANCOVA), taking body mass index (BMI), age and sex as covariates; and it was performed by using Statgraphic Plus 5.0 software (Manugistic, Inc., Rockville, MD, U.S.A.).

Results

Table 1 summarizes characteristics of population included in the study: i.e. patients with bone metastasis, respectively from breast and prostate cancer (Ca).

Tab 1	Breast Ca (media +/- DS)	Controls (media +/- DS)	Ca prostata (media+/- DS)	Controls (media +/- DS)
Age	56,5±8,2	58,5 ± 5,21	58,7±12,6	60,3±8.7
Weight (Kg)	64,4±9,8	74,7 ± 21,96	71±13,61	80±10,75
Height (m)	1,58±0,073	1,55 ± 0,06	1,64±1.06	1,72±1, 35
BMI	25,7±4,40	30,89 ± 9,5	26,9± 5.5	27,5±6.2
T-S lumbar (DS)	-1,59±1,16	-1,03 ± 0,90	-1.4±0.3	-0.9±0.9
Z-S lumbar (DS)	-0,368±1,41	0,075 ± 0,87	-0.2±0.5	0.2±1.3
T-S Femoral (DS)	-0,452±0,94	-0,825 ± 0,87	-0.9±0.75	-0.4±1,03
Z-S Femoral (DS)	0,452±1,10	0,068 ± 0,82	0.1±0.92	0.51±0.85
sRANKL (pmol/L)	2,25±0,5	0,89±0,6	1,32±0,3	0,77±0,4
OPG (pmol/L)	9,5±0,35	5,5±0,5	10,2±0,46	4,2±0,65
sRANKL/OPG ($x10^2$)	23,68±13,69	16,18±2,75	12,94±3,65	18,8±2,75
CTX (ng/ml)	0,65±0,1	0,63±0,25	0,73±0,28	0,55±0,25
OC (ng/ml)	12,95±2,87	21,65±5,4	16,5±7,4	18,7±5,4
BALP (IU/L)	49,88±14,27	22,7±10,62	48,6±31,5	25,4±10,6 2

Analysis of variance, performed on densitometric data, shows lumbar density values, expressed by T-score, which appear reduced in breast cancer patients compared to controls (fig. 1)

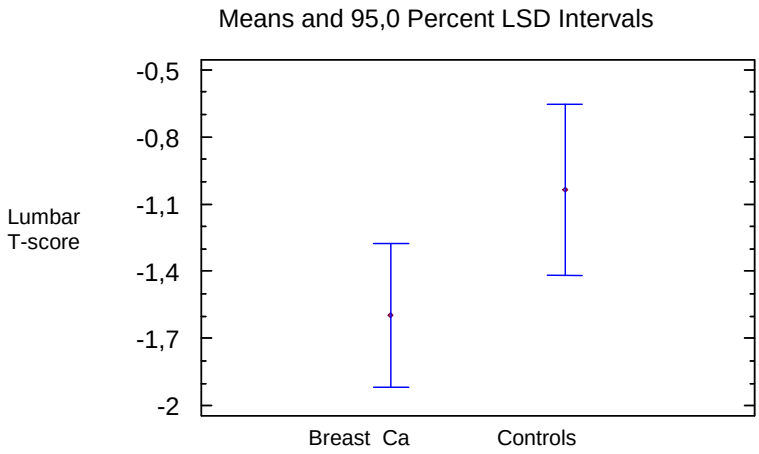

Fig. 1 Analysis of variance for the lumbar T-Score variable in the two study groups

The same trend has emerged from the analysis of variance of the lumbar Z-score variable (Fig 2)

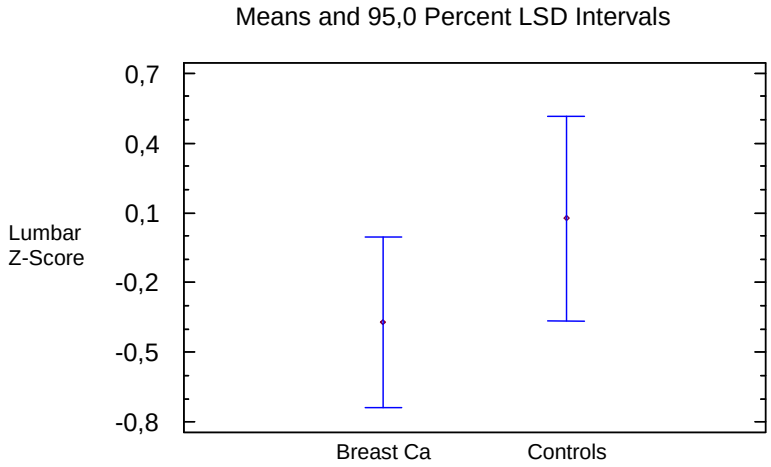

Fig. 2 Analysis of variance for the lumbar Z-Score variable in the two study groups

While analysis of variance performed on densitometric data, obtained from femoral site, has given opposite outcomes (Fig 3-4)

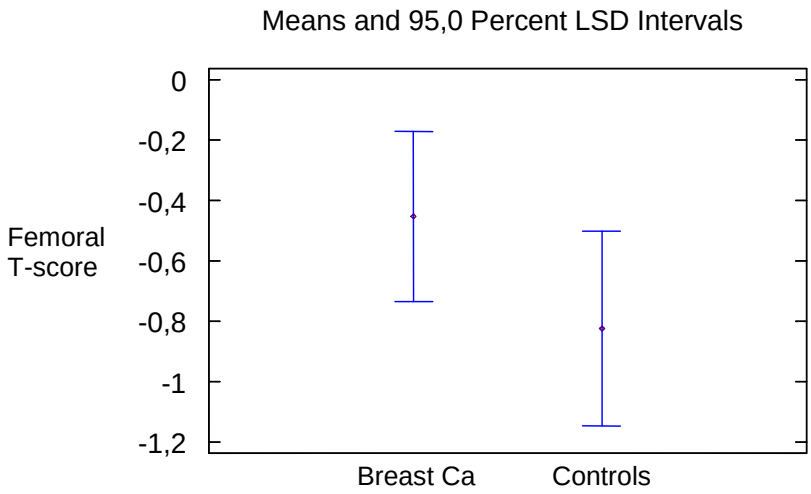

Fig. 3 Analysis of variance for the Femoral T-Score variable in the two study groups

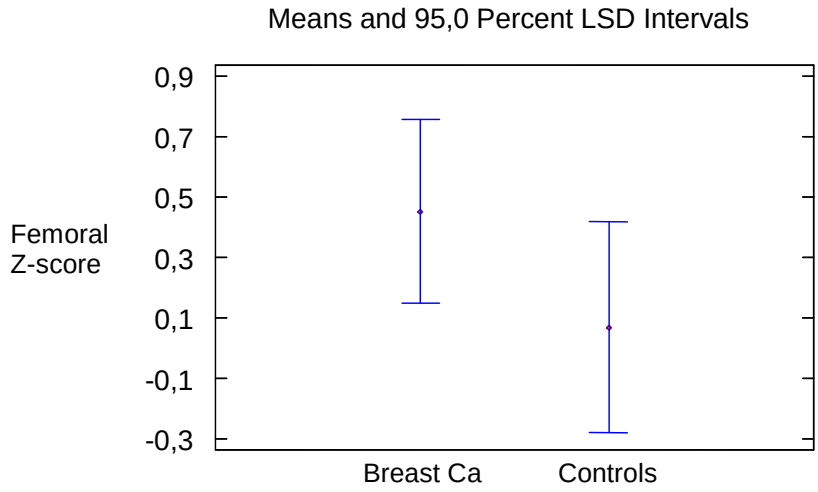

Fig. 4 Analysis of variance for the Femoral Z-Score variable in the two study groups

The dosage of bone metabolism markers has showed that bone alkaline phosphatase serum concentration is significantly higher ($p < 0.001$) in patients with bone metastases from breast cancer then in the healthy controls. While an opposite trend has been highlighted with

regard to Osteocalcin, even if difference shown does not reach noteworthy values (fig. 5).

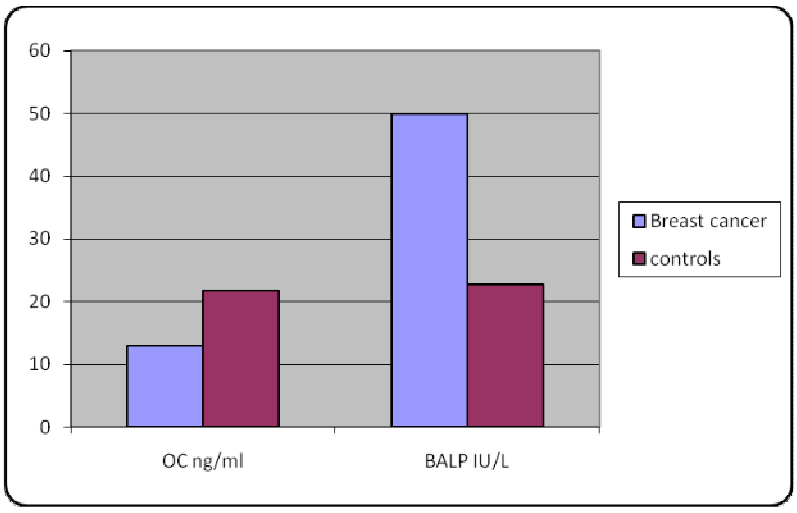

Fig. 5: OC and BALP serum levels of in patients with metastases from breast CA and in healthy subjects.

The measure of RANK-RANKL-OPG pathway factors has highlighted a sRANKL/OPG ratio significantly increased in patients with bone metastases from breast cancer compared to controls ($p < 0,05$) (fig. 6).

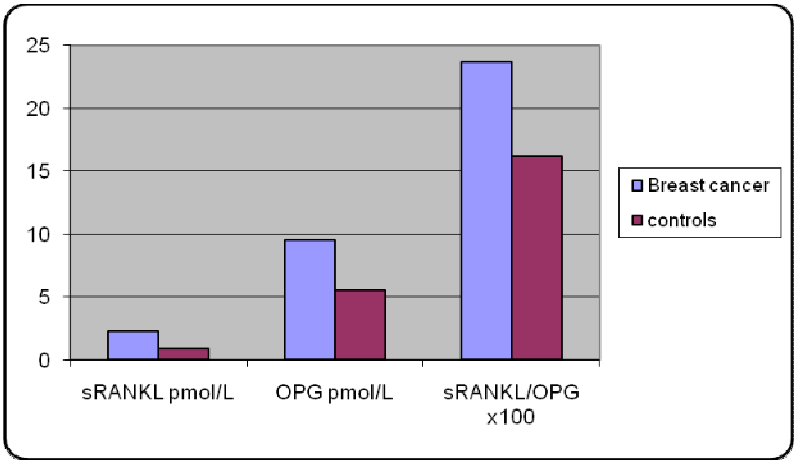

Fig. 6 s-RANKL and OPG and s-RANKL/OPG serum levels in patients with metastases from breast CA and in healthy subjects

Densitometric follow-up after 18 months treatment with zoledronic acid i.v. has showed a significant increase of the densitometric parameters, particularly of the lumbar T-score (p = 0.04) (Fig.7 and 8), whereas it has not revealed significant differences at the femur (Fig. 9 and 10).

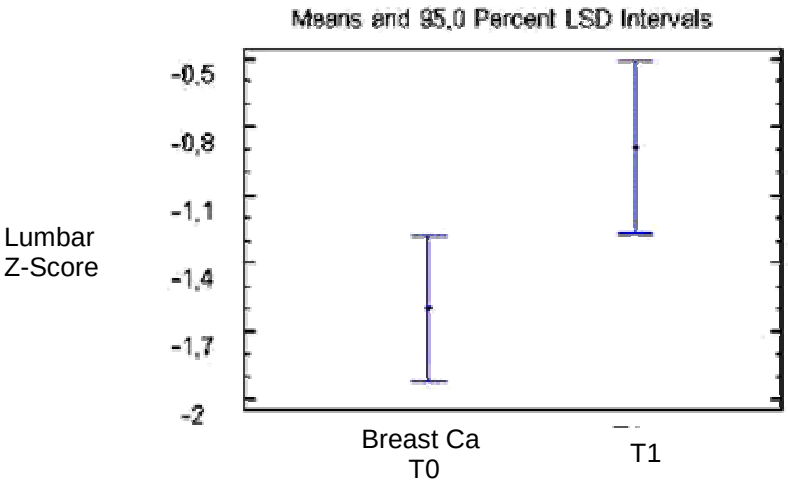

Fig 7 analysis of variance for the lumbar T-score before (T0) and after (T1) treatment with zoledronic acid 4 mg/4 weeks iv in patients suffering from breast cancer metastasis

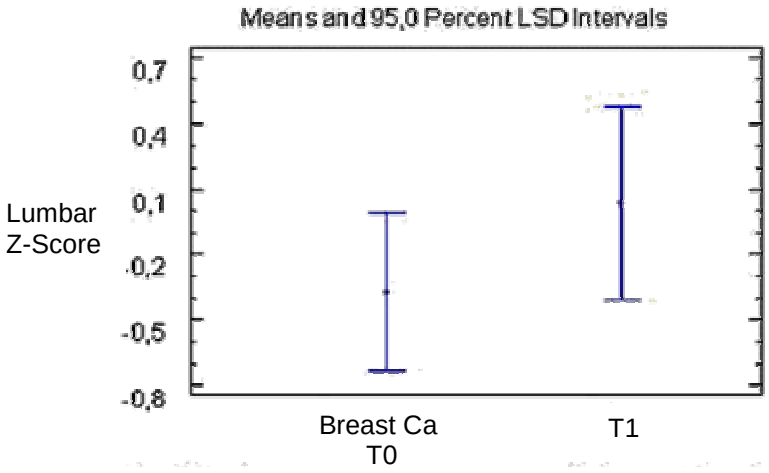

Fig 8 analysis of variance for lumbar Z-score before (T0) and after (T1) treatment with zoledronic acid 4mg/4 weeks i.v.

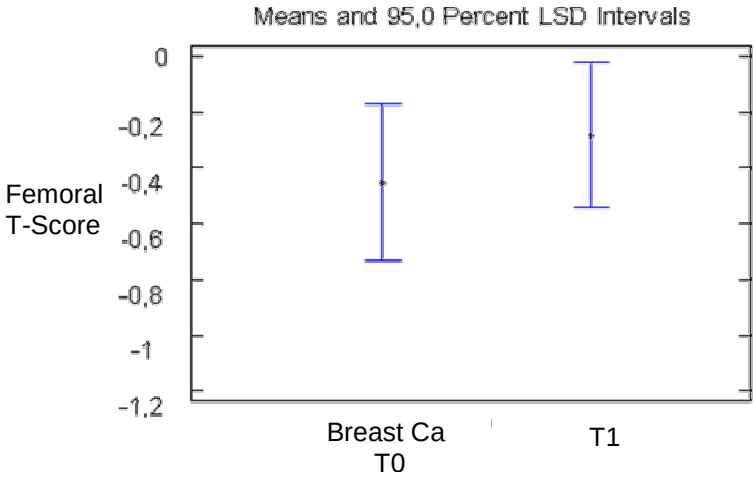

Fig 9 analysis of variance for the femoral T-score before (T0) and after (T1) treatment with zoledronic acid 4 mg/4 weeks i.v. in patients suffering from Breast cancer metastasis.

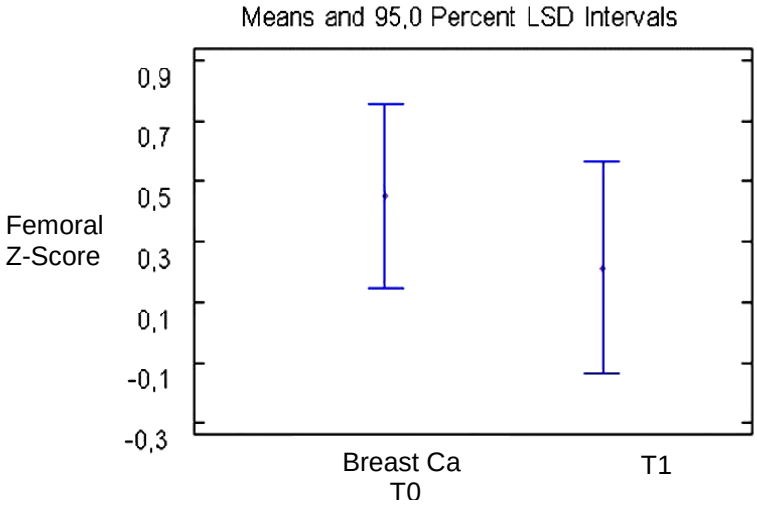

Fig 10 analysis of variance for the Femoral Z-score before (T0) and after (T1) treatment with zoledronic acid 4 mg/4 weeks i.v. in patients suffering from Breast cancer metastasis

The treatment resulted in clear reduction of serum CTX and BALP, but hasn't reached statistical significance level (Fig 11 and 12)

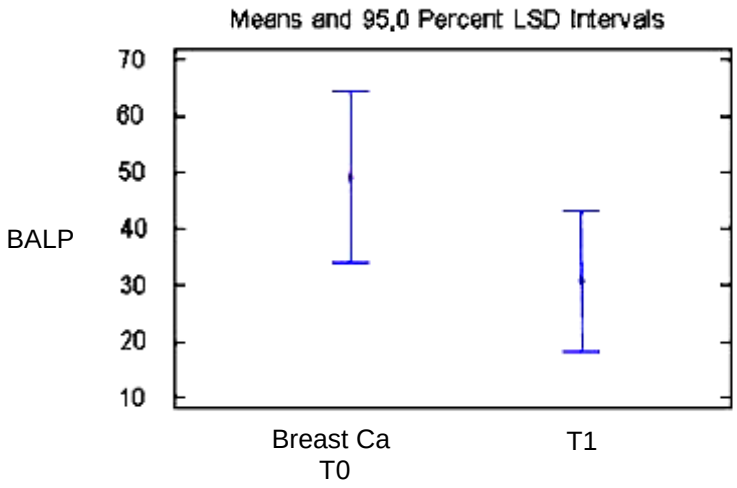

Fig.11 Analysis of variance of BALP variable before (T0) and after (T1) treatment with zoledronic acid 4 mg/4 weeks i.v. in patients suffering from Breast cancer metastasis.

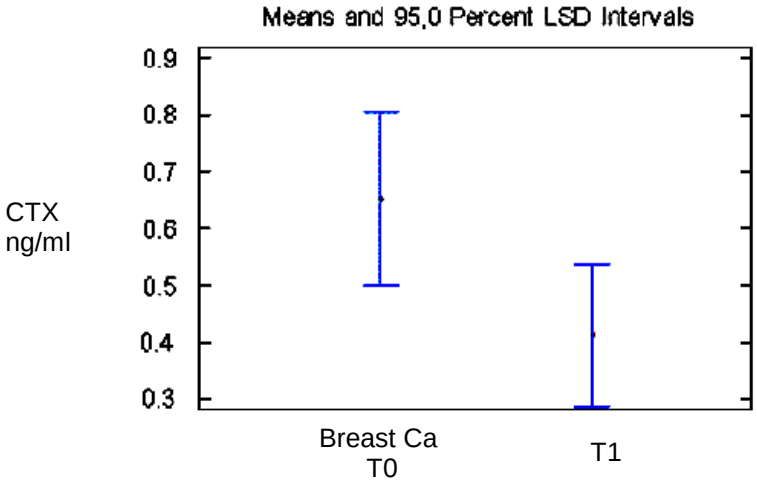

Fig. 12 Analysis of variance of serum CTX, before (T0) and after (T1) treatment, in patients suffering from Breast candcer metastasis

The determination of serum sRANKL/OPG ratio has outlined a decreasing trend, but hasn't reached levels of statistical significance (Fig. 13).

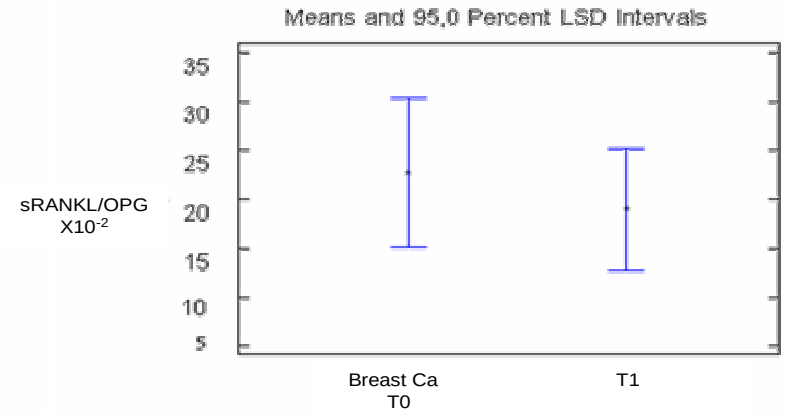

Fig. 13 Analysis of variance of sRANKL/OPG ratio in patients suffering from breast cancer metastasis before (T0) and after (T1) treatment.

With reference to the bone mass of patients with bone metastases from prostate cancer, the analysis of variance has showed lower bone mass in patients than in controls (Fig. 14,15).

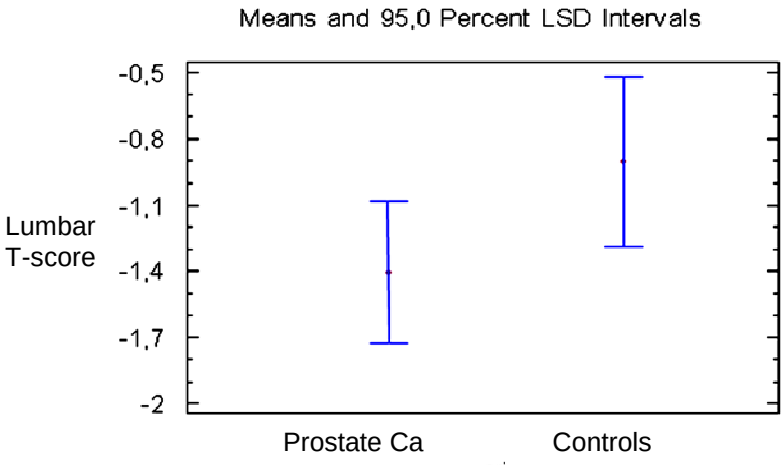

Fig. 14 Analysis of variance of the lumbar T-score variable in patients suffering from bone metastasis from prostate cancer and healthy controls

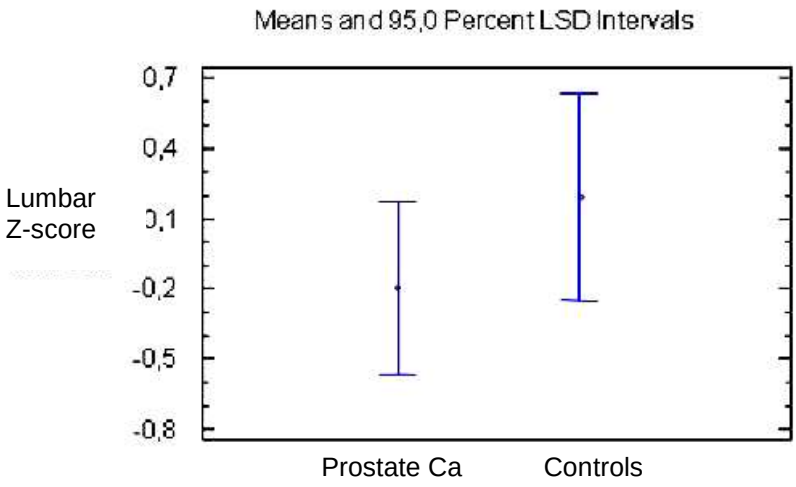

Fig. 15 Analysis of variance of the lumbar Z-score variable in patients suffering from bone metastasis from prostate cancer and healthy controls

A similar trend has benn highlighted at the femoral level, but neither it hasn't reached statistical significance (fig. 16)

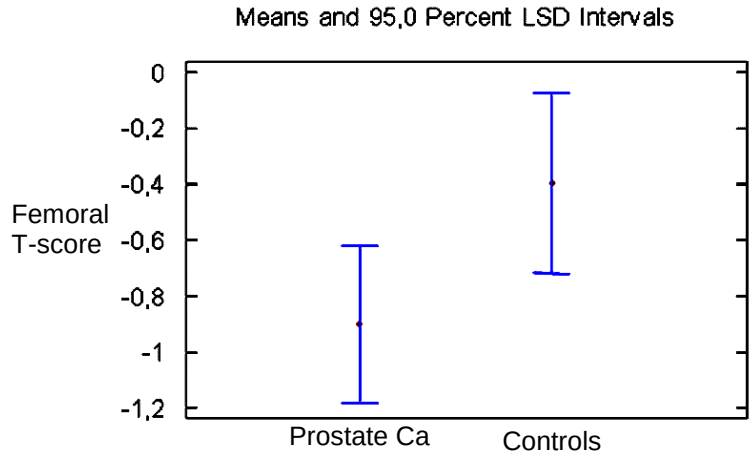

Fig. 16 Analysis of variance of the femoral T-score between patients suffering from bone metastasis from prostate cancer and healthy controls

Statistical analysis of blood parameters under consideration has revealed that serum levels of OPG are increased in patients, compared to the control group ($p < 0,05$), whilst sRANKL/OPG ratio is reduced ($p < 0,05$) (Fig 17)

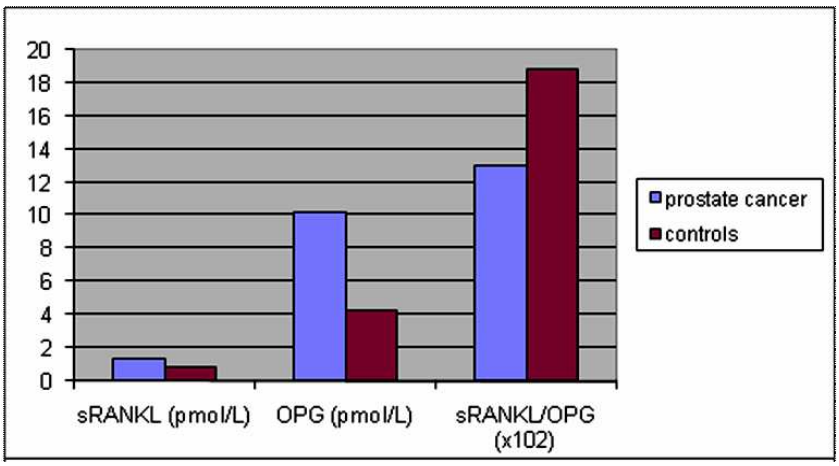

Fig. 17 Statistical analysis of serum OPG/sRANKL in patients suffering from bone metastases from prostate cancer compared to healthy controls

Analysis of markers of bone metabolism has outlined increasing values for both bone resorption (CTX) and formation (BALP) ($p<0,05$) in patients suffering from bone metastasis from prostate cancer, if compared to controls (fig. 18-19).

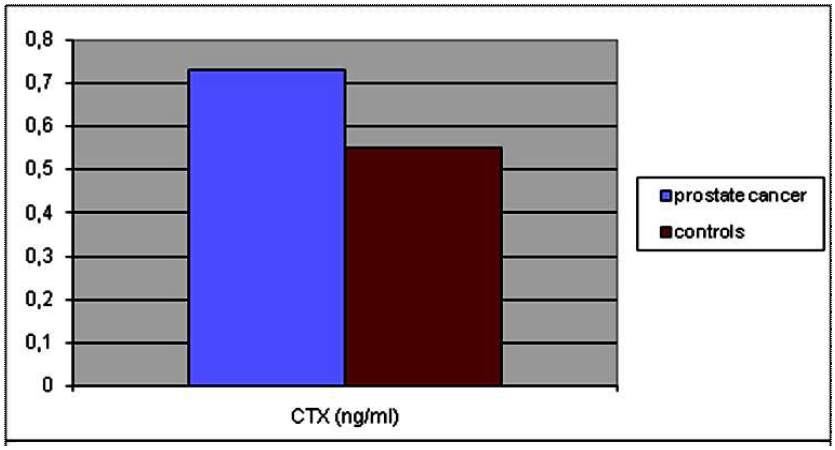

Fig. 18 Serum CTX in patients suffering from bone metastases from prostate cancer compared to healthy controls

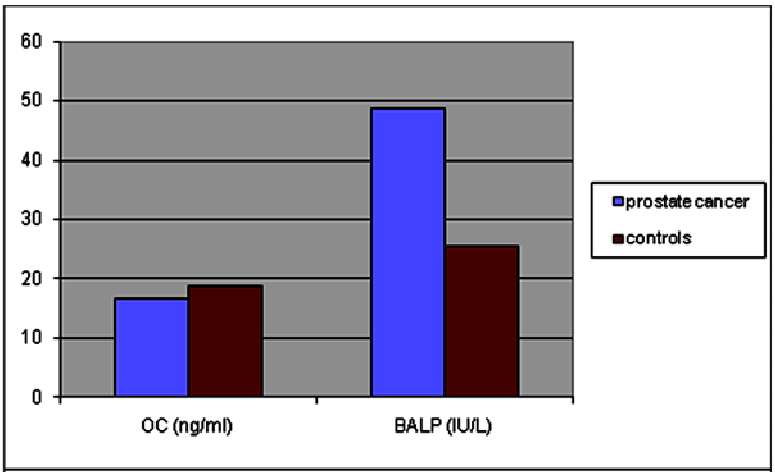

Fig 19 Serum OC and BALP concentrations in patients suffering from bone metastasis from prostate cancer compared to healthy

The following table (table 2) summarizes variations in parameters after the treatment with zoledronic acid IV for 18 months.

Tabella 2	Ca prostate (media +/- DS)	Controllo a 18 mesi (media +/- DS)
T-score lombare	-1.4±0.3	-1,33±0.8
Z-score lombare	-0.2±0.5	0.19±1.2
T-score femore	-0.9±0.75	-0.89±1,45
Z-score femore	0.1±0.92	0.09±0.73
sRANKL (pmol/L)	1,32±0,3	1,56±0,2
OPG (pmol/L)	10,2±0,46	17.72±0.85
sRANKL/OPG ($x10^2$)	12,94±3,65	8,80±2.65
CTX (ng/ml)	0,73±0,28	0.45±0,25
OC (ng/ml)	16,5±7,4	11,42±5.4
BALP (IU/L)	48,66±31,5	35,62±15.6

Densitometric follow-up after 18 months from the beginning of treatment with zoledronic acid IV therapies has not showed significant changes in bone density at both lumbar (Fig 20 and 21) and femoral level (Fig 22 and 23).

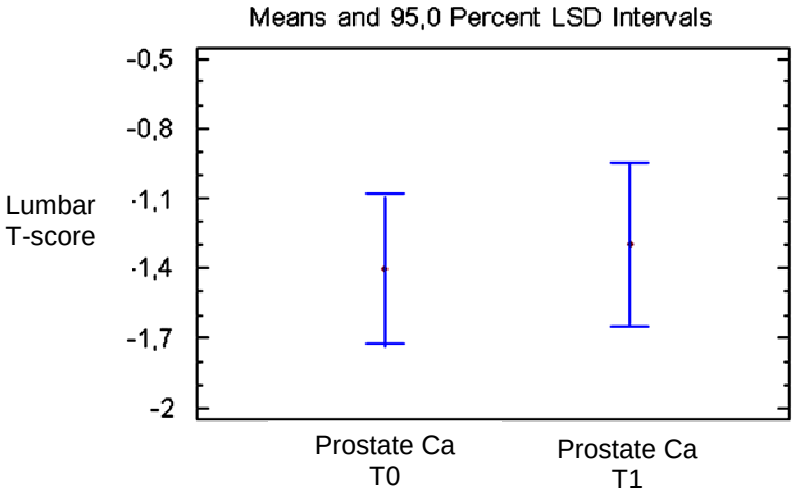

Fig 20 Analysis of variance of the lumbar T-score variable before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate

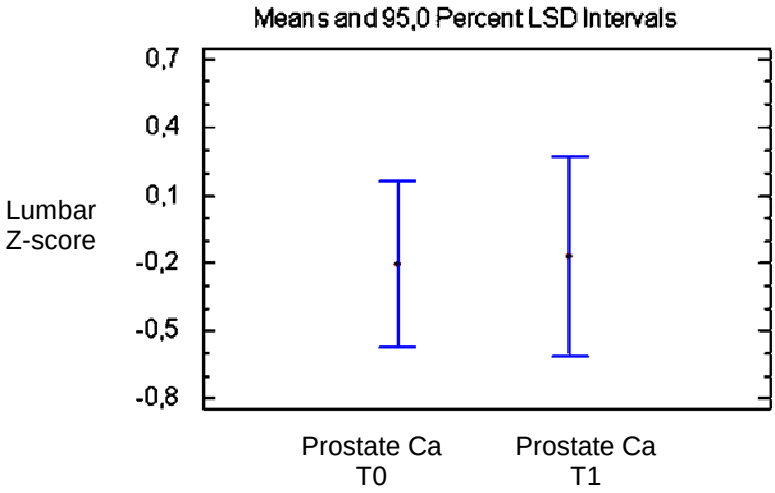

Fig 21 Analysis of variance of the lumbar Z-score variable before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate cancer

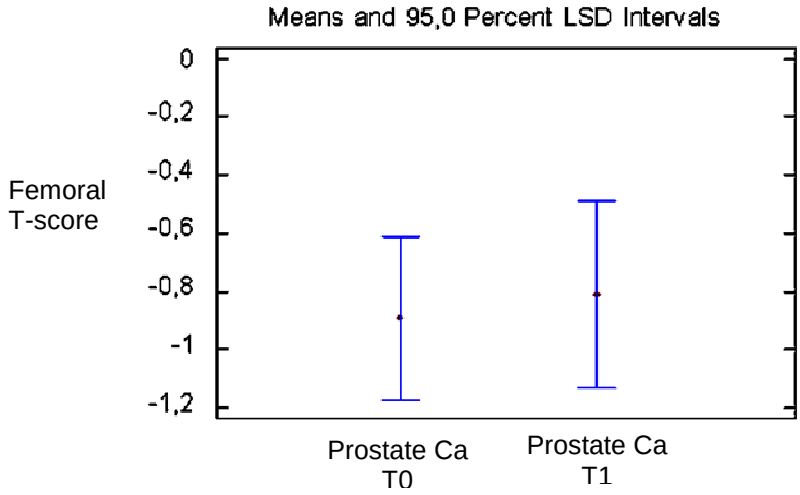

Fig 22 Analysis of variance of the femoral T-score variable before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate cancer

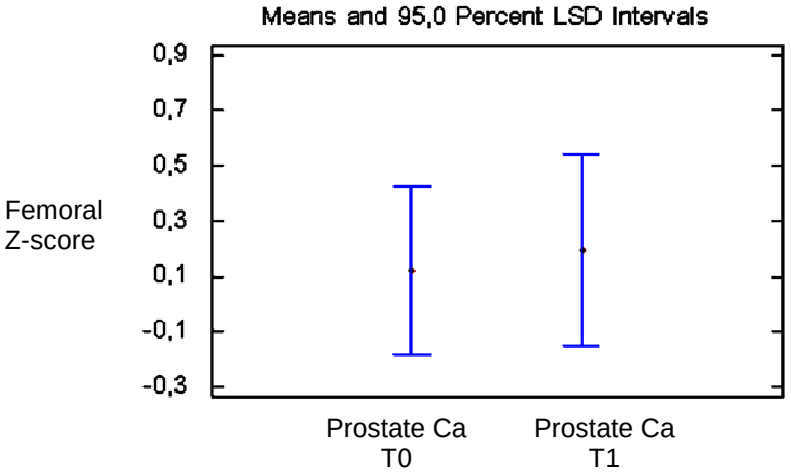

Fig 23 Analysis of variance of the femoral z-score before (T0) and after (T1) therapy in patients suffering from bone metastasis from prostate cancer

The dosage of of bone metabolism markers, after 18 months of treatment, has showed a definite reduction of serum CTX ($p < 0,05$),

BALP and sRANKL/OPG, although the latter two have not reached statistical significance (Fig 24, 25, 26)-

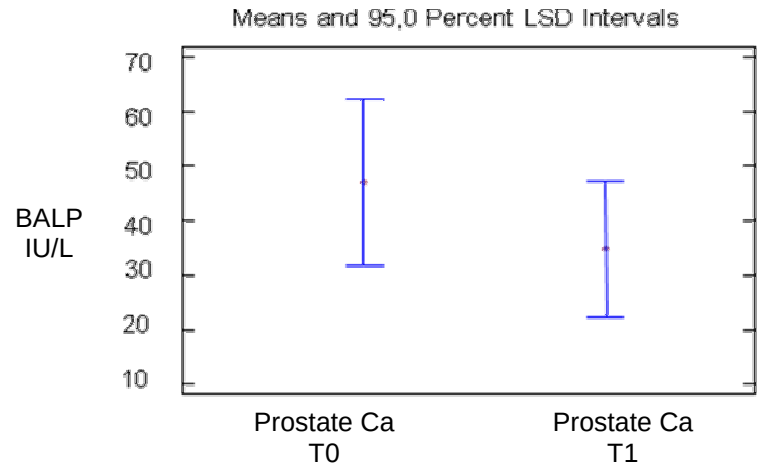

Fig 24 Analysis of variance of serum BALP before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate cancer

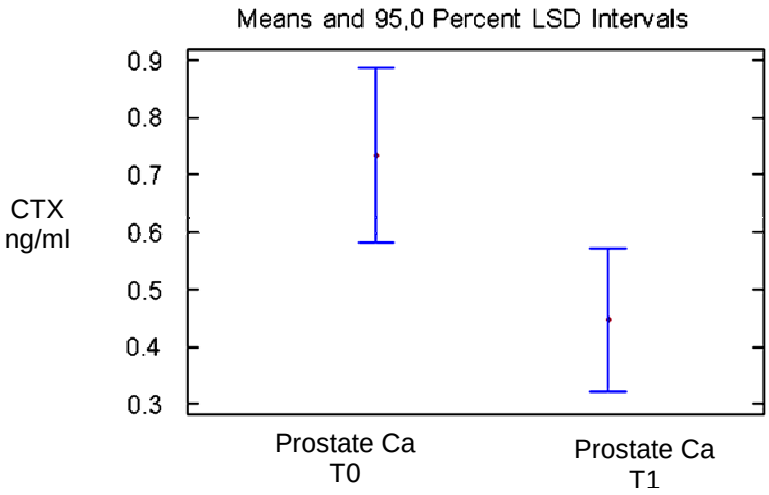

Fig 25 Analysis of variance of the serum CTX before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate cancer

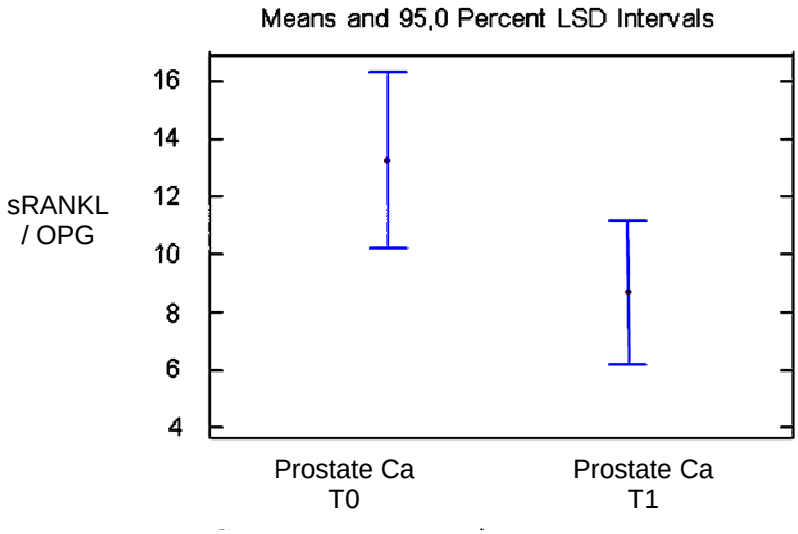

Fig 26 Analysis of variance of the sRANKL/OPG before (T0) and after (T1) 18 months therapy in patients suffering from bone metastasis from prostate cancer.

Discussion

An emerging bulk of evidence emphasizes the crucial role of feedback interactions between tumor cells and bone marrow microenvironment leading to the establishment of a vicious circle which acts by upregulating the physiological mechanisms that normally favor bone resorption. Many researchers have reported that tumor cells mainly express RANKL when they adhere to the bone microenvironment (33,34). The nuclear factor kappa B, which is the one of the final transcriptional targets of the RANKL/RANK pathway, plays a key role in the induction of pro-inflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors and enzymes (35)

In the present study, our results suggest that imbalance of the physiological bone remodeling process is mediated by severe disruption of the sRANKL/OPG system towards either osteolysis or bone formation resulting in subsequent changes in the levels of the bone turnover markers.

Our results also suggest that in patients with breast cancer there is a severe disruption in RANKL/OPG axis in favor of RANKL leading to increased osteoclast function.

Subsequent rise in the levels of CTX reflect excessive bone resorption in this subset of patients, whereas increas in BALP and OPG levels possibly represents a compensatory effect of reactive bone formation, which, however, cannot counterbalance the increased bone destruction.

On the contrary, OC was found to be down regulated, especially in the breast cancer subgroup, probably reflecting inefficient osteoblastic activity in these patients. Nevertheless, all the measured patient and control values of OC were within normal limits according to the manufacturer.

Patients with prostate cancer metastatic to the skeleton seem to follow a rather different pattern of bone turnover with predominance of bone formation, reflected by increased levels of BALP, resulting in the well-defined osteoblastic lesions. Prostate cancer cells seem to provoke profound elevation of OPG only, resulting in moderate suppression of the sRANKL/OPG ratio with subsequent increase in bone formation markers. It is thought today that the tumor microenvironment can release high amounts of OPG to counterbalance the high RANKL concentration produced by tumor cells. OPG acts in this case as a ‘‘decoy’’ receptor of RANKL and must therefore be considered as a ‘‘protector’’ of bone (36). Recently, promising results of a phase I study using recombinant OPG in patients with multiple myeloma or patients with breast cancer-related bone metastases were reported (37) and the future will show whether OPG has a therapeutic potential in this area.

Several considerations have been raised regarding the results of bone markers’ measurements. It must be noted that absolute changes in marker values are often misleading if the interpretation does not take

into account the respective marker's analytical and biological variability. It has recently been reported that serum levels of CTX and OC follow a circadian rhythm as a result of diurnal variation of cortisole in both breast cancer patients and healthy controls (38). This fact could possibly explain the failure of these two markers to correlate adequately with increased osteoclastic and osteoblastic activity, respectively, in the present study. Fohr et al. suggested that a change of 30% in a bone formation marker should be considered significant, whereas for most bone resorption markers, the least significant change should be at least 60% because of their higher coefficient variation (39).

A consideration of this study is the low number of patients.

The impact of previously received or concurrent chemotherapy (CT) and whether or not hormonal supplements or targeted hormonal therapies (estrogens, anti-estrogens, anti-androgens, aromatase inhibitors and LHRH analogs) were included in the treatment regimen is also an issue of major concern, due to the profound effects of hormonal intervention onto bone metabolic process (40). Marker levels might also be expected to change during the course of the disease, either in response to the effects of antineoplastic therapy- which may obscure the original differences in marker levels between patients and controls- or due to disease progression or regression (41). In patients with breast cancer metastatic to bone, a rise in serum OC or BALP after CT has been associated with local recalcification and therefore considered as a sign of therapeutic success (42). Recent data indicate that high baseline levels of OC could be predictive of better progression-free survival in patients with hormone-refractory prostate cancer (43). Increased uNTX levels have been recently shown to correlate negatively with clinical outcome and with 2-fold increase in the risk for skeletal complications and disease progression (44).

Recently, promising results of a phase I study using recombinant OPG in patients with multiple myeloma or patients with breast cancer-related bone metastases were reported (37) and the future will show whether OPG has a therapeutic potential in this area.

Our results suggest that antiresorptive therapy may affect bone metabolism through its action on OPG/RANKL/RANK pathway.

However more large cohort studies are needed to confirm this concept.

Conclusion

Skeletal morbidity remains a major problem in cancer patients and many aspects of the pathophysiology of malignant bone disease have not yet been fully clarified. Although the central role of the sRANKL/OPG system in both physiological and pathological bone remodeling process has been elucidated, it remains unlikely that a single marker has sufficient diagnostic or prognostic value in malignant bone disease.

Further larger-scale studies to assess the clinical utility of the combination of these markers with other laboratory tests (e.g. tumor markers) or imaging techniques are therefore required. Furthermore, important clinical questions regarding the use of these markers in monitoring bisphosphonate therapy or their prognostic value in predicting response to antineoplastic chemotherapy remain to be addressed.

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