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ZOLENDRONATE E ALENDRONATE DOSING IN BONE SEQUESTRA FROM BISPHOSPHONATE-RELATED OSTEONECROSIS OF THE JAWS: A MULTICENTER OBSERVATIONAL STUDY.

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1. *Introduction*

Bisphosphonates (BPs) are a class of drugs characterized by an antiresorptive effect on the bone mainly mediated by decreased function of osteoclasts [Russell RG *et al*., 1999].

These molecules are used in many clinical settings, including prevention and treatment of primary and secondary osteoporosis, hypercalcemia, heritable skeletal disorders in children, postmenopausal and glucocorticoid-induced osteoporosis, and bone metastases in patients with malignant tumors [Coleman RE *et al*., 2004]. BPs, indeed, efficaciously reduce the incidence of pathologic bone fractures in those patients especially affected by multiple myeloma or bone secondarisms from breast, prostate, pancreatic or lung cancers [Coleman RE *et al*., 2005]. Thus, the use of BPs in oncology has been preeminent as the drugs of first choice in clinical practice to counteract bone problems [Body JJ *et al*., 2004].

To date, BPs acquired the role of the most commonly used analogue of inorganic pyrophosphate, able to strongly bind the bone mineral portion, i.e. calcium phosphates and hydroxyapatite, and reduce its resorption. As result, BPs are among the most prescribed drugs; approximately 17 million prescriptions are recorded per year in the US alone and more than 300 million prescriptions of BP have been issued so far worldwide [Sharma D *et al.,* 2013].

However, the use of BPs may have several adverse effects (AEs). Most frequently, they include gastrointestinal effects, such as nausea, diarrhea, esophageal ulcers, and, more rarely, atypical femoral fractures, atrial fibrillation, ocular inflammation [Orozco C *et al*., 2012], but the most serious complication of BP therapy remains the bisphosphonate-related osteonecrosis of the jaw (BRONJ). In 2003, the first description of this AE was reported in patients with BP treatment [Marx RE *et al.,* 2003]. This complication has been reported with both oral and intravenous BP therapy and is characterized by painful and usually exposed, non-vital jaw bone in the oral cavity [Ruggiero SL *et al.*, 2006]. The precise pathogenic mechanisms underlying BRONJ is still unclear and both a specific cure and universally accepted protocols are lacking.

High intravenous dose of BPs have been linked to BRONJ with an incidence rate of 3%–15%, depending on the type of BPs used in therapy [Jadu F *et al*., 2007]. Similar complications have also been observed in patients receiving lower dosages of BPs for osteoporosis [Solomon DH *et al*., 2013], but with an lower incidence rate, ranging from 0.03% to 4.3% [Fellows JL *et al*., 2011; Sedghizadeh PP *et al* (a), 2009].

In 25% to 40% of cases, BRONJ can arise spontaneously, not related to any particular trauma or to BP treatment [Marx RE *et al.*, 2005; Bagan JV *et al*., 2006]. Spontaneous cases may be attributed to

anatomic and physiologic traits, because they normally occur in posterior portion of inferior jaw, where the mucosa is thinner than the anterior one. This region, together with posterior maxilla, is the most affected regions after dental extraction [Marx RE, 2003].

The most frequent symptoms are burning or sensation paresthesia and gradually the pain became more intense and it is usually caused by necrotic bone infection by oral bacterial flora. All of these signs precede clinical evidence of pathology, thus, it is essential to recognize them in order to prevent a progressive of BRONJ [Chiandussi S, 2006].

The International Task Force on Osteonecrosis of the Jaw [Aliya AK *et al.,* 2015] defines BRONJ as:

(1) exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider;

(2) current or previous treatment with bisphosphonates;

(3) no history of radiation therapy to the craniofacial region.

Histopathologically, BRONJ is characterized by avascular necrosis; infection of the exposed bone, abscess, fistulas, and pathologic fractures typically complicate the clinical picture, leading the patient to suffer with chronic pain [Leite AF *et al.,* 2006; Migliorati CA *et al.,* 2006].

BRONJ influences severely the quality of life of affected individuals.

Considering the increasing use of BPs due to their great efficacy, BRONJ is expected to gain more and more importance under several points of views, from prevention to pathogenesis and treatment. In this perspective, the aim of this narrative review is to current knowledge on the pathogenic mechanisms behind bisphosphate therapy of BRONJ, also providing details on the incidence, treatment and prevention of the condition. To address these objective, a broad search was conducted by consulting PubMed and EMBASE databases, retrieving the most updated evidences on BPs and BRONJ and identifying relevant papers published in English between 1980 until today.

2. Bisphosphonates

2.1 Historical Background

Bisphosphonates are synthetic analogues of inorganic phyrophosphates with a high affinity for calcium. When circulating in the circulatory system, they easily reach the mineral componet of the osseous structure; the excess of the active ingredient is eliminated with urine.

Bisphosphonates, once known as diphodphonates, were discovered around the half of the XIX century; the first synthesis leads back to 1865, in Germany. They were mainly used in the industrial field and later were introduced to the medical field thanks to their ability in restricting the precipitation of calcium carbonate. This feature was accidentally discovered in the '30s by Rosenstein who noted that small amounts of phosphates, introduced into irrigation pipes, were able to avoid the formation of crystals usually obstructing the same pipes [Blomen *et al.* 2005]

First descriptions about the biological characteristics of these compounds were made in 1968 by Herbert Fleish who as able to isolate pyrophosphate from urine. He supposed that pyrophosphate, detectable in plasma as well, was able to prevent the calcification of tissues and that alkaline phosphatase, in the osseous tissue, was able to locally destroy pyrophosphate, so allowing the amorphous phase of calcium phosphate to crystallize and form new bone tissue.

The first clinical trials, dating back to the '70s, were run in treating fibrodysplasia ossificans progressiva and Paget's disease with etidronate, revealing itself much more efficient compared to previous treatments [Smith *et al.* 1971*;* Basset *et al.* 1969]. As studies proceeded, it became clear the inhibiting action of pyrophosphate on the dissolution of calcium phosphate. In vivo studies showed the ability of this compound in preventing the ectopic calcification, not being able, however, to participate to the processes of mineralization and remodeling of the bone, these effects probably determined by the local action of phosphatase. On these bases, a synthesis of analogues of pyrophosphate resistant to enzymatic digestion was easily reached, compounds that are nowadays called bisphosphonates [Feish *et al.* 2002]. These molecules establish, in this very moment, a valuable therapeutic possibility in preventing skeletal complications associated with bone metastasis of osteolytic or osteoblastic type [Ross *et al.* 2003*;* Bhandari 2003], in postmenopausal and glucocorticoid-induced osteoporosis and in osteogenesis imperfecta. These substances, as well, take part in the maintaining of the serum balance of calcium [Hilner *et al.* 2000]. Bisphosphonates have become the standard treatment for patients suffering from multiple myeloma and bone metastasis secondary to breast cancer or other solid tumors Filleul *et al.* 2010]. The interruption of the therapy with bisphosphonates is advisable after two years if the osseous pathology can be considered stabilized and there is evidence of actual response to the treatment; in the opposite situation, carrying on with the therapy with bisphosphonates must be carefully evaluated by the practitioner [Durie 2007*;* Kyle *et al.* 2007].

The fundamental property of bisphosphonates, used by clinical pharmacology, is the ability of these compounds to bound to the surface of crystals and form complexes with cations in the solution next

to the interface between solid and liquid phase or directly on this latter. Being these compounds synthetic analogues of pyrophosphate, they chemically act in a similar way, but have a stronger stability. The are so able to inhibit in vitro calcification and in vivo osseous reabsorption.

2.2 Chemical structure of BPs

Structurally, bisphosphonates are synthetic derivatives of inorganic pyrophosphate (PPi), with a P–C–P bond instead of the P–O–P bond of inorganic pyrophosphates (Figure 1). The BPs have an extremely long half-life and are able to accumulate in the bone matrix [Cremers SC *et al*., 2005], because they are resistant to enzymatic hydrolysis, unlike pyrophosphates.

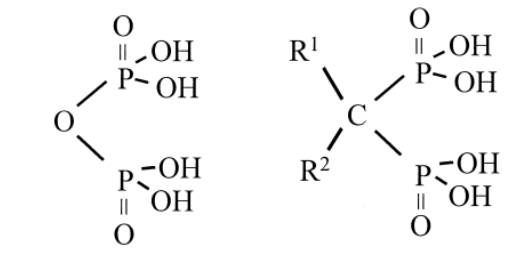


Figure 1: structural formula of pyrophosphate and bisphosphonate Cancer 2000; 88: 2961-78

Two lateral chains (R_1 and R_2) are attached to the central carbon and changes of those groups can lead to a great number of possible variations. (Figure 2) The two flanking phosphate groups are essential for binding to the bone mineral, such as hydroxyapatite, whereas the hydroxyl (OH) group or amino group in R_1 side position, increase the affinity for calcium and thus for bone mineral [Russell RG *et al*., 2007], and both act as "*bone hooks*". The R_2 chain determine the potency for inhibition of bone resorption [Nancollas GH *et al*., 2006].

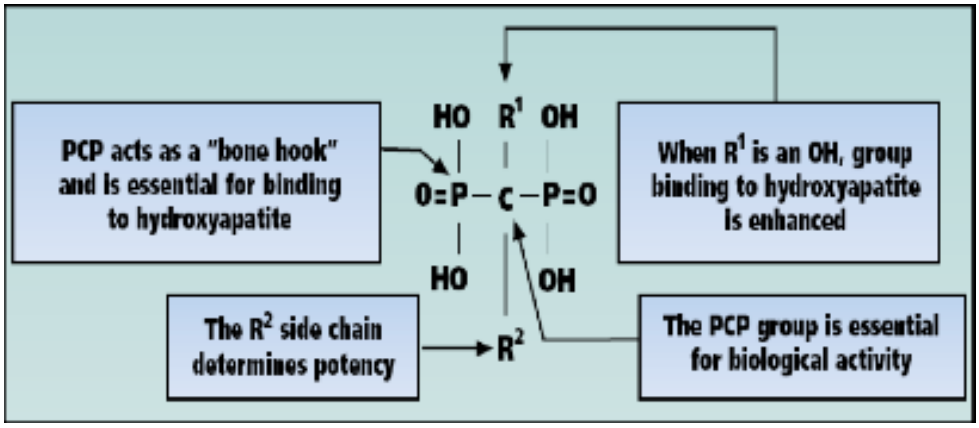


Figure 2: Biological activity and pharmacological effect of bisphosphonates are influenced by modifications of their basic chemical structure JADA 2005; 136: 1658-68

Bisphosphonates can be classified into two major groups based on their distinct molecular modes of action:

- non-amino-BPs (non-nitrogenated), which are considered the first-generation of bisphosphonates (etidronate, clodronate, and tiludronate); they can be metabolized by ATP analogues containing methylene; this leads to an accumulation of non-hidrolyzable ATP analogues in the osteoclasts and the subsequent inhibition of metabolic enzymes as phosphatase and pyrophosphatase. amino-BPs (nitrogenated), which are the second and third-generation bisphosphonates (alendronate, risedronate, ibandronate, pamidronate, and zoledronic acid). They mainly interact inside the osteoclasts in the mevalonate pathway [Vannucchi *et al.* 2005] inhibiting the key enzyme of the process, farnesyl diphosphate synthase 14, causing the interruption of a series of cellular functions essential for the survival and the activity of osteoclasts and, so doing, leading to an unbalanced homeostasis of the bone turnover [Bhandari *et al*. 2003; Green *et al.* 2003; Russel *et al*. 1999; Conte *et al*. 2004; Corey *et al*. 2003]. Amino-bisphosphonates have greater affinity for the bone and a strength from 100 to 1000 times greater compared to non amino-BPs. NBP are nowadays the solely category of bisphosphonates for which an association with BRONJ has been identified [Migliorati, Epstein *et al*. 2011]; the same fact can not be affirmed for bisphosphonates not containing amino-groups, exception made for few case reports.
- This class can be further divided based on the characteristics of the amine in the radical group:

- Amino-bisphosphonates with primary amine: alendronate, pamidronate;
  - Amino-bisphosphonates with secondary amine: icandronate;
  - Amino-bisphosphonates with tertiary amine: olpandronate, ibandronate;
  - Amino-bisphosphonates with aromatic ring: zolendronate, risendronate.

Bisphosphonates can be also classified into:

- 1° generation: without nitrogen atom;
- 2° generation: with nitrogen atom/s;
- 3° generation: with heterocyclic chain.

A new classification based on the mechanism of action sets the subdivision in:

- bisphosphonates not containing nitrogen (non N-BPs);
- alkyl-amine bisphosphonates;
- heterocyclic bisphosphonates containing nitrogen.

Not N-BPs act thanks to the incorporation of ATP, Alkyl-amine BPs interfere with the farnesyl pyrhophosphate sinthase enzyme (FPPS), while heterocyclic bisphosphonates, containing nitrogen, inhibit as well the FPPS enzyme and increase their efficacy stabilizing structural modifications [Russel *et al*. 2007].

These different molecular structures reflect different mechanisms of action.

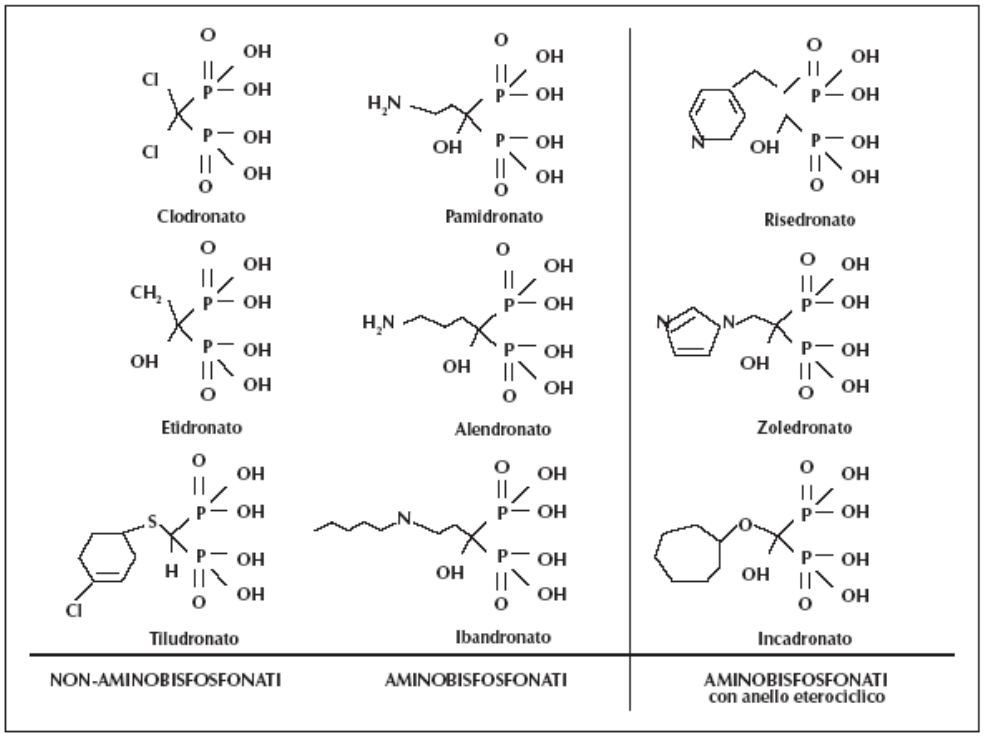


Figure 3: Synthesis molecules deriving from bisphosphonate Reumatismo 2005; 57(3): 142- 53

2.*3 Mechanism of action of BPs*

The fundamental biological action of all bisphosphonates is the inhibition of the osseous turnover and, therefore, of the reabsorption, so reducing the serum levels of circulating calcium.(figure 4)

The anti-osteoclastic and anti-reabsorption actions are powered by the inhibiting effects on the osteoclasts, on which bisphosphonates are likely to cause an irreversible cellular death. After administration, the drug quickly binds to the mineral crystals of the whole bone tissue and, following multiple doses, accumulates in the osseous matrix. During regular bone remodeling, osteoclasts reabsorb mineralized bone tissue and bisphosphonates enter these cells; the effect of this drug is similar to the one caused by isoprenoid diphosphate lipids. These substances are essential for farnesylation and geranilation of the ezymes of triphosphate guanosin (GTPase) having a preventive function about osteoclasts'apoptosis. Microscopically it can be observed that osteoclasts lose their characteristic wrinkled brush border usually localized in the Howship reabsorption lacunae, they withdraw from the osseous surface and undergo necrosis. Without reabsorption and, at the same time, the releasing of proteins that induce osteogenesis, like the bone morphogenetic protein (BMP) and insulin-like growth factors 1 and 2 (ILG1 and ILG2), the pre-existing bone is not removed and new osteoid substance is not formed.

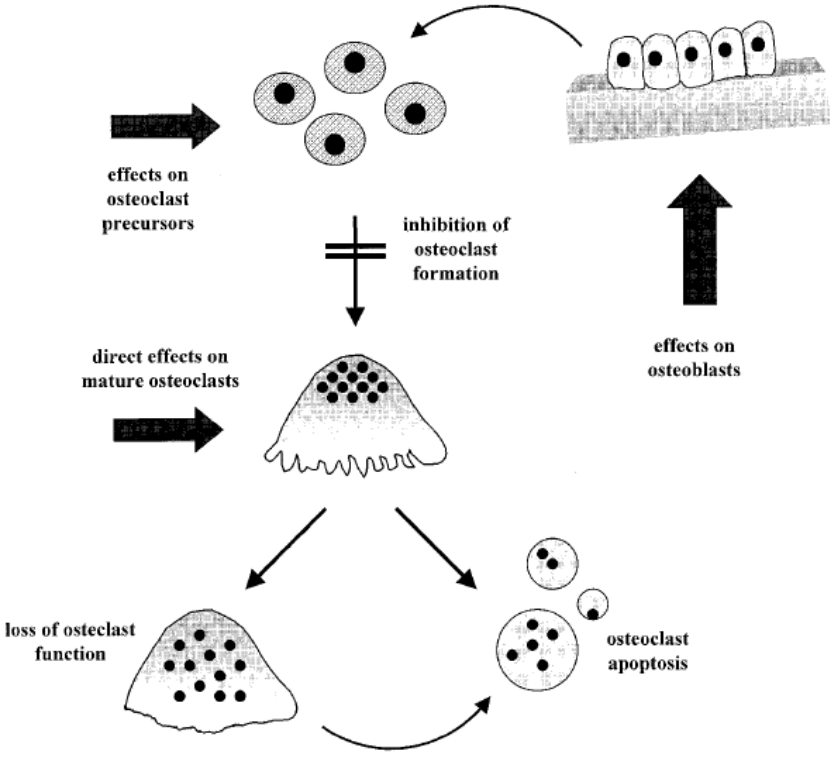


Figure 4: Inhibition pathways of osseous reabsorbtion mediated by osteoclasts with bisphosphonates. Cancer 2000; 88:2961-

The old bone, therefore, survives beyond its programmed vitality. Since osteocytes are not immortal cells, they will undergo death leaving a non-vital bone surrounding them. It is important to notice that the main function of osteocytes is not the formation of new bone, but to act as mechanoreceptor

in order to mantaing the existing mineral matrix of the bone. If the osteocyte does not control the regular remodeling, an extra production takes place of more not-vital mineralized matrix. This hyper-mineralization is radiologically highlighted by the sclerosis of the lamina dura, followed by the generalized osteo-sclerosis of the alveolar bone. This phenomenon is the principal cause of osteo-sclerosis.

The alteration of the homeostatic equilibrium induces the impossibility of forming new vital bone and, therefore, areas of not vital tissue, of different size, appear, leading to a classic osteonecrotic pattern.

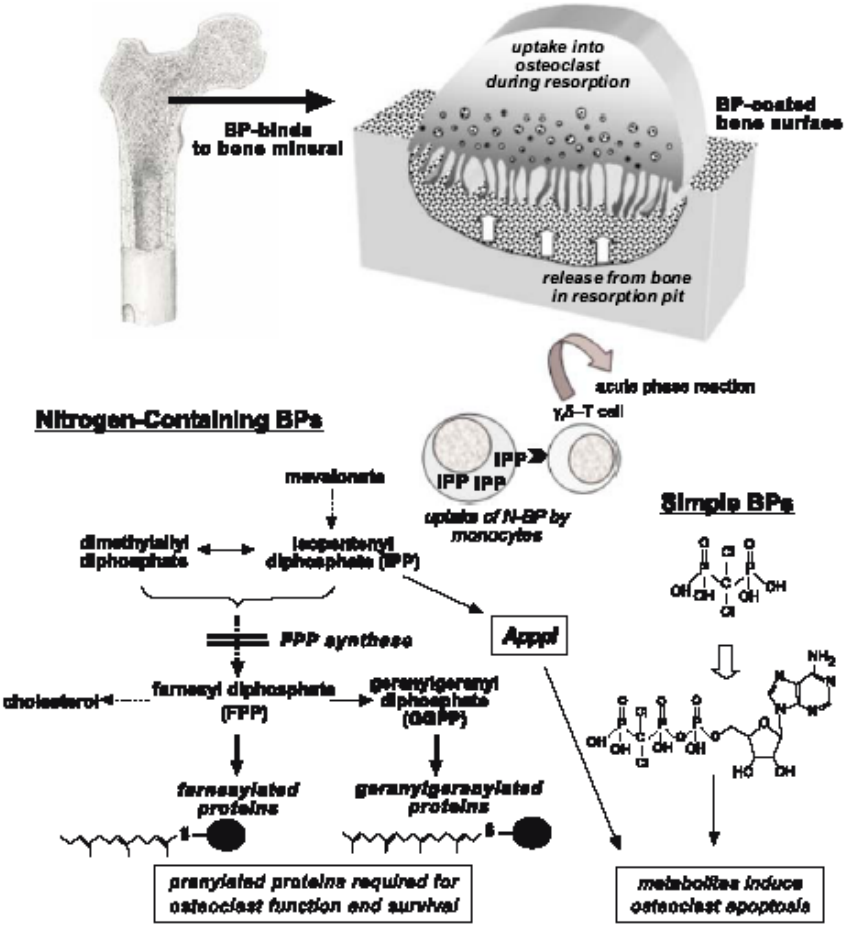


Figure 5: Mechanism of action of bisphosphonates on osteoclasts. Mechanism of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy, Osteoporos Int.,2007

The non-amino BPs are incorporated as non-hydrolyzable analogues of newly formed adenosine triphosphate (ATP) after osteoclast uptake form bone mineral [Russell RG *et al*., 2006]. Intracellular accumulation of these non-hydrolyzable ATP analogues interferes with mitochondrial

function, leading in the end to osteoclast apoptosis due to inhibition of multiple ATP-dependent cellular processes [Roelofs AJ *et al*., 2006].

The amino-BPs are more potent because the presence of amino group increases the bisphosphonate's antiresorptive potency by 10 to 10,000 folds [Russell RG *et al.,* 2006***;*** Dunford JE *et al.,* 2001]. Amino-BPs inhibit farnesyl pyrophosphate synthase (FPPS) within osteoclasts, a key enzyme of the mavalonate/cholesterol biosynthetic pathway, critical for the production of cholesterol, sterols and isoprenoid lipids [Dunford JE *et al*., 2001; Kavanagh KL *et al*., 2006]. These compounds are essential for the post-translational modification of small GTP-binding proteins (which are also GTPases) such as Rab, Rho, and Rac, which play central roles in the regulation of osteoclast cellular activities including, osteoclast morphology, membrane ruffling and survival. The inhibition of protein prenylation and the loss of function of key regulatory proteins, leads to osteoclast apoptosis [Luckman SP *et al*., 1998].

However, in general BPs act on both osteoblast and osteoclasts [Im GI *et al*., 2004]. In fact, BPs have a target on osteoblast cells, which in turn influence the osteoclasts. Several studies have showed experimentally that bisphosphonates inhibit the expression of the receptor activator of NF-kappa B ligand (RANK-L) and up-regulate osteoprotegrin (OPG) in osteoblast cells, which is one of the mechanism by which BPs, indirectly induce the resorption [Pan B *et al*., 2004; Gracis S *et al.*, 2013].

2.4 Pharmacokinetics (PK) and Pharmacodynamics (PD)

2.4.1Pharmacokinetics (PK)

Bisphosphonates are synthetic compounds absorbed, stored and excreted unaltered by the organism. They have a very short biological half-life: between 20 minutes and 3 hours, depending on the type of drug and the individual clearance. On the contrary, because of their great affinity for calcium phosphate crystals, a percentage between 20% and 50% of the absorbed dose is intercepted by the bone in the first 12-24 hours and there is stored for years. The release of bisphosphonates takes place mostly when the bone where they are stored is undergoing reabsorption and this accounts for their long half-life; because they are not metabolized by the organism, they are excreted unaltered and are, therefore, pharmacologically active.

2.4.2 Absorption

To discuss the mechanisms of absorption and evaluate them it is necessary, first of all, to introduce

the topic of oral bio-availability, defined as the fraction of the oral dose that reaches the systemic circulation.

After the oral ingestion of bisphosphonates, especially for the most powerful, the plasmatic concentration of the drug results to be too low in order to asses a real quantification: this fact forces to use other means to measure oral absorption. Because of the kinetic characteristics of these compounds, the selective renal bonding, it is possible to asses a urine or osseous concentration in order to evaluate the oral absorption. Several in vivo studies, on animal first and volunteers after, unanimously consider the average intestinal absorption extremely low, with measures not over 10%. There are differences in intestinal absorption between Amine-bisphosphonates and Non-Amine-bisphosphonates. N-BPs like aledronate, risendronate and ibandronate have an absorption (F) of about 0.7%. Bisphosphonates not containing an atom of nitrogen in their chain, like clodronate and etidronate, seem to have a greater absorption, equal to 2-2.5%. Absorption is strongly prevented by drinks (water excluded) and food, in particular by those containing calcium (milk and dairy products) but could increase with elevated gastric pH [Cremers *et al*. 2011].

Several attempts to increase intestinal absorption have been made but without satisfactory results.

These data have led to the conclusion that oral bio-availability is similar among all molecules and is therefore not fit for deciding the kind of bisphosphonate best to administer. It is known that electrically charged molecules, and bisphosphonates among them, because of their low lipophility, hardly pass across the cellular membrane, obliging to an epithelial junction passage. The most efficient ways of administration are, for sure, the intravenous and the intramuscular: under these circumstances, it is possible to have the greatest bio-availability and, therefore the efficacy of the active ingredient.

Bisphosphonates are currently administered intravenously (IV) , orally or intramuscular. Oral BPs are absorbed into the bloodstream from gastrointestinal lumen (GI) by two ways: 1) through epithelial cells into the blood (transcellularly); 2) via the circulation by the tight junctions between epithelial cells [Porras AG *et al*., 1999]. When administered orally BP absorption is low, in rates equal to or below 1% of the total dose. The poor absorption of BPs can probably be attributed to their very poor lipophilicity, which prevents transcellular transport across epithelial barriers. The low oral bioavailability also decreases in presences of food or calcium, aluminum or magnesium containing drinks and is enhanced with elevated gastric pH [Dunn CJ *et al.,* 2001; Gertz BJ *et al*., 1995]. In fact, if the BPs are taken with food, the absorption may be reduced to zero [Fogelman I *et al*., 1986].

When given IV, they are rapidly removed by the plasma and show a 40% renal excretion rate in the first 24 h, without being metabolized. While the half-life of BPs in the plasma is short, in bone it lasts for about 10 years [Walter C *et al.,* 2007]. IV administration of BPs, with the use of 14C and 99mTc-labeled BPs in animals and humans, showed that the bone uptake of bisphosphonates is rapid and most of drug that is not excreted in the urine within 24 h either is bound to bone or is in the extracellular matrix about to bind to bone [Hyldstrup L *et al.,* 1984].
The structure of BPs influence also the absorption: amino-BPs have absorption of ~0.7%, while non-amino-BPs appear to have a slightly higher absorption (2–2.5%) [Cremers SC *et al.,* 2005].

2.4.3 Plasmatic proteins binding

It is universally known the importance of lipophilicity for the bonding with plasmatic proteins; a similar key role is played by ionic bonding and electrostatic forces.

Bisphosphonates at plasmatic pH (7.4) are completely ionized and possess, therefore, sufficient requirements in order to make the bonding possible. To analyze this pharmacokinetic aspect, several studies have been made using a molecule of alendronate. From these researches it resulted that bisphosphonates bond mainly with albumin: this protein undergoes saturation after infusion of high concentrations of the drug. The affinity of alendronate for plasmatic proteins increases greatly alkalinizating the plasmatic pH and the same happens in presence of calcium ions with a mechanism not clarified yet.

2.4.4 Distribution

Although the uptake and distribution of BPs have been widely investigated, knowledge about distribution of BPs in humans is still limited. Several studies of radio-labeled compounds showed that BPs are not immediately excreted by kidney, but are taken-up and absorbed primarily by bone and some are also delivered to soft tissue such as kidney, liver and spleen [Russell RG *et al.*, 2008]. In addition, the binding affinities of BP to bone crystals has been extensively studied *in vitro* and *in vivo* revealing important differences [Russell RG *et al*., 2008; Lawson MA *et al.*, 2010; Nancollas GH *et al*., 2006]. Binding to the bone mineral is stronger for amino-BPs risedronate, ibandronate, pamidronate, alendronate and zoledronate, and weaker for non-amino BPs.

2.4.5. Tissue distribution

Not calcified tissue

After intravenous infusion, bisphosphonates are quickly delivered to all corporeal districts, even not calcified, exception made for kidneys where the excretion of the drug takes place. The concentration of the molecule in not-calcified tissues decreases rapidly (5% of the dose after 1 hour) as it does in blood plasma. Therefore, thanks to the limited exposure to the drug, side effects, like tissue necrosis, take place only if doses are too high or infusion was too quick. In the bone tissue, however, the concentration of the drug keeps increasing reaching its peak an hour after the ingestion.

Calcified tissue

The conduct of the drug in the bone it is different depending on the interested tissue: compact bone (cortical) and spongy bone (trabecular), (figure 6) Both types are vital and undergo continuous remodeling but the cortical portion, even though is the 80% of the skeletal mass, is having a turnover 5 times slower compared to trabecular bone. Bisphosphonates, accumulating in areas metabolically active, are not distributed homogenously in all calcified tissues but more in the spongy bone. [Sato *et al*. 2002] demonstrated that hydroxylapatite exposed in reabsorption sites is subjected to these molecules. Other factors, however, can participate to this non-homogeneous distribution: blood circulation and bone surface/volume ratio play in favor of trabecular bone.

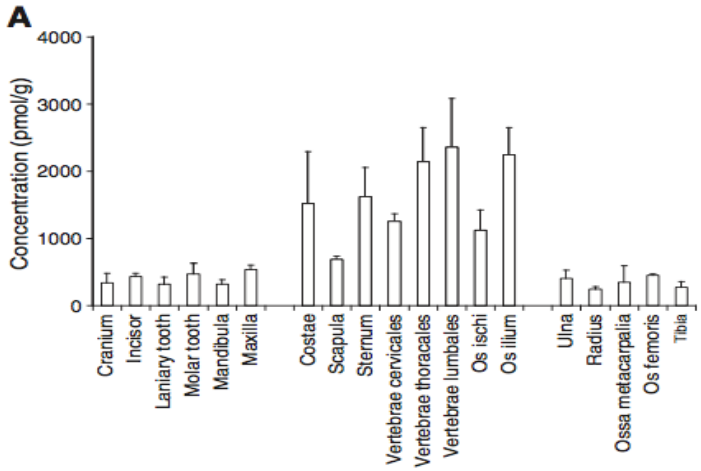


Figure 6: Zolendronate's concentration in tissue after 96 hours from administration of a single intravenous dose of 0.15 mg/kg in dogs. (Cremers et al, Bone 2011)

The mechanism thanks to which the drug passes from systemic circulation to the bone is still not clear: it is thought that bisphophonates enter the extracellular space of the bone thanks to a paracellular transport and bound to free hydroxylapatite on the surface. There are no studies analyzing this important phase of biodistribution; on the contrary, the last phase of this process, the

bounding between drug and hydroxylapatite crystals, has been accurately studied, highlighting substantial differences in the affinity of bounding among different bisphosphonates. Crystallography, NMR, in vitro molecular and dynamic modeling have demonstrated, in these past years, that phosphonates groups and R1 and R2 groups have an important role in the bonding. As previously discussed, R1 can be formed by H, Cl or OH (with a bonding affinity in this order) while R2 can be formed by Cl or by complex organic structures (those containing nitrogen and that showed the potential to influence the bonding of BP to calcium's crystals included).

The bonding is weaker for non N-BP like etidronate, clodronate and tiludronate and considerably stronger for N-BP like risedronate, ibadronate, pamidronate, aledronate and zolendronate.

Differences among various N-BP are substantially fewer compared to differences between N-BP and non N-BP [Cremers *et al*. 2011].

The bone tissue seems to be saturable by these drugs (after massive administration); it is thought that the bone maximum capacity in taking up these chelating compounds might be around 105 nmol/mg and this would explain the competitively phenomenon going on in case of simultaneous assumption of high doses of different bisphosphonates.

Moreover, the distribution of BPs in bone is not homogeneous. The uptake of BPs in the femur neck and spine is higher than in femur shaft [Carnevale V *et al*., 2000], suggesting that BPs bind preferentially to bones with high turnover. In fact, studies have indicated that the bone turnover rates may differ between young male and female rats, but not between older male and female animals [Lin JH *et al.,* 1992]. Skeletal uptake and retention of BPs depends on host factors, such as binding site availability and renal function [Cremers SC *et al.,* 2005].
Clinical studies have indicated a very wide range of rate retention among BPs. For example, the retention of pamidronate range between 47 and 74% in patients with osteoporosis [Cremers S *et al.,* 2002], while in patients with Paget's disease the skeletal retention of olpadronate range between 10 and 90% [Cremers SC *et al.,* 2003] and between 12 and 98% in patients with breast cancer and bone metastases [Cremers SC *et al.,* 2005].

2.4.6 Metabolism

As already outlined, thanks to the P-C-P bonding, bisphosphonates have demonstrated to be quite resistant to hydrolysis compared to the analogue pyrophosphate. In vitro and in vivo studies [Rodan GA 1996], Merck research laboratories have demonstrated the extreme stability of these compounds and, moreover, have not revealed the presence of active metabolites (experiments were run on

pamindronate and etidronate), usually accountable for pharmacological toxicity.

2.4.7 Excretion

Bisphosphonates are excreted unmodified in the urine, studies assessed with C14 have demonstrate a small percentage of BPs (parenterally administered) is excreted with bile as well. In pharmacological doses, renal excretion is divided in three fundamental phases: glomerular filtration (GFR), tubular secretion (CLs) and tubular lumen reabsorption (CLra).

At the moment, it is safe to claim that renal excretion of bisphosphonates is concentration-dependent and saturable, features that lead to the conclusion that a system of active transport might exist. The human renal clearance of bisphosphonates is in a range of values between 0.9 and 1.5 mL/min kg.

2.4.8 Elimination

BPs are excreted unchanged by glomerular filtration in urine, except a very small percentage of parenterally administered BPs, which are excreted in the bile. After they attach to bone, BPs are desorbed from hydroxyapatite during bone resorption and are taken-up by osteoclasts, and they are liberated again only when the bone in which they are present is resorbed. This explains the very slow and long elimination of all BPs from the skeleton [Drake MT *et al.,* 2010]. Therefore, the rate of the bone turnover influences the half-life of this drug [Lin JH *et al.,*1996].
However, it is unclear what percentage of the dose is metabolized and how BPs are exactly excreted.

Plasma elimination

As already stated, the permanence of bisphosphonate in blood plasma is very short (1 or 2 hours). The removal of this drug from systemic circulation is mediated by the seizing action of bone tissue, storing it, and by the kidney, causing its removal.

Bone elimination

This topic has been frequently discussed by several researchers and results seem to suggest that bisphosphonates, once absorbed by the bone, are released only when the bone undergoes reabsorption. Thanks to these results it is possible to understand that the biological half-life totally depends on the osseous turnover frequency, a phenomenon frequently slowed down in patients treated with these substances, sometimes because of age. Some Authors claim that bisphosphonates

might persist in the bone tissue for more than 10-12 years [Lin JH, Russel G *et al*. 1999; Melo MD *et al*. 2005] even though, as stated in a review published on Bone in January 2011, until today no measurement of concentration on human bone biopsies has been assessed [Cremers *et al*. 2011], but in man the quantity of BP in bone has been calculated referred to the administered dose and the concentration detected in serum and urine.

A biological half-life (t1/2γ) of approximately 11 years, similar to those of calcium and other minerals in bone, has been reported in a study carried out on 21 female patients undergoing menopause treated with aledronate for osteoporosis [Khan SA *et al* 1997].

This study, carried out in 1997 by a team of English researchers and published on the Journal of Bone and Mineral Research is the only one to have a long follow-up: 18-24 months. The 21 women enrolled in the study (mean age 66 years old) had received 30 mg of intravenous aledronic acid for 4 consecutives days (7.5 mg/die); urinary excretion of the drug has been monitored in the following 18-24 months. About 54% of the total dose of aledronate was excreted, with urine, in the first week after intravenous administration; as a consequence, 7 days after administration, approximately 6% of the total drug was stored in the skeleton. From this moment on, the release of aledronate from bone is characterized by a slower elimination phase. At the end of the 18 months, approximately 30% (9 mg) of the administered dose was not detected in urine, Authors had therefore supposed that it was accumulated in the skeleton. The terminal elimination half-life was estimated by a logarithmic-linear regression of the percentage held back compared to the temporal curve between 240 and 540 days, replacing the declivity of the line of regression in the equation: $t1/2\gamma = -\log 2/\text{slope}$. Data were sufficient for the analysis of the pharmacokinetic in 11 patients. Based on the analysis of data between 240 and 540 days, it was calculated, as previously stated, a half-life of approximately 11 years.

In our knowledge, there are no other works in which the elimination of bisphosphonates was monitored for more than some weeks after the administration of the drug.

On this topic, a study published on Clinical Drug Investigation [Lasseter, Porras *et al.* 2005] calculated the terminal half-life of aledronic acid using the results of the first 30 days of the just mentioned study and observing and half-life of only 11 days. This work highlights the importance of an adequate length of follow-ups with the purpose to carefully estimate the real elimination half-life of bisphosphonates. The relatively short (days instead of years) terminal half-life reported for some bisphosphonates, based on only 30 days or fewer of follow-up, might indeed underestimate greatly the real half-life.

2.5 Pharmacokinetic/Pharmacodynamic *(PK/PD) models*

PK/PD modeling integrates pharmacokinetic and a pharmacodynamic models into one set of mathematical expressions to clarify the time course of effect intensity in response to administration of a drug dose.

Exact pharmacodynamics information has been obtained for the first time in humans during treatment of Paget's disease, which has demonstrated that the initial effect of BPs treatment is the decrease of bone resorption.

Gasser *et al.,* using different IV doses of bisphosphonates, showed that BPs effect was dose-dependent [Gasser JA *et al*., 2008]. Many preclinical studies have demonstrated different potency of BPs. The FACT study showed that alendronate 70 mg decrease biochemical markers of bone turnover and increase BMD (Bone Mineral Density) significantly more than risedronate 35 mg, in women with postmenopausal osteoporosis [Rosen CJ *et al*., 2005]. Moreover, the nature of bone disease is a further determinant of the response of biochemical markers of bone resorption to BPs. For example, single IV dose of 75 mg of pamidronate, could provoke variable responses depending on the disease. The effect lasts longer in patients with Paget's disease of bone, but only a few days in patients with hypercalcemia of malignancy.

Therefore, the complete effect of BPs on bone resorption depends on many factors: a) potency of BPs; b) pharmacokinetics; c) the initial dose; d) the bone disease to be treated.

To better understand the right efficacy of a certain dose regimen of a BP, quantitative PK/PD studies are essential.

Side effects and way of use

Bisphosphonates can be administered orally (os), intravenously (ev) or via intramuscular injection (im). Oral ingestion, even though very simple, doesn’t allow an absorption superior to 5%, it is therefore necessary the employment of high doses with the purpose to increase the bioavailability and obtain the desired effect. The gastric absorption of bisphosphonates is affected by the simultaneous ingestion of beverages (exception made for water) and food (especially those containing calcium); it is therefore recommended a fasting ingestion. The use of high doses of this drug leads to side effects among which the most fearsome for incidence, the gastrointestinal effects, like esophagitis, nausea, vomiting and diarrhea. For this reason, it is considered advisable a sitting or standing position for 30 minutes after the ingestion of the drug and is also advisable to avoid the prescription of these products on patients with esophageal problems [Ross *et al*. 2004].

Minor gastrointestinal side effects can be experienced more frequently because of the big capsules

and tablets of less effective bisphosphonates like etidronate and clodronate. Amine-bisphosphonates, if taken orally, are associated to more serious side effects: occasionally erosive esophagitis and gastritis can be reported [Brown *et al*. 2004].

These effects can be dose-dependent and related to the direct contact of the undisolved crystals of the drug with the mucous membrane covering the gastrointestinal tract. A synergic action between nonsteroidal anti-inflammatory drugs (NSAIDs) and bisphosphonates has been noted: this phenomenon could accelerate the gastric ulcerative process. Bisphosphonates, moreover, can lead to a deleterious effect on the healing of gastric ulcers [Carter *et al.* 2005]. Cases of ulcers affecting the oral mucous membrane following the ingestion of aledronate, probably because of a mis-ingestion of the dose, have been reported. Patients reported suction and/or chewing of the pills, this causing a direct and prolonged contact with the mucous membrane, producing an irritating local process. Oral therapy with bisphosphonates is associated to a high percentage of non-compliance and give-ups due to the complex modality of ingestion and gastrointestinal side effects [Conte 2004].

Bisphosphonates parenterally administered, certainly more efficient, have a greater bioavailability and are commercialized in pharmaceutical formulations with doses and infusion times shorter and shorter in order to avoid unpleasant side-effects related to kidney functionality that seem to depend from both dosage and administration velocity of the drug [Berenson *et al*. 2004]. The strongest bisphosphonates, belonging to the so-called third generation, allow to further reduce the amount and the intervals of administration, reducing the risks of developing renal pathologies (i.e. zolendronic acid, infusion of 4 mg in 15 minutes). Patients receiving bisphosphonates for long-term therapies or at risk for renal pathologies have greater possibility of developing a degeneration of the renal functionality. Usually, however, the increasing of the serum creatinine is transient and of modest or moderate amount. In many patients treated with zolendronic acid the spontaneous decay of the renal functionality resulted to be less frequent and, in half of the cases, happened in patients affected by multiple myeloma: this pathology, by itself, is associated with an increase of the risk for renal failure [Berenson *et al*. 2005].

Other side effects, caused by bisphosphonates and emerged in literature, are flu-like symptoms, asthenia, fatigue, bone pain and, with a minor incidence, dyspnea, lymphocytosis, anemia, intestinal edema and serum electrolytic balance subversion, a circumstance that can be corrected with simultaneous administration of Ca^{2+} and Vit. D [Migliorati *et al*. 2005]. Infrequently, bisphosphonates can have ocular side effects: conjunctivitis, uveitis, scleritis, episcleritis, photophobia, pain, ocular visual impairment. Under these circumstances it is advisable to be visited

by an ophthalmologist and evaluate the suspension of the drug (which is necessary for scleritis). A side effect that must be taken under serious consideration is the maxillary osteonecrosis [Migliorati *et al*. 2005], a topic that will be discussed in the following chapters.

3. Osteonecrosis

3.1 Definition and histopathological carachteristics

Ostenonecrosis, also called avascular necrosis (aseptic), is the death of the bone tissue in any skeletal section. The necrosis of the bone and of the bone marrow is a phenomenon moderately frequent in the human organism. The common pathognomonic pathway is represented by the insufficient vascular supply, anyway the etiology can be various: mechanical vascular interruption in fractures, chronical use of corticosteroids, thrombosis and embolisms, vascular damage in vasculitis induced by radiant therapy, intra-osseous pressure increase with vascular compression, venous hypertension. The cause of necrosis, often, is not determined but fractures excluded, the greatest part of osteonecrosis events are of idiopathic origin.

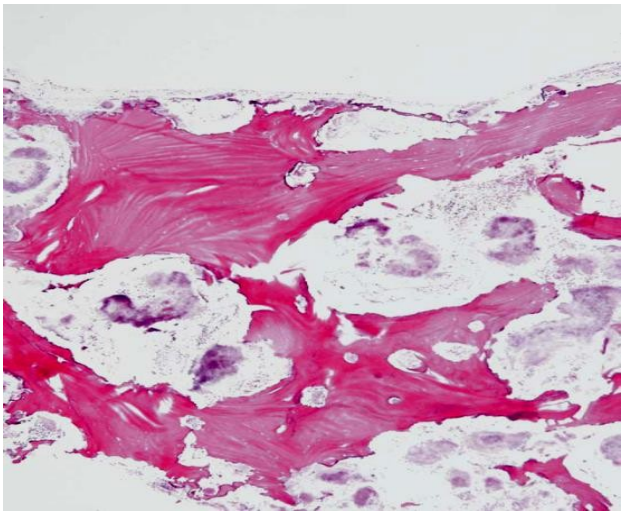


Figure 7: Necrotic bone with multiple inflammatory cells and bacterial colonies Hellstein and Marek Letter to the Editor J. Oral Maxillofac Surg 2004

Regardless of the etiology, the histopathological characteristics of necrosis are similar. In bone marrow infarctions, necrosis has a map pattern and involves the trabecular bone and the bone

marrow; the cortical area is usually spared thanks to collateral vascular flux. Vital bone areas show a vascularized connective tissue with veins congested by red blood cells while, in the infected areas, the major protagonists are inflammatory cells and bacterial colonies (Melo et al. 2005; Hellstein et al 2005).

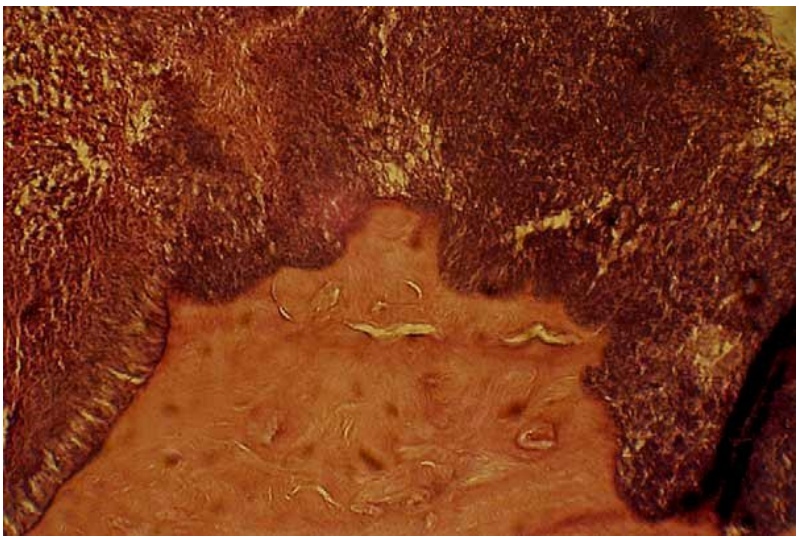


Figure 8 : Necrotic bone fragment with colonies of Actinomices Lugassy, Shaham Am J Med 2004

Non-vital bone is recognizable by the presence of empty lacunae, is surrounded by necrotic adipocytes that frequently undergo damage, releasing fatty acids able to form, with calcium, insoluble soaps that can persist in loco for the entire life of the subject. Those cases undergoing healing, osteoclasts reabsorb the necrotic trabeculae, those remaining work as framework for the deposition of new vital bone thanks to a process known as crawling substitution.

Symptoms associated with osteonecrosis are different and depend on the localization and extension of the infarction. Typically, subchondral infarctions cause chronic pain, initially associated with movement, then stable in time, even during rest. On the contrary, bone marrow infarctions are clinically silent and usually stable in time, exception made for very extended or infectious lesions. Osteonecrosis sites, in fact, can undergo superinfection, often bacterial, causing therefore an osteomyelitis. Staphilococcus aureus is responsible of about 80-90% of all the cases of bacterial ostemyelitis (in all those cases where a correct identification of the organism causing the infection was possible). Once penetrated in the bone, bacteria reproduce inducing an acute inflammatory response that leads to cellular death. The affected bone undergoes necrosis in the first 48 hours, bacteria and inflammation spread along the bone axis and can penetrate through the Havers' channels reaching the periosteum; it is therefore possible to have the formation of sub-periosteal abscess. The raising of the periostium compromises the hematic flux in the afflicted regions and

consequently it is possible to have segmentary bone necrosis. The periosteal membrane can be perforated by the infectious process, leading to an abscess of the soft tissues and, eventually, to the formation of a fistula.

The attempt by the organism to limit the infectious process can cause the formation of a sequestrum. This term defines a portion of the bone delimited by reactive granulation tissue. The necrotic portion of the bone is then separated from the vital portion, delimiting the spreading of the infection. Otherwise, the process could spread to the neighboring osseous areas.

The osseous sequestrum must be removed in order to stop the inflammatory reaction and to allow the reparation process. If it remains, sequestra can break apart, forming free foreign bodies that can come out through the fistulous tract.

In peculiar categories of patients, the infective process can become chronic furtherly complicating the treatment. Chronic osteomielytis, called osteitis as well, is one of the possible consequences of acute osteomyelitis (not treated or treated in an inadequate way) but can also develop from a low grade and long persisting inflammatory reaction that have never faced a relevant acute or clinically evident phase. The success of the therapy depends by the virulence of the microorganism, by the extension and localization of the pathology and by the health conditions of the patient. Age and pre-existing systemic factors, as Paget's disease and sickle-cell anemia, must be considered as well.

3.2 Maxillae's osteonecrosis

The osteonecrosis of the maxillae is a potential complication that can affect patients suffering from neoplasms and are undergoing radiant therapy, chemotherapy or other specific treatments or patients that showed embolic events due to tumoral or infective causes. An association between avascular necrosis of the maxillae bones and several risk factors has been recognized. Among these there are traumatic events, women, old age, edentulous patients, combined anti-neoplasm therapies, blood dyscrasia, metastasis, coagulopathies, dental surgeries, alcohol and tobacco, infections. A correlation between therapy with bisphosphonates and maxillary osteonecrosis seems to be well-founded [Damato *et al*. 2004].

The irradiated bone, for the treatment of malignant tumors of the head and neck district, is particularly susceptible to infections. Radiations have deleterious effects on osteocytes, osteoblasts and endothelial cells, causing a reduction of the bone capacity to react to injuries. As a consequence of the diminished vascularization and the demolition of the osteocytes, almost 20% of patients that underwent radiant therapy develop osteonecrosis, usually followed by a secondary infection.

Factors that frequently cause this condition are periapical inflammation originated from a non-vital dental element, traumatic events, extractions, advanced periodontal disease, fractures or osseous expositions adjoining with the dermis or with the mucosae membrane of the oral cavity. Other important factors are oral hygiene, nutritional conditions and alcoholism. Identification of a specific infective agent thanks to microscopy or microbiological techniques is difficult. Bias linked to sampling is frequent, due to the small size of the bacterial foci, difficult to reach, and due to contamination by the resident oral microbial flora; moreover, the administration of antibiotics may reduce the possibility to isolate the culpable agent. From the collected data, however, it seems that the greatest part of the cases of osteonecrosis is caused by bacteria (staphylococci, streptococci, bacterioides, actinomyces).

The mandible, especially in its posterior portion, is more frequently affected by osteonecrosis compared to the maxillae. Pain, in various degrees, is a frequent condition, not necessarily related to the extension of the lesion. It is frequent the finding of a maxillary tumefaction and dental mobility, fistulous tracts and, rarely, paresthesia.

Radiologically, ostenecrosis displays, in the majority of cases, as a radiolucent material with delimited opaque areas. The radiolucent image of the lesion is described as weaved; these weaves can be of big dimension and often have undefined edges. The inflammatory reaction in chronic osteomyelitis can range from extremely light to intense. In less serious cases, histological diagnosis can be difficult due to the similarities between osteo-fibrous lesions, like ossifying fibroma and fibrous dysplasia. Signs of osteoblastic and osteoclastic activity can be present, associated to irregular osseous trabeculae. Inflammatory cells are more copious and osteoclastic activity is enhanced.

Treatment consists in the identification (thanks to antibiotic sensibility tests and culture tests) of the pathogens responsible and in the administration of appropriate antibiotics, if necessary surgery of the lesion can be performed. The association of more antibiotics showed a greater effectiveness compared to treatment with only one active ingredient. In cases of bone sequestrum treatment with surgery is able to accelerate the healing process. It has been suggested the use of surgery in order to resolve the fistulous tract and eliminate the scar tissue. In cases where pathological fracture is a risk, it is necessary the immobilization of the maxilla. If chronic osteomyelitis is present or radiation-related osteonecrosis resistant to treatment, a substantial benefit comes from the use of hyperbaric medicine with oxygen, taking advantage of the capacity of oxygen in stimulating vascular proliferation, collagen synthesis and osteogenesis. Side effects include: viral infections, optic

neuritis, remains of malignant neoplastic tissue and some lung dieseases. Therapy consists in cycles of treatment of two sessions for two hours in a hyperbaric chamber (100% oxygen at 2 atmospheres) that can last for several weeks. Hyperbaric medicine was indicated as a useful preventive measure in patients undergoing radiant therapy to the head and neck district [Marx *et al*. 1985], but a more recent study has evaluated the benefits of this treatment in these types of lesions as not more significant compared to the administration of a placebo [Annane *et al*. 2004].

3.3 Osteonecrosis and denosumab

Denosumab is a monoclonal antibody recommended for the treatment of post-menopausal osteoporosis and for prevention and treatment of osseous metastasis. This drug (monoclonal antibody) has high affinity and specificity for the RANKL receptor. It works inhibiting the reabsorption of the bone, inhibiting osteolysis and preventing the bonding between RANKL and RANK (a receptor activating NF-kB, found on osteoclasts) blocking the differentiation and the activation of osteoclasts. These new drugs have a different mechanism of action compared to bisphosphonates since they prevent the formation, differentiation and the function of osteoclasts acting on the RANKL receptor [Camila-Carvalho de Oliveira *et al*, 2016].
Denosumab as well has side effects, more bearable compared to bisphosphonates, like fever, arthralgia and renal toxicity. Among the most severe side effects, osteonecrosis of the maxillary bones can be found.
According to prospective longitudinal studies [Henry *et al*, 2011] there are no differences of incidence for osteonecrosis between patients treated with zolendronate 4 mg (1.3%) and denosumab 120 mg (1.1%). A study authored by Stopeck et al. evaluated the incidence of osteonecrosis in patients with breast cancer treated with zolendronate (1.4%) and with denosumab (2%) [Stopeck et al, 2010].

3.4 Medication-related osteonecrosis of the jaw (MRONJ)

There is still not an agreement upon a term universally accepted to identify this new condition. This side effect has been called BRONJ (bisphosphonates-related osteonecrosis of the jaw), BRON (bisphosphonates- related osteonecrosis), BAOJ (bisphosphonates-associated osteonecrosis of the jaw) or simply ONJ (osteonecrosis of the jaw). The definition of bisphosphonates osteonecrosis was formulated for the first time in 2007 by the American Association of Oral and Maxillofacial Surgeons (AAOM) as the "presence of necrotic bone exposed in the oral cavity for more than 8 weeks in a patient undergoing bisphosphonates therapy that never underwent head and neck radiant

therapy". According to AAOM, therefore, patients affected by this pathology must have these following 3 characteristics:

1. Current or previous therapy with bisphosphonates;
2. Exposed necrotic bone in the maxillary-facial area for more than 8 weeks;
3. Absence of current or previous radiation therapy in the maxillary area.

The definition adopted by the most influent international scientific societies has remained unchanged. The same definition was adopted by the Italian Ministry of Health in the "Recommendations for prevention of osteonecrosis of maxilla/mandible caused by bisphosphonates". This definition of BRONJ is presented in a descriptive form, identifying the most typical clinical manifestation of the disease in patients considered at risk, which means the expositions of the bone in the oral cavity. Therefore, the diagnosis of this pathology remains exclusively based on the clinical observation of this sign in the oral cavity. The definition of this pathology appears to be limited. The recent introduction of a "stage 0" of disease in the 2009 AAOMS recommendations [Ruggiero, Dodson *et al*. 2009], including patients with signs and symptoms of the disease but without osseous exposition, is in contrast with the definition of BRONJ itself.

In a recent publication supported by SIPMO and SICMF of 2013 (Clinical-therapeutical recommendations on osteonecrosis of the maxillary bones associated to bisphosphonates and its prevention) the Authors tried to find a new definition of the disease supported by the latest knowledge on the topic [Bedogni, Fusco *et al.* 2012], and identified the following criticalities:

- The presence of "exposed necrotic bone in the oral cavity" is only one of the possible clinical manifestations of the pathology; therefore, to consider as affected by BRONJ only those patients that are presenting the exposed bone in the oral cavity prevents the diagnosis of BRONJ in and potentially large number of patients [Bagan, Jimenez *et al*.2009]

- The presence of "exposed necrotic bone in the oral cavity" is often a late sign of the pathology; the presence of this clinical sign in order to formulate a diagnosis can be the reason of a diagnostic delay that could lead to a tardive beginning of the fundamental therapy, considerably limiting the effectiveness. The new proposed definition of BRONJ [Bedogni, Fusco *et al*. 2012] denotes the previous considerations and is stated as follow:

> "the osteonecrosis of the maxillae associated to bisphosphonates is a drug-related side effect characterized by the progressive destruction and necrosis of the mandible and/or the maxilla in subjects exposed to treatment with amine-bisphosphonates, in absence of previous radiant therapy".

This latest definition, however, does not include all the new drugs inhibiting the osseous reabsorption documented to be causing osteonecrosis and, therefore, in 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) proposed a new nomenclature, from Osteonecrosis of the Jaw (BRONJ) to Medication-related osteonecrosis of the jaw (MRONJ) in order to include the increasing number of osteonecrosis cases caused by anti-reabsorption drugs (like denosumab) and by anti-angiogenetic therapies [Rosella *et al*, 2016; Ruggiero *et al.,* 2014)] Therefore, the MRONJ is described as a severe side effect consisting in a progressive destruction of the bone in the maxillary-facial area (Rosella et all, 2016) and must present the following characteristics:

1) Current or previous therapy with drugs inhibiting the osseous reabsorption or angiogenetic agents

2) Extra-oral osseous exposure or necrotic bone with intra-oral or extra-oral fistula in the maxillary-facial area for more than 8 weeks

3) Absence of current or previous radiation therapy

3.4.1 Epidemiology of BRONJ

In December 2002, the manufacturing company received the first spontaneous warning of an event of osteonecrosis of the jaw ascribed to pamidronate or zolendronic acid. After this warning and after publication [Marx *et al*. 2003], by a maxillary-facial surgeon in Florida, of 36 cases of avascular osteonecrosis of the mandible, the description of similar cases during therapy with bisphosphonates increased, both as spontaneous warnings to several agencies for pharmacovigilance and as articles and case reports on medical journals. The National Network of Pharmacovigilance, taking into consideration the warnings for side effects (Adverse Drug Reaction-ADR) received from 2001 to mid-2007, noted 690 advisories of ADR caused by bisphosphonates administered intravenously or orally.(figure 9)

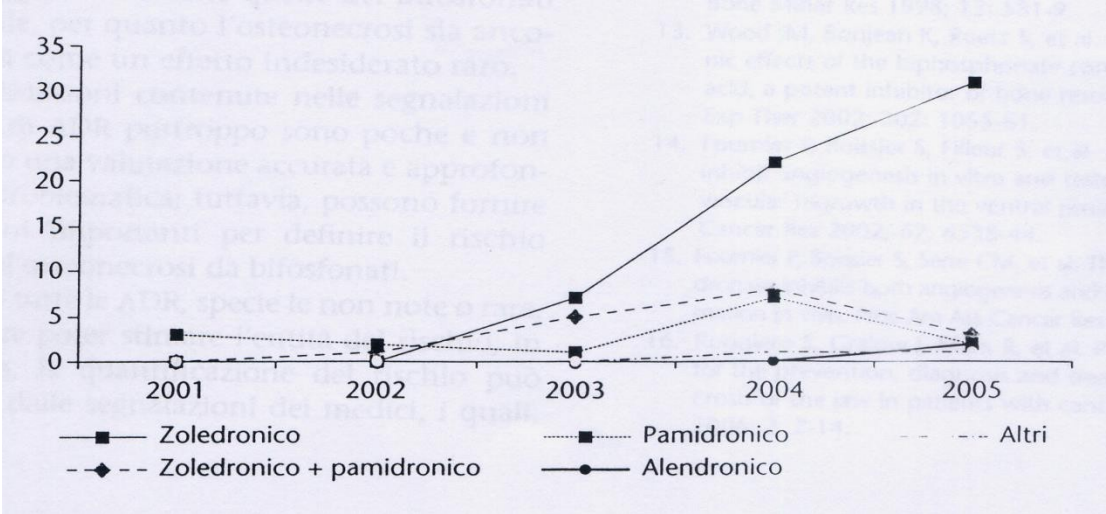


Figure 9 : Temporal trend of bisphosphonates osteonecrosis'warnings

The first reviews on osteonecrosis caused by bisphosphonates were published in 2003 by Marx with 36 cases [Marx *et al*. 2003]. Since then, a study with 63 cases, held by Ruggiero [Ruggiero *et al*. 2008)] by Bagan *et al*. (10 cases) and by Marx *et al* (119 cases), together with a series of case reports and case series, have added further information on the characteristics of this effect.

Currently, despite osteonecrosis can be considered as a side effect scarcely associated to therapy with bisphosphonates, a worldwide survey has estimated its effect, on 1203 patients undergoing intravenous administration of bisphosphonates for the treatment of multiple myeloma (904 patients) or breast carcinoma (299 patients), as around 12.8% in patients with myeloma and 12% on patients with carcinoma [Durie *et al*. 2005]. After 36 months of therapy, the effect of osteonecrosis resulted to be 10% among patients treated with zolendronate and 4% among patients treated with pamidronate. (Figure 10)

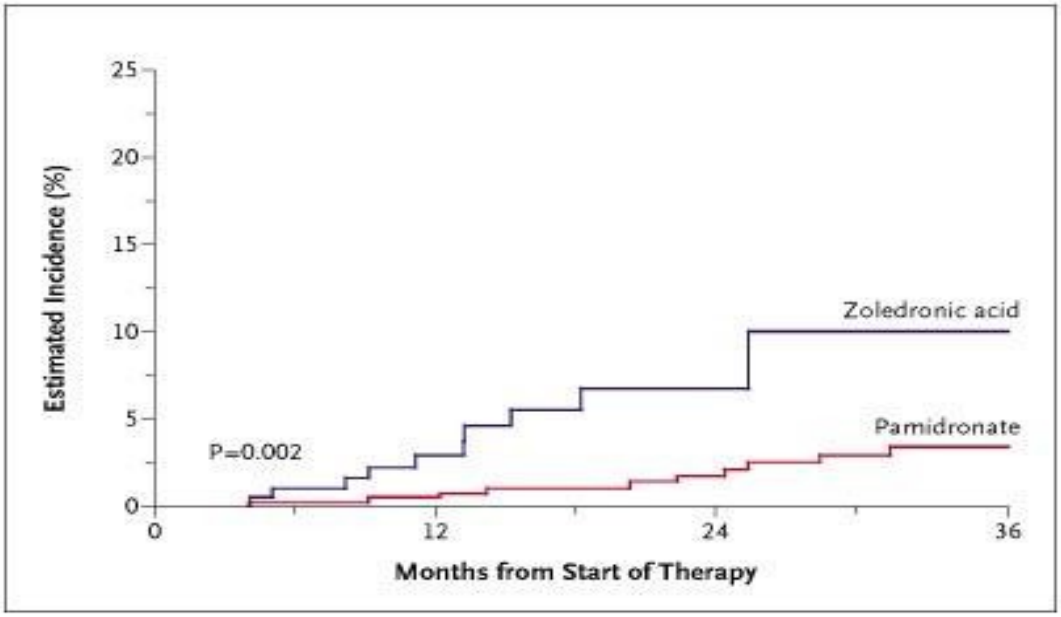


Figure 10 : Time of onset of osteonecrosis caused by bisphosphonates in patients treated with zolendronic acid or pamidronate (Durie BGM et al. N Engl J Med 2005; 353: 99-102)

According to some studies on a cohort of patients affected by multiple myeloma and in treatment with zolendronic acid administered intravenously, the prevalence of osteonecrosis of the maxilla is about 1.9%. The onset of osteonecrosis of the maxilla was more frequently associated to chemotherapy, treatment with corticosteroids, old age, in women, anemia, parodontopathy and dental extractions [Pozzi S., Baldini L. *et al*. 2007]

Other Authors state that the incidence of developing ONJ in patients undergoing therapy with bisphosphonates intravenously varies from 0.4% to 12% [Tirelli *et al*. 2009] According to other Authors, it ranges from 0.8% to 12% [Gaudin *et al*, 2015].

According to Grbic [Grbic et al 2011] in a study published on November 2010, the incidence of osteonecrosis in patients undergoing therapy with zolendronic acid (5mg ev per 1vv/aa) results to be less than 1 per 14200 patients under therapy/year.

Walter conducted a study on a population of patients suffering from multiple myeloma that were administered with zolendronate and reported a prevalence of ONJ of 20.5%; in all these cases a surgical traumatism as a triggering factor was observed [Walter *et al.* 2010].

One of the main risk factors for osteonecrosis is the duration of the therapy with bisphosphonates.

The risk is about 1% after the first year and 21% after 3 years of treatment with colendronic acid. On the other side, it's around 0% for the first year and 4% after 3 years for therapies with alendronate. Lesions were more commonly observed in the jaw compared to the maxilla (2:1 ratio). Between 1998 and 2004 researchers of the Research on Adverse Drug Events and Reports (RADAR) have identified 561 cases of osteonecrosis of the maxilla in patients with cancer treated with zolendronate. From 2003 to March 2006, 261 cases were reported in literature, 141 of which were ascribed to pamidronate, 101 to pamidronate and zolendronate, 21 toalendronate, 1 to risendronate and 4 to combined therapy (1 for alendronate, pamindronate and zolendronate; 1 for zolendronate and ibandronate; 1 for alendronate and zolendronate)

Conte-Neto, in a publication observed two cases of jaw osteonecrosis in women undergoing therapy with aledronate for 3 years for rheumatoid arthritis that developed spontaneous jaw lesions; the Author has therefore supposed a possible synergism between the effects of the drug and rheumatoid arthritis in developing osteonecrotic lesions [Conte- Neto *et al*. 2011]

Cases of patients showing osteonecrosis not related to cancer have been reported: 19 were suffering from osteoporosis (17 treated with alendronate, 1 with risendronate and 1 with alendronate and zolendronate) and 9 affected by Paget's disease (4 in treatment with alendronate, 4 with pamidronate and 1 with alendronate and pamindronate). Probably this data are an underestimation of the real incidence of such a side effect in patients treated with oral administration of bisphosphonates. In a review by the American Dental Association, cases of osteonecrosis associated to oral use of bisphosphonates worldwide resulted to be 170 for aledronate, 12 for risedronate and 1 for ibandronate [Parish *et al*. 2009].

It has to be noted, however, that in the 2009 guidelines of the American Association of Oral and Maxillofacial Surgeons [Ruggiero *et al.* 2009], it is repeated several times that the risk to develop osteonecrosis related to oral administration of bisphosphonates increases with a duration of the therapy superior to 3 years; in fact, the risk of developing this side effect would be of 1/1000000 when the therapy is shorter than 3 year, while increases to 1/10000 when exceeding 3 years of therapy [Gaudin *et al*. 2015]. Patients in therapy with oral bisphosphonates have a minor risk, however, of developing osteonecrosis compared to patients treated intravenously [Migliorati *et al*. 2005; Ruggiero *et al*. 2004; Marx *et al*. 2005; Ruggiero *et al*. 2006]. On this matter, a study of the Pennsylvania University [Akintoye *et al*.] published in 2012 on the Journal of Evidence Based Dental Practice, took into consideration 191 patients affected by osteonecrosis caused by bisphosphonates and associated to 573 case-control patients. This study highlighted that ONJ can

happen at any moment of the therapy with bisphosphonates (oral or parenteral) but a significant risk of BRONJ increased after 2 years of therapy.

In a work published in 2011, patients treated with oral alendronate had an incidence of osteonecrosis lower than 0.004% for patients with age-related osteoporosis or with osteoporosis caused by hormonal metabolic variations, while the incidence was greater than 0.1% in patients with iatrogenic osteoporosis glucocorticoid-related [Malden *et al.* 2011].

In 2007, the Food and Drug Administration approved the use of zolendronic acid (Zometa) once a year for the treatment of osteoporosis alternatively to oral bisphosphonates (USA FDA 2009).

Since 2003, more than 1000 cases of BONJ have been reported and/or documented [Leite AF *et al.,* 2006; Migliorati CA *et al.,* 2006; Badel T *et al.,* 2013; Hasmim M *et al.,* 2007]. The prevalence of BRONJ in patients with oral BPs treatment for osteoporosis is between zero to 0.04% [Malden N *et al.,* 2102*;* Taylor T *et al.,* 2013]. Results showed that prevalence of BRONJ in patients under treatment with BPs form more than 2 years, ranges from 0.05% to 0.21% and appeared to be related to duration of exposure [Felsenberg D, 2006]. Furthermore, it has been evaluated that prevalence of BRONJ in those prescribed high dose IV BPs is significantly higher than that seen with low dose IV or oral BPs, with prevalence rates of zero to 0.35% [Fellows JL *et al*., 2011; Cartsos VM *et al*., 2008]. Instead, for oncology patient, who have received higher doses of IV BPs than osteoporotic patient, prevalence ranges from zero to 0.19% [Cartsos VM *et al*., 2008; Assaf AT *et al*., 2013]. The incidence of BRONJ in patients prescribed oral BPs to treatment osteoporosis varies between 1.04 to 69 per 100,000 patient-years [Khan AA, *et al*., 2011; Tennis P *et al*., 2012; Ulmner M *et al*., 2014], while for those receiving IV BPs ranges from 0 to 90 per 100,000 patient-years [Lyles KW *et al*., 2007; Powell D *et al*., 2012]. Research about use of alendronate, the most widely prescribed oral-BP, showed that the incidence of BRONJ is 0.7 cases per 100,000 person-years [American Dental Association Council on Scientific Affairs, 2006].

In patients with cancer, the incidence of osteonecrosis of the jaw appears to be related to dose and duration of BP exposure, in fact, in those patients treated with IV BPs, incidence ranges from 0.8% to 12% [American Dental Association Council on Scientific Affairs, 2006].

However, there is considerable variability in the reported incidence and prevalence rates of BRONJ, especially because the incidence changes in association with monthly administration of IV BPs [Saad F *et al*., 2012; Thumbigere-Math V *et al*., 2012; Bonomi M *et al*., 2010].

3.4.2 Risk factors

Several risk factors have been investigated in last decades and they are summarized in Table 1

Risk factors	-Drug-related risk factors;
	Local risk factors;
	-Demographics;
	-Systemics;
	- Other possible risk factor associated to the onset of osteonecrosis

Table 1

Not all patients taking bisphosphonates develop osteonecrosis. Scientific literature is trying to study the possible risk factors. Five categories have been identified:

Drug-related risk factors:

• Pharmacological potency: a classification of the pharmacological potency has been made: the one with the greatest potency is zolendronate (100.000 pr its relative potency) followed by ibandronate (10.000), risedronate (5.000 pr), alendronate (1.000 pr) and as last pamidronate (100 pr) [Hilner *et al.* 2000];

• Duration of therapy: the risk increases with the increasing of the duration of therapy after treatment with zolendronate, the risk is around 1% for the first year and 15% after 4 years [Dimopoulos *et al*. 2006].

Local risk factors:

• Dental-alveolar surgery: extractions, implants insertion, endodontal and parodontal surgery; about 60% of all cases are associated with dental extractions. On this matter, a study demonstrated that the prevalence of osteonecrosis in patients with cancer that did not undergo dental extraction was 1%, while patients that had dental extractions had a prevalence of 7-9%. Patients taking intravenous bisphosphonates and undergoing dental-alveolar surgery are 7 times more at risk compared to patients that do not undergo surgery;

• Anatomy: many cases are located on the tongue-side of the jaw (where the mucous membrane in particularly delicate), in the context of tori (palatine or lingual) or exostoses. The majority of cases affects the jaw compared to the maxilla (2:1 ratio);

• Other oral problems: patients with history of oral inflammatory pathologies, as dental or

periodontal abscesses, have a risk 7 times greater of developing osteonecrosis; a severe periodontal disease has been considered as a risk factor for ONJ [T. Yamazaki *et al*. 2012].

Demographic risk factor:

• Age: at the passing of each decade, risk increases of 9% for patients treated with IV bisphosphonates;

• Race: Caucasian race is a risk factor according to a 2006 study [Badros *et al*. 2006].

Systemic risk factors:

• Cancer diagnosis: risk is greater for patients with a diagnosis of multiple myeloma compared to breast cancer;

• Osteopenia/osteoporosis: associated to cancer diagnosis;

•I n contrast to what stated in 2007, the American Association of Oral and Maxillofacial Surgeons (AAOMS), in its 2009 report, showed that some studies had highlighted an increasing of the risk of onset in patients undergoing chemotherapy with cyclophosphamide, erythropoietin and steroids [Ruggiero *et al*. 2009; Jadu *et al*. 2007; Zervas *et al*. 2006].

Other possible risk factors associated to the onset of osteonecrosis:

• Therapy with corticosteroids;

• Diabetes;

• Alcohol use;

• Poor personal hygene.

Khamaisi *et al.,* in a study conducted on 31 patients affected by maxillary osteonecrosis and with therapy with bisphosphonates, highlighted that 18 of these patients (58%) resulted to be suffering from diabetes mellitus. This percentage, much higher of the incidence of diabetes in the general population (14%), is greater than the percentage of patients with diabetes in the control group (composed by oncological patients treated with bisphosphonates and not affected by osteonecrosis

(12%). According to this study, diabetes can be considered as a risk factor for the onset of osteonecrosis in patients taking bisphosphonates [Khamaisi *et al*. 2007].

Lazarovici *et al*. evaluated 101 patients affected by osteonecrosis, 85 of which under parenteral therapy with bisphosphonates and 16 with oral therapy. This study states that no statistically significant association can be made between osteonecrosis of the maxilla and diabetes, corticosteroids therapy, anti-angiogenetic drugs and periodontal pathologies [Lazarovici *et al*. 2009].

A case report published by our team at the end of 2011 on the Journal of Oral and Maxillofacial Surgery [Lodi *et al*. 2011] identified in photodynamic therapy (PDT) for the treatment of oral lesions a potential risk of onset for BRONJ.

Further studies are, at present time, necessary in order to validate the association of these possible risk factors with the onset of osteonecrosis.

Clinical risk factors (e.g. surgery to the jawbones) were initially suggested to predict the likelihood of BRONJ development, but more recent studies have demonstrated they are not reliable predictors as only 50% of patients with BRONJ report a history of dental surgery/infection as trigger of their necrotic bone disease.

BRONJ is usually associated with nitrogen-BPs and its risk is incremental with the duration of BPs therapy. In particular, the risk increases with potent IV preparations, which are normally used in the treatment of metastatic bone cancer or hematological disease. The reported rates of incidence for utilization of BPs in the first or second year is zero, but this increases after four years of use to about 0.21% [Kwon YD *et al*., 2012; Park W *et al*., 2010].

The bioavailability of these BPs is 70% in the bone, compared to less potent oral preparation, that are used most frequently in postmenopausal osteoporosis therapy [Drake MT *et al.,* 2008], which have a bioavailability in the bone of only 0.4–0.6 % [Marx RE, 2008; Ficarra G *et al.,* 2007; Marx RE *et al.,* 2005].

In most patients, tooth extraction, periodontal disease, dento-alveolar surgery or ill-fitting dentures seem to be the dominating event to development of BRONJ [AAOMS, 2007; Ficarra G *et al.,* 2007; Aghaloo TL *et al.,* 2011; Anavi-Lev K *et al.,* 2013].

Other risk factors that predispose patients to BRONJ are human immunodeficiency virus infection, diabetes and corticosteroid treatment. Moreover, BPs dose-reduction preventive strategies have also been suggested as risk factors (e.g. for individuals with multiple myeloma), yet in the absence of risk stratification models, their impact on BRONJ development/risk is unclear.In addition, the use

of steroids influences the development of BRONJ, because the long-term use of steroids decrease immune cells and delay wound healing. The risk of BRONJ shows an increasing trend also in diabetic and older patients [Barasch A *et al*., 2011; Peer A, *et al*., 2015], with the highest prevalence seen in patients 75 to 79 years [Lee JK *et al*., 2013]. Others risk factor are anemia [Barasch A *et al*., 2011], hyperthyroidism [Thumbigere-Math V *et al*., 2012], and dialysis [Jadu F *et al*., 2007].

There are reports that certain single nucleotide polymorphisms (SNPs) are related to a higher incidence of BRONJ; e.g. in the farnesyl pyrophosphate synthase [Marini F *et al*., 2010] or in the cytochrome P450 CYP2C8 genes [English BC *et al*., 2010; Sarasquete ME *et al*., 2008]. Studies on the correlation between the genome sequence and the risk of developing BRONJ are under investigation.

Thus, there are many proposed risk factors of developing ONJ, but the most common risk factor is dental trauma (dental extraction or previous dento-alveolar surgery) [Migliorati CA *et al*., 2005; Badros A *et al*., 2006; Woo SB *et al*., 2006].

3.4.3 BRONJ pathogenesis

Despite it is clear that the pathogenesis of BRONJ is multifactorial, the exact mechanisms has not yet clearly been understood. This dramatic lack of evidence is mainly related to a strong void of scientific knowledge about molecular basis of this disease. However, as BRONJ occurs only in a subset of BP users, it is likely that genetic variations among individuals confer susceptibility or resistance to its development.

3.4.4 Pathogenic theories

Several pathogenic theories for BRONJ have been proposed. The leading theory suggests that it may be caused by cessation of bone remodelling and turnover. BP can cause a marked over-suppression of bone metabolism which may result in accumulation of physiologic micro-damage in the jawbones, compromising biomechanical properties and ability to repair injury (e.g. tooth extraction) [Badel T *et al.,* 2013]. Even though some clinical observations seem to support this theory (almost 50-70% of cases described since 2003 present a history of trauma, dental surgery or dental infections as triggering factor) [Hasmim M *et al.,* 2007], evidence or experimental data, currently available, only demonstrate that this theory is putative.

On the other hand, other pathogenetic theories have been supposed and investigated, even if to a lesser extent. One of the proposed mechanisms suggest an “inside-out” process in which a low bone turnover induced by BPs leads to bone cells necrosis due to a decreased of blood flow; together

with infection, this leads to the development of exposed bone areas in the mouth [Ruggiero SL *et al,*. 2004; Marx RE, 2003]. However, other data suggest an "outside-in" process in which the first event is a mucosal damage, which is then followed by infection and consequently by bone necrosis. To date, available data suggest five main theories (Table. 2) regarding the development of BRONJ, which have been discussed as follows.

Hypothesis	Description	References
Inhibition of angiogenesis	Inhibition of vascular endothelial growth factor; vessel obliteration.	Croucher P *et al.,* 2003; Deckers MM *et al.,* 2002; Giraudo E P *et al.,* 2004; Hansen T *et al.,* 2006, 2007; Hunziker EB, 2000.
Alteration in bone turnover	Reduction of bone turnover; high remodeling rate of jaw; high numbers of osteoclast cells.	Garetto LP *et al.,* 1998; Hansen T *et al.,* 2006; Huja SS *et al.,* 2006; Russell RG *et al.,* 2007, 2008; Vignery A *et al.,* 1980.
Infection	Actinomyces species found in 73.2% of BRONJ cases; 90% of positive response to antibiotics versus Actinomyces spp.; presence of biofilm; stimulation of bone resorption; bacterial adhesion to nitrogen-BP coated HA.	Abu-Id MH *et al.,* 2008; Biasotto M *et al.,* 2006; Bisdas S *et al.,* 2008; Diel IJ *et al.,* 2007 Hansen T *et al.,* 2006, 2007; Kumar SK *et al.,* 2011 Nair SP *et al.,* 1996 Pap T *et al.,* 2003 Sedghizadeh PP *et al.,* 2008, 2009.
Bisphosphonate toxicity to bone	Accumulation of these toxic molecules within bone cells; 50% of IV BPs are internalized and retained indefinitely;	Khan SA *et al.,* 1997; Reid IR *et al.,* 2002; Reid IR *et al.,* 2007; Thompson K *et al.,* 2006.
pH	Acidic pH stimulates the release of toxic derivatives of BPs.	Abu-Id MH *et al.,* 2008; Sato M SL *et al.,* 2004.

Table 2 Different hypothesis of BRONJ pathogenesis.

Inhibition of angiogenesis

The necrotic process behind BRONJ may result from vascular insufficiency with damage to the microcirculation resulting, in predisposed individuals, in bone ischaemia and subsequent necrosis. This pathogenic model has already been hypothesized by other researchers who suggested that BP might inhibit angiogenesis in the jaws, leading to loss of blood vessels and a tendency to avascular ischaemic bone necrosis. [Croucher P *et al.,* 2003; Deckers MM *et al.,* 2002; Giraudo E P *et al.,* 2004; Hunziker EB, 2000], although there are conflicting results in the current literature.

In fact, the comparison of histopathology features of BRONJ and avascular necrosis of the hip showed that the former is often associated with inflammation, infection and soft tissue breakage whereas the latter consists of aseptic bone necrosis. This has led to suppose as the bone necrosis may be a phenomenon secondary to gingival ulceration and infection (osteomyelitis) of the jawbones, rather than being a primary event. Moreover, the intra-bony matrix necrotic changes observed in dogs exposed to long-term BP therapy is histopathologically different from traditional aseptic avascular necrosis of the hip as some normal vessels could still be observed.

These observations suggested that bone matrix necrosis due to BP could be a primary aseptic process (i.e. without soft tissue breakage and infection), but the pathogenic mechanisms may be different from that of femoral osteonecrosis. Osteonecrosis, or "avascular necrosis", normally occurs in the knee or hip and it is never associated with infection. Moreover, no cases of BRONJ have been reported. If BRONJ was just an avascular necrosis, it would be expected to be present in both the hip and knee, but there are not cases of BRONJ in none of these. However, there are some evidence that show an inhibition of vascular endothelial growth factor, which could lead to the development of BRONJ, subsequently to vessel obliteration [Hansen T *et al.,* 2006, 2007].

Some other studies, in contrast with those above mentioned, suggest that in BRONJ the vasculature remains intact [Hellstein JW *et al.,* 2005]. Because only a minority of BP users develop bone necrosis, other factors are likely to play a role in conferring susceptibility [Durie BG *et al.,* 2005; McMahon RE *et al.,* 2006; Bouquot JE *et al.,* 2008; McMahon RE *et al.,* 2007]. One of these variations/susceptibility factors may be the dysfunction of the coagulation system, as thrombophilia/hypofibrinolysis conditions. This hypothesis suggests the intra-bony ischaemic process of avascular necrosis might occur when an anti-angiogenetic factor (BP therapy) further complicates a pre-existing already-altered bone microenvironment characterized by impaired venous circulation, venous thrombosis, increased intra-osseous venous pressure, reduced arterial flow, and hypoxic bone due to hereditary/acquired thrombophilia/hypofibrinolysis.

To date, it is still unclear whether jaw bone necrosis in individuals on BP therapy is a primary ischemic bone phenomenon (causing eventually bone exposure and infection) or a process

secondary to gingival ulceration, exposure of jaw bone to the oral cavity, bone infection (osteomyelitis) and consequent necrosis. Looking at available data, none of these theories can be strongly supported and both are worth investigating.

Acquired/hereditary capillary and venous micro-thrombotic events may represent a risk/predisposing factor, leading to elevation in local intraosseous pressure and secondary sub-clinical arterial ischemia. Further exposure to BP, with consequent osteosclerosis and anti-angiogenesis, might then precipitate the ischemic process. Oral surgery could finally trigger the clinical manifestations of osteonecrosis as it leads to infection and colonization of already avascular bone.

BRONJ pathogenesis may be associated with a decrease in intra-osseous blood flow is supported by the following evidence:

1. Pamidronate and zoledronate inhibit capillary neo-angiogenesis and experiments have shown that BP: (i) decrease capillary tube formation and vessel sprouting, both in vitro and in a rat model; (ii) inhibit endothelial proliferation in cultured human umbilical vein and rat aortic ring cells; (iii) are able to reduce vessel sprouting in the chicken egg chorioallantoic membrane assay; (iv) inhibit angiogenesis both in bone and prostate tissues in a murine model; (v) cause a significant decrease of circulating VEGF levels in patients with advanced solid cancer and bone metastases [up to 21 days from a single infusion]; (vi) inhibit endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways [Hasmim M *et al.,* 2007]. Moreover, a recent study has shown that patients with BRONJ have reduced circulating endothelial cells [Allegra A *et al.,* 2007].
2. Histopathological examination of BRONJ has shown the presence of necrotic avascular bone.
3. Patients with sickle cell anemia can be affected by bone/bone marrow infarction of the mandible as a consequence of microvascular occlusion. [Kavadia-Tsatala S *et al.,* 1996].
4. Animal studies have shown that long-term BP therapy leads to intra-bony necrotic changes of otherwise healthy jawbones, even though some specific histopathological features seem to be different from those of femoral osteonecrosis. Allen MR reported that female beagles were treated for 1-3 years with oral doses alendronate and histological sections of mandible showed regions of bone matrix necrosis in 25% of those dogs treated with the lower dose of alendronate and 33% of dogs treated with higher dosage 33%. Similar necrotic regions were noticeably absent from all control animals. These findings were observed also in dogs treated with intravenous BP for few months and also in other skeletal sites (e.g. ribs) [Allen MR *et al.,* 2007].

5. Magnetic Resonance Imaging studies have shown that focal areas osteonecrosis are present in regions of the jawbones where no gingival ulceration/exposed bone/infection are present. These are considered areas of early osteonecrosis before the occurrence of an adjunctive factor that causes exposure of the focal lesions and consequent infection [Garcia-Ferrer L *et al.,* 2008; Chiandussi *et al.,* 2006].

Nonetheless, the vascular hypothesis seems to be more likely in consideration of the following issues:

1. About 30% and 50% of the osteonecrotic processes in patients on IV and oral BP respectively occur spontaneously without previous infection/trauma/soft tissue breakage, namely when the jaw bones seem to be otherwise healthy (infection-free).
2. The consequential process of (i) aseptic ischemic bone necrosis followed by (ii) infection/bone exposure is not novel in the jaw bones. Osteonecrosis of the jawbones due to radiotherapy (osteoradionecrosis) is associated with ischemic intra-bony changes (vessel obliteration due to radiation-induced endarteritis followed by hyalinized narrowing of the vessels) and can occur irrespectively of any bone trauma/infection or gingival ulceration, with infection being usually a secondary phenomenon [McMahon RE *et al.,* 2000]
3. Biopsy specimens of BP-associated osteonecrosis of the jawbones are usually taken when bone exposure and infection has been present for a significant amount of time. This can explain the presence of bacterial contamination, soft tissue breakage, and inflammation and does not necessarily mean that these are primary events and bone necrosis occurs eventually as secondary phenomenon. Aseptic jaw bone necrosis before exposure and infection has been suggested to represent an early mildly-symptomatic stage of BOJ and indeed the presence of toothache-like pain in the jaws weeks-months before the occurrence of trans-mucosal exposure of bone and infection has been reported by clinicians [Bouquot JE *et al.,* 2008; McMahon RE *et al.,* 2007; Ruggiero SL *et al.,* 2007].

The histopathological characteristics of necrotic bone observed in animal experiment (e.g. persistence of bone vessels) does not rule out the vascular hypothesis as (i) they might represent early pathological changes, (ii) animals had no thrombophilic tendency (which is the major predisposing factor in the vascular pathogenic hypothesis of BRONJ).

Alteration in bone turnover

Alteration in bone turnover has been assumed by some authors to be the cause of BRONJ because the bisphosphonate's mechanism consists in the reduction of the bone turnover. In fact, the hypothesis of the suppression of remodeling induced by BPs in the jaw could be justified because of the high remodeling rate of the jaw [Vignery A *et al.,* 1980; Garetto LP *et al.,* 1998; Huja SS *et al.,* 2006] compared with other skeletal parts. This could explain the fact that BRONJ appears only in the jaw [Russell RG *et al.,* 2007, 2008].

Although the bone turnover in the jaws is higher, there is no evidence that BPs accumulate at higher concentrations in this bone compared with other sites. Moreover, if low bone turnover rate was part of the pathophysiology of BRONJ, it could be expected that it occurred in other conditions in which reduced bone turnover is usually chronic, such us hypoparathyroidism or treatment with other anti-remodeling agents. However, any BRONJ lesion has been reported in these type of cases.

Furthermore, it has been showed that osteoclast numbers in BRONJ patients were four-fold higher compared to the control group [Hansen T *et al.,* 2006].

Infection

Infection could be closely related to the pathogenesis of BRONJ, and several data have shown bone bacterial colonization in patients affected by BRONJ [Hansen T *et al.,* 2006, 2007; Sedghizadeh PP *et al.,* 2008, 2009].

A variety of species have been implicated, but specific staining of the detected bacteria has revealed that Actinomyces spp. is a common oral organism found [Abu-Id MH *et al.,* 2008; Hansen T *et al.,* 2006; Bisdas S *et al.,* 2008; Biasotto M *et al.,* 2006] in the 73.2% (407 of 556) of BRONJ cases reported in literature, most commonly *Actinomyces israelii* [Yeung MK, 1999].

Actinomyces spp. are human commensal which normally colonize human and animal mucous membranes, mostly tonsillar crypts, gingival membranes and dental caries [Pulverer G *et al.,* 2003; Sedghizadeh PP *et al.,* 2008, 2009; Smego RA *et al.,* 1998].

However, is still unclear if Actinomyces spp. infection is a primary event in BRONJ pathophysiology or whether bisphosphonated-related osteonecrosis leads to a secondary Actinomyces spp. infection. The role of infection in pathogenesis of BRONJ is supported by numerous reports that showed a positive clinical response in 90% of the patients to an antibiotic therapy directed to Actinomyces spp. with the consequent elimination of BRONJ symptoms.

Another clinical problem related with BRONJ and the possibility of infection, is the presence chronic microbial biofilm at the bone level. Microbial biofilms consist of a dense layer of mixed microorganisms attached to a surface and surrounded by a polysaccharide matrix secreted by the

bacteria that are living in it [Hall-Stoodley L *et al.,* 2009]. In this condition, the bacteria, protected by this layer, could become resistant to antibodies and phagocytes secreted by host cell and to antibacterial agents. Recently, some authors have showed that microbial biofilm was present in all of four BRONJ-patients analyzed [Sedghizadeh PP *et al.,* 2008]. Microbial biofilms are probably associated with BRONJ lesions as a result of the chronicity of lesions and the presence of BPs on the bone surface likely contributes to development of biofilms.

The hypothesis of biofilm as a pathogenic event contributing to the development of BRONJ, could be explained because many cases of bone necrosis secondary to BPs treatment have appeared mostly in the jaws, where the concentration of oral bacteria is higher than in other sites of the body. [Kumar SK *et al.,* 2011]. Furthermore, bacteria have the capacity to directly stimulate bone resorption with the release of various proteases and acids. An example is the lipopolysaccharides from gram-negative bacteria which is likely to stimulate cytokine production [Sedghizadeh PP *et al.,* 2008; Nair SP *et al.,* 1996, Pap T *et al.,* 2003], with the consequent alteration of bone remodeling. Moreover, Ganguli *et al*. have shown that the *in vitro* adhesion of *S. aureus*, determined by the Modified Vortex Device (MVD), to hydroxyapatite (HA) pamidronate-coated (nitrogen-BP) was significantly greater (60-fold increase) than bacteria adherent to uncoated HA or than clodronate-coated HA (chlorine-based BP) (90-fold increase), suggesting that BPs not only are able to increase the capacity of microbial adherence, but that nitrogen-BPs are most associated with BRONJ than others with different molecular structure [Ganguli A *et al.,* 2005], as documented in various reports [Diel IJ *et al.,* 2007].

Bisphosphonate toxicity to bone

Normally, the only cells that are able to internalize toxic rates of BPs are osteoclast. However, BPs if used at high frequent IV dosing or high concentrations could be toxic to other bone cells, probably acting on the mevalonate pathway. In fact, at least 50% of intravenous dose of bisphosphonates are internalized and retained indefinitely [Reid IR *et al.,* 2002; Khan SA *et al.,* 1997]. A study showed that an approximately amount of 70 nmol/g of bone skeletal content can be achieved after using 4mg/month of zoledronate for about 4 years [Reid IR *et al.,* 2007].

Some authors, in fact, have shown that the pathogenesis of BRONJ could be caused by direct toxicity of BPs to bone cells. In particular, these cells, with the exception of osteoclast, could internalize irreversibly BPs by fluid-phase endocytosis which in turns lead to a gradual accumulation of these toxic molecules [Thompson K *et al.,* 2006]. Furthermore, it has been hypothesized that bone accumulated BPs can inhibit the epithelialization, a crucial step in wound healing after an intervention.

Thus, BPs could prevent normal healing of soft tissue, leading in the exposure of the bone, which later could necrotize [Reid IR *et al.,* 2007, 2009]. However, some clinical evidence showed that not in all cases of necrosis and BRONJ pathology an exposed bone is present and this condition can occur even when the mucosa is still intact [Ruggiero SL *et al.,* 2004; Otto S *et al.,* 2009].

Role of pH on BRONJ

The uptake of BPs to the bone normally occurs when pH is neutral and they are released in the milieu at an acidic pH. This process take place during bone resorption, when the pH became more acid and consequently BPs decrease the affinity to hydroxyapatite, moving into the environment. During infection or wound healing after surgical intervention (e.g. tooth extraction or dental implantation) the pH in the milieu is acid and therefore a greater amount of BPs is released [Sato M SL *et al.,* 2004 *et al.,* 1991]

Furthermore, as a result of tissue's acidification, the release of toxic derivatives, resulting from transformation of nitrogen-containing groups, such NH2 to NH3 can occur. Only nitrogen-containing BPs are subjected to this process activation and this is in accordance with clinical observations in which BRONJ has been caused exclusively by nitrogen-containing drugs [Abu-Id MH *et al.,* 2008]. Despite the exact role of pH on the pathogenesis of BRONJ is still unclear, this hypothesis might explain not only why the jaw is the only bone affected, but also why BRONJ occurs using nitrogen-containing BPs.

3.4.5 ***Staging***

Ruggiero and colleagues, in 2006, described four clinical stages of BRONJ [Ruggiero SL, 2006], currently being used and adopted by the AAOMS as a useful system to identify an appropriate diagnosis and management [Ruggiero SL, 2009; Advisory Task Force, 2007].

BRONJ progression was firstly indicated as three-staged, based on clinical signs and symptoms; phase zero has been recently added in order to include high-risk patients with no clinical evidence of necrotic bone, but with unspecific clinical signs and symptoms [Ruggiero SL *et al*., 2009]. The staging of the disease was made according to the recommendations of the AAOMS 2014 [Ruggiero SL, 2014], as outlined below:

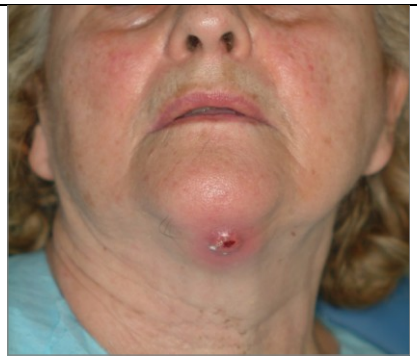
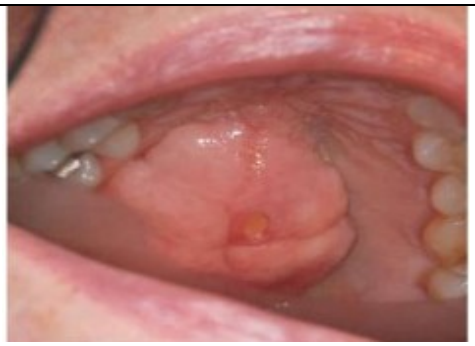
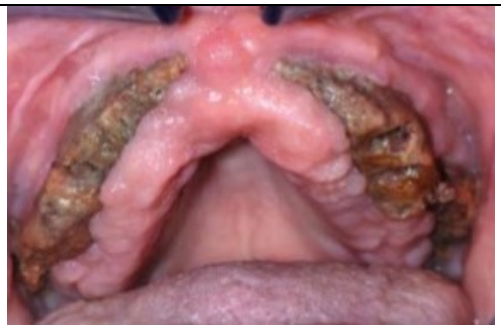
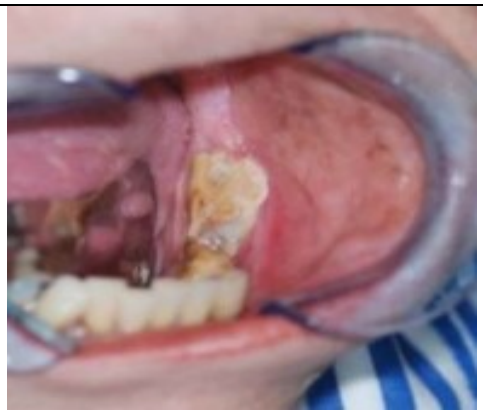
Stage 0	*no clinical evidence of necrotic bone but nonspecific clinical findings, radiographic changes, and symptoms*;	
Stage 1	*exposed and necrotic bone or fistulas that probe to bone in patients who are asymptomatic and have no evidence of infection*	
Stage 2	*exposed and necrotic bone or fistulas that probe to bone associated with infection as evidenced by pain and erythema in the region of exposed bone with or without purulent drainage*;	
Stage 3	*exposed and necrotic bone or a fistula that probes to bone in patients with pain, infection, and ≥1 of the following: exposed and necrotic bone extending beyond the region of alveolar bone (i.e., inferior border and ramus in mandible, maxillary sinus, and zygoma in maxilla) resulting in pathologic fracture, extra-oral fistula, oral antral or oral nasal communication, or osteolysis extending to inferior border of the mandible or sinus floor*	

Table 3

3.4.6 Prevention

Before treatment with intravenous BPs, a patient should undergo thorough intraoral examination followed by comprehensive dental treatment, because invasive dental procedures have been related to osteonecrosis of the jaw in more than 70% of cases [Cheng A *et al*., 2005; Badros A *et al*., 2006].

There are different guidelines about prevention in patients taking bone resorption inhibitors:

a. the American Dental Association (ADA) Council on Scientific Affair, in 2011 has suggested that patients with a bisphosphonates treatment period of less than 2 years can undergo dental operations without any period of drug holiday [Hellstein JW *et al*., 2011];
b. the International ONJ Task Force guidelines in 2011 recommended for patients with a bisphosphonates treatment period of more than 4 years or in presence of other risk factors, drug holyday [Khan AA *et al*., 2015];
c. the American Association of Oral and Maxillofacial Surgeons (AAOMS) recommended for patients with evidence in bone pathology a drug holiday of 2-4 months [Damm DD *et al*., 2013].

In cases of high dose IV BPs treatment, there is a little evidence on whether a drug holiday is needed in advance for prevention.

3.4.7 Diagnosis of BRONJ

Diagnosis of BRONJ includes previously defined clinical conditions (gingivitis/periodontitis, alveolar osteitis, periapical lesions, forms of cement-osseous dysplasia), while infections and bone inflammation appear in a more advanced stage. At first, necrotic bone remains asymptomatic for long periods, even years [Allen MR *et al*., 2009]. Before the development of clinically detectable osteonecrosis, patients may have such symptoms such as mucosal swelling, ulceration, pain and tooth mobility [Fedele S *et al*., 2010]. In advance stages, BRONJ lesions occur in 65% of cases in the mandibular; 28.4% in maxilla and just in 6.5% of cases occurs in both [Ruggiero SL *et al*., 2004, 2006; Marx RE *et al*., 2005]. Furthermore, the exposed bone is surrounded by purulent discharge and infection is usually present. However, different oral pathogens often coexist and the selection of an appropriate empiric therapy could be difficult [Sedghizadeh PP *et al*. (b), 2009, 2012].

Radiologic and nuclear medicine imaging may be valuable in recognizing and defining bone lesions in patients undergoing BPs therapy [Chiandussi S *et al.,* 2006]. However, radiographic features of BRONJ remain relatively nonspecific.

In current diagnosis various techniques are available, each of which present advantages and limitation. Computed tomography (CT) may allow a greater definition in characterizing the features of osteonecrosis. Common CT findings include osteosclerosis, cortical erosion, necrotic areas and their relationship with neighboring anatomic structures, leading to quantify the status of bone [Borgioli A *et al.,* 2009]. Scintigraphy exams (Tc99-scan) is the most sensitive diagnostic strategy to identify edema and vascular changes even in the early stages of the disease, although this

technique has limitations such as the inability to distinguish osteonecrosis from metastatic processes [Chiandussi S *et al*., 2006; Hermans R *et al*., 1996].

As regards to the use of biomarkers to identify osteonecrosis, there are controversial hypothesis. Marx and colleagues [Marx RE *et al*., 2007] suggested that quantification of bone by C-terminal Telopeptide (CTX, used as a biomarker in the serum to measure the rate of bone turnover) value, may be useful for prognosis. However, Atalay and colleagues [Atalay *et al.,* 2011] found that CTX is unable to predict treatment prognosis in cancer patients. Thus, although low levels of CTX normally indicate a recent treatment with BPs, current data do not establish if it is useful in managing patients with BRONJ. The relation between excessive suppression of C-terminal telopeptides of type I collagen (CTX), a bone resorption marker, and osteonecrosis incidence has been reported in several studies. However, a correlation between CTX level and severity of osteonecrosis has not been observed [Kwon YD *et al*., 2009, 2011]. Therefore, the most accurate tool for diagnosis of BRONJ is an extensive clinical examination, which could associate to the patient anamnesis the most accurate technique for the given context.

3.4.8 BRONJ management

To date no standard therapy has been established for BRONJ, in particular regarding surgical procedures, and there are just recommendations in the literature. Many factors may contribute to the administration of the most accurate treatment, such as taking into consideration age, sex, extent and type of lesions and stage of the pathology.

Two main treatment approaches have been developed:

i) conservative;

ii) surgical.

For patients with a documented diagnosis of BRONJ, the treatment goals are the alleviation of pain, the reduction of infection's burden and the prevention of necrosis progression [Ruggiero SL *et al.,* 2009]. In 2009, the position paper of AAOMS (*American Association of Oral and Maxillofacial Surgeons*) has recommended to limit surgical protocol to Stage III only, though others studies showed good improvements of pathology also in early stages of BRONJ. Debridement surgery is not suggested because the necrotic bone itself does not cause pain and usually jaw still preserves its normal function.

Only after an infectious process, following to fistula formation, patients can experience a painful condition. Thus, surgical treatment could be reserved only for advanced stages or in those patients in which the *sequestrum* is well defined and can be easily separated from the healthy tissue in order to avoid a constant source of soft-tissue irritation. Therefore, any teeth within exposed bone should

be removed, because it seems likely that the extraction will contribute to stop the necrotic process. [Carlson ER *et al.,* 2013]. Another study, conducted by Montebugnoli *et al.*, showed that there were not statistically significant differences between a group of subjects submitted to antibiotics therapy compared with a group managed with surgery [Montebugnoli L *et al.,* 2007]. Discontinuation of IV BP therapy does not offer short-term benefit normally, whilst only if the systemic condition is permissive, it has been showed that discontinuous therapy could be improve clinical symptoms. However, subjects that usually are under IV therapy are oncologic and need BPs to control bone pain and incidence of fractures.

As concern oral BP treatment, this has been correlated with improvement of clinical symptoms [Weinreb M *et al.,* 1994]. In fact, there are evidence that a discontinuous period of 6-12 months may result in the spontaneous resolution after debridement surgery.
The position paper of AAOMS suggests also that several treatment strategies have to be considered for the different stages of the pathology (Table. 2) [Ruggiero SL *et al.,* 2009].
The use of oral antimicrobial rinses is recommended just for stage I, using 0.12% chlorohexidine, but without any surgical treatment, while a systemic antibiotic therapy is suggested for subjects in stage 0, II and III. Empirical antimicrobial treatment usually includes penicillin, quinolones, doxycycline, erythromycin and metronidazole [Ruggiero SL *et al.,* 2009]. In patients allergic to penicillin, some authors suggest the use of ciprofloxacin or erythromycin together with metronidazole [De Ceulaer J. *et al.,* 2014].
There is clear evidence that long-term antibiotic therapy, in combination with surgery when it is necessary to retrieving sequesters, is a consolidated treatment [AAOMS 2007; Ficarra G *et al.,* 2007; Marx RE *et al.,* 2005; Mücke T *et al.,* 2011; Lee CY *et al.,* 2011].

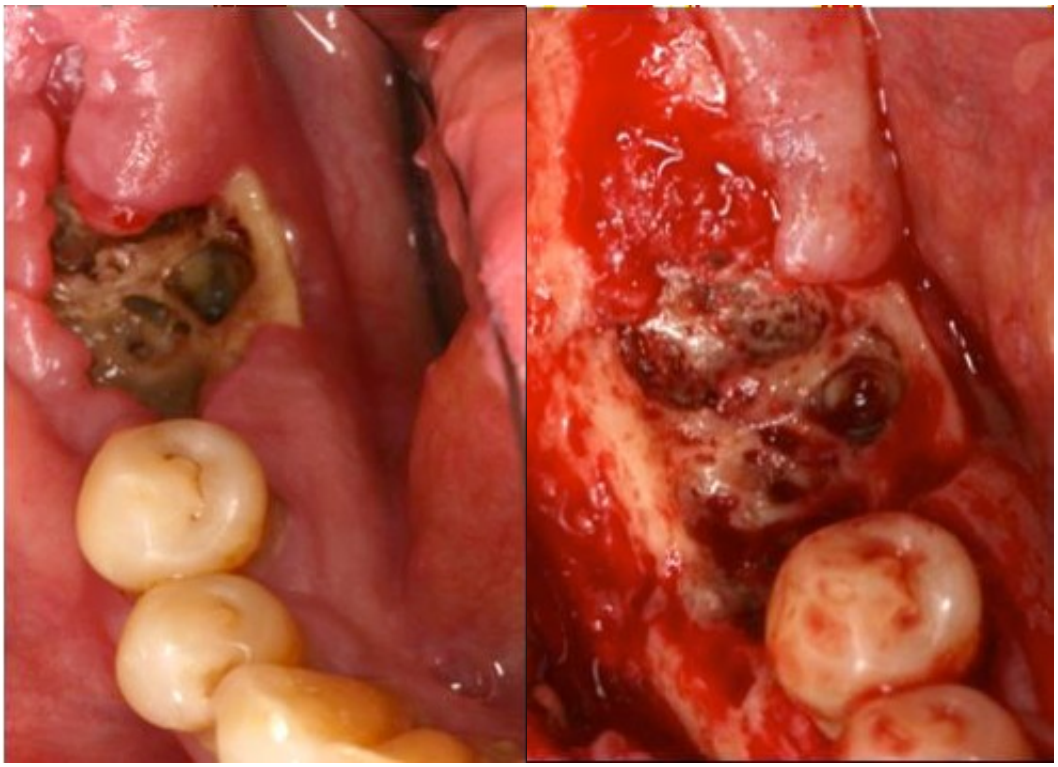


Figure 11 described surgical steps of sequestrectomy.

Furthermore, an observational trial on 119 patients affected by BRONJ, showed suppression and net reduction of main symptoms in 90% of patients with exposed bone and pain which were under Actinomyces spp antibiotic therapy [Marx RE *et al.,* 2005], confirming the crucial role of these microorganisms in the pathogenesis of BRONJ.

In conjunction with antibiotic treatment, it has been documented a reduction of exposed bone area and symptoms of BRONJ in the early stages of this pathology when using pentoxifylline and α-tocopherol [Epstein MS *et al.,* 2005]. Recent case studies have shown that teriparatide (TP) which, increases bone diameter and mass (anabolic effect), have an healing effect in BRONJ patients [Subramanian G *et al*., 2012; Hokugo A *et al*., 2010]. Harper and Fung [Harper RP *et al*., 2007] have demonstrated that patients with 3 months of TP treatment showed healing of soft-tissue. Additionally, in a case study, Ohbayashi *et al* [Ohbayashi Y *et al*., 2013] demonstrated bone regeneration 6 months after TP therapy in a refractory BRONJ patient.

In 2015, Ikeda *et al.* showed remission or total heling in 95% of subjects suffering of osteoporosis using sitafloxacin (STFX). 19 out of 20 patients in stages II-III, with persistent infected bone or fistulas that did were not resolved common antibiotic therapy, showed with STFX a high infection control [Ikeda T *et al.,* 2015].

Moreover, studies *in vitro* using geranylgeraniol (GGOH) have shown that this molecule seems to have a mechanism of mucosal healing in stage I of BRONJ, stimulating the migration of cells such as fibroblast, endothelial cells and osteoclast [Ziebart T *et al.,* 2011].

Finally, alternative therapies, such as treatment with Hyperbaric Oxygen Therapy (HBO) and Ozone therapy (OT) have been reported in literature. These therapies could stimulate soft tissue healing with subsequent reduction of pain [Ripamonti CI *et al.,* 2011; Freiberger JJ *et al.,* 2009]. In addition, laser applications at low intensity (Low Level Laser Therapy - LLLT) has proved to improve the reparative process by stimulating lymphatic and blood capillaries growth (Table.3) [Vescovi P *et al.,* 2006, 2008].

In summary, conservative management with optimal oral hygiene, topical and systemic antibiotic therapy, is recommended in the absence of asymptomatic, non-infectious lesions, but antibiotics should promptly administered with the first signs and symptoms of infection. For advanced stages, surgery is an alternative therapy. Further studies are needed to better identify the right etiology of BRONJ and to establish an effective treatment for this severe condition.

BRONJ stage	Description	Treatment strategies
At risk category	No symptoms of osteonecrosis in patients who have been treated with either oral or IV bisphosphonates	No treatment Patients education
Stage 0	No clinical evidence of necrotic bone, but nonspecific clinical findings and symptoms (toothache, bone pain, dysesthesia)	Conservative treatment (pain control). Systemic therapies including pain medications and antibiotics
Stage I	Osteonecrosis with no infection evidence in patients with bone exposure but without asymptomatic lesion	Antibacterial mouth rinse. Clinical follow-up
Stage II	Bone necrotic exposure, pain, infection and erythema in exposed area.	Antibacterial oral rinse and antibiotics. Pain control with analgesics. Superficial debridement to relieve soft tissues irritation
Stage III	Exposed and necrotic bone with signs of infection, pain, pathological fractures, cutaneous fistulas, maxillary sinus involvements.	Antibiotic therapy, pain control, antibacterial oral rinse. Surgical debridement/resection.

Table 4 *- Clinical classification of BRONJ and treatment strategies. Modified from Ruggiero et al., 2006*

Alternative therapies	Description	References
Geranylgeraniol (GGOH)	Stimulating the migration of cells such as fibroblast, endothelial cells and osteoclast in stage I patients	Ziebart T *et al.,* 2011
Hyperbaric Oxygen Therapy (HBO)	Stimulate soft tissue healing	Freiberger JJ *et al.,* 2009; Ripamonti CI *et al.,* 2011.
Ozone therapy (OT)	Stimulate soft tissue healing	Freiberger JJ *et al.,* 2009; Ripamonti CI *et al.,* 2011.
Low Level Laser Therapy (LLLT)	Stimulate lymphatic and blood capillaries growth	Vescovi P *et al.,* 2006, 2008

Table 5 *– Alternative therapies from literature.*

AIM OF THE STUDY

4. Aims of the study

Bisphosphonates (BP), synthetic analogues of inorganic pyrophosphate able to inhibit the osteoclastic activity, quickly bond to the mineral component of the bone structure and, consequently, experience a progressive accumulation in the osseous tissue and where they can remain for many years.

Nevertheless, in literature a direct measuring of the dose of these drugs in the human bone tissue is not available. In man, the concentration of BPs stored in bones is estimated on basis of administered dose and amount of drug detected in serum and, more frequently, in urine.

Considering the complete absence of information on this topic, the aim of this study is to investigate the presence of BPs in bone sequestra (surgically removed or naturally exfoliated) in patients with ONJ caused by BPs, in the Oral Pathology Unit of the San Paolo Hospital, University of Milan, and in the Dental Unit of the Papa Giovanni XXIII Hospital of Bergamo, by the developmet and validation, of the first analytical method on bone matrix, able to quantify the presence of BPs

This is the first study able to directly detect and quantify the BFwithin the bone sequestrations of patients receiving therapy. The method was based on the high resolution and sensitivity of LC-MS/MS, with a simple SPE purification procedure required for the sample preparation.

High performance was demonstrated in terms of precision, sensitivity and accuracy, making the proposed method useful to detect and quantify other classes of bisphosphonates from human bone tissues. The method showed to be a valuable tool for clinical studies on BRONJ patients, to better clarify the pathophysiological role of BF bone concentration in the development of BRONJ and related prognosis.

MATERIALS AND METHODS

5. Experimental study

Starting from April 2005, a new service for the treatment and prevention of osteonecrosis caused by bisphosphonates is operating at the Unit of Oral Pathology, Medicine and Geriatrics of the University of Milan.

Since April 2008, patients in current or previous therapy with bisphosphonates, undergoing spontaneous or surgical removal of bone sequestra, are being recruited among the subjects sent to the Odontostomatology II Unit of the ASST Santi Paolo e Carlo-San Paolo Hospital, University of Milan and the Dental Unit of the Papa Giovanni XXIII Hospital of Bergamo (Italy). Personal and clinical data have been collected and physical examination of the oral cavity carried out. Diagnosis of BRONJ is based on the current criteria [Migliorati *et al*. 2011].

For each patient a clinical chart was filled out with particular attention to general records, basic pathologies, type of bisphosphonates used, dosage and administration duration, time of onset of the osteonecrosis, surgery characteristics and controls. If possible, we investigated the duration of the treatment with bisphosphonates and the cumulative dose, getting in touch with the Oncologist of every patient.

These patients were enrolled according to the following characteristics: osseous exposure, drainage from mucous or cutaneous fistulae, suppuration, intense pain. These patients were often reported by other colleagues or facilities for suspect osteonecrosis.

Patients in our study group were selected among this subjects according to the following inclusion criteria:

• History of therapy with zolendronate and alendronate;

• Presence of exposed necrotic bone for at least 8 weeks and/or presence of osteolytic lesions highlighted thanks to imaging and not related to other pathologies;

• Negative medical history for head and neck radiant therapy.

Patients of the control group were selected according to the following inclusion criteria:

• Absence of previous therapy with drugs that limit the osseous turnover, associated with osteonecrosis of the maxilla or previous therapy with drugs for the bone tissue different from alendronate or zolendronate (other bisphosphonates and denosumab) able to cause osteonecrosis or

medical history positive for radiant therapy of the head and neck region not associated to therapy with bisphosphonates;

• Presence of necrotic exposed bone for at least 8 weeks and/or presence of ostelytic lesions highlighted thanks to imaging and not associated to other pathologies;

• Necessity of sequestrectomy with therapeutic purpose;

• Spontaneous detachment of the necrotic bone.

Bone samples were stored in sterilized plastic containers at a temperature of -80°C in order to be analyzed by the Independent Section of Forensic Toxicology, Institute of Legal Medicine of Milan.

Criteria for surgical sequestrectomy were:

• Presence of exposed osteonecrotic lesions for at least 8 week and belonging to Stage 3 of the 2009 Ruggiero Classification, so with presence of festering drainage and progressive osseous destruction;

• A period of at least 3 months of conservative therapy (cycles of systemic antibiotics, domestic oral antiseptics, non-surgical debridement of the exposed bone, irrigation of the necrotic area with saline solution) without benefit for the clinical presentation;

•Presence of lesions observed thanks to imaging techniques (oral rx, panoramic radiograph, maxillary TC) showing a necrotic aspect with decreased bone density, irregular cortical portion and, sometimes, presence of an osseous portion detached from the surrounding bone tissue (the same aspect that is seen in osseous sequestrum);

• Presence of lesions not wide enough to be considered for more invasive surgery, not possible in day hospital sessions or requiring general anesthesia, as in segmental resections or en bloc; these patients were sent to the Maxillo-Facial Surgery ward of the San Paolo Hospital of Milan.

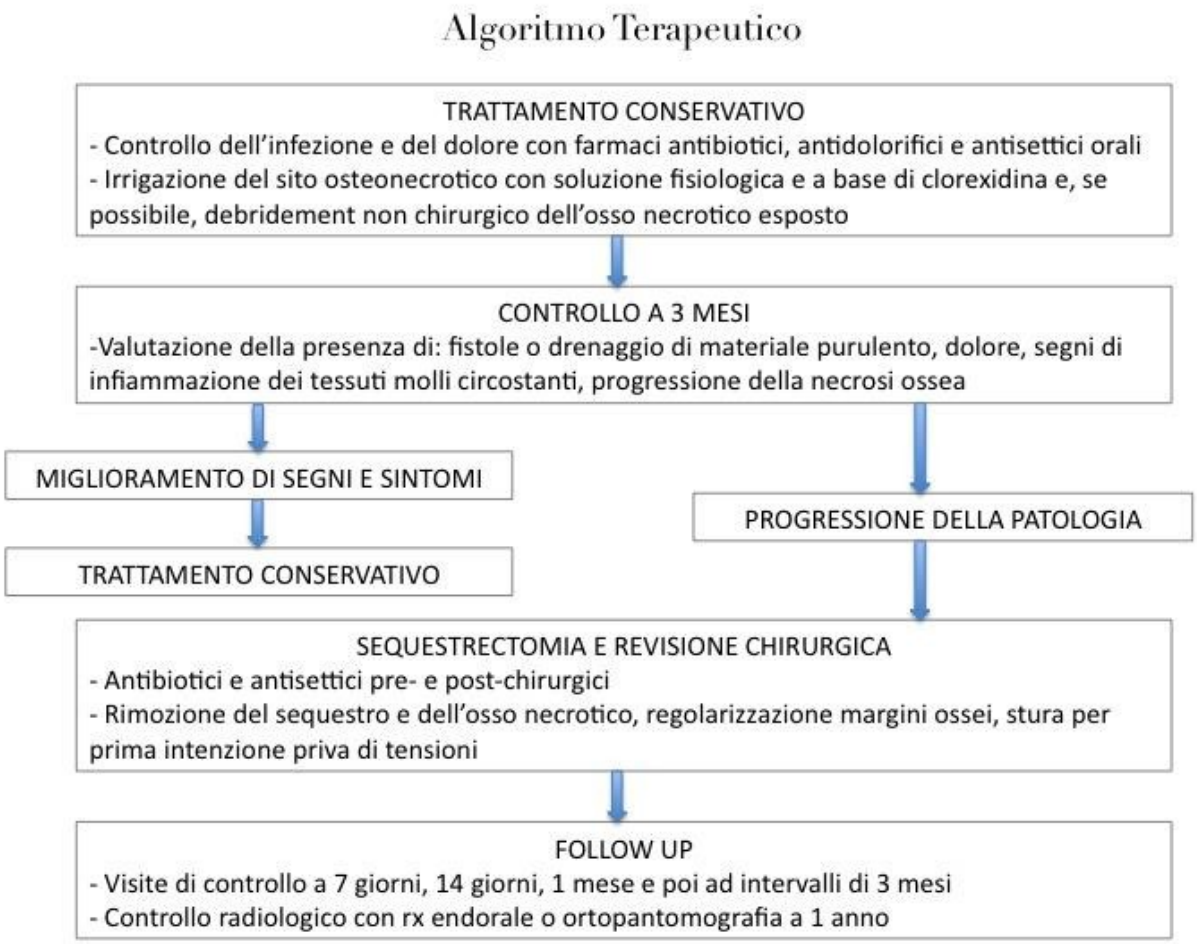


Figure 12: Therapeutic algorithm adopted in this study

5.1 Surgical procedure in case of sequestrectomy

Following the article [Lodi *et al.* 2010] describing the acquired experience in the application of the preventive procedure for extraction of patients under current or previous therapy with IV bisphosphonates, we have adopted this procedure to the surgical intervention of removal of the osseous sequestrum in patients that had already developed osteonecrosis caused by BPs,.

Pre-operating phase:

- Oral hygiene session no more than 2 weeks before surgery;
- Rinsing with chlorhexidine mouthwash 0.2% every 12 hours in the 2 weeks before surgery and chlorhexidine gel 1% to be used every 12 hours;

Systemic antibiotic therapy to be started at least 15 days before surgery.

Operating phase:

1) Surgical intervention trying to minimize the trauma to soft and hard tissues;
2) Removal of osseous sequestrum and necrotic tissue sparing noble anatomical structures nearby;
3) Surgical seaming for primary intention of the surgical wound;
4) Storage of the sample of necrotic bone in a sterile plastic container at -80°C.

Post-operating phase:

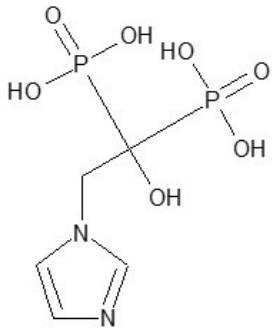
5) Chlorhexidine mouthwash 0.2% rinsing and use of chlorhexidine gel 1% on the wound for at least 2 weeks after surgery;
6) Systemic antibiotics for at least 15 days after surgery;

Post-operating supervision until total remission of the pathology.

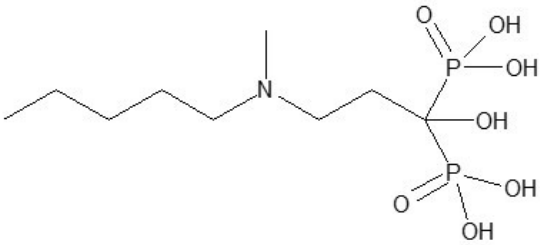
5.2 Methods and equipment used for the analysis of the bone samples

Chemicals

Zoledronic acid (1) hydrate, ibandronate sodium salt used as internal standard (2) , LC-MS grade acetonitrile, water, trimethylsilyl diazomethane (TMS-DAM, 2% in ether) and phosphoric acid were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). HyperSep™ SAX, strong anion exchange cartridges for solid phase extraction (SPE) were purchased from Thermo Scientific (Waltham, Massachussett, USA).



(1)



(2)

Chromatography

A gradient was developed to separate ZA tetramethyl phosphonate and IS from the background interference on a QTRAP 5500 System (500 Old Connecticut Path Framingham MA 01701, USA). The LC-MS/MS was equipped with a temperature-controlled autosampler, a direct infusion syringe pump, an inline vacuum degasser and binary pump. One µL of each sample was injected into a Kinetex™ 2.6 µm C8 100 Å, LC column 30 x 2.1 mm, Ea column. The mobile phase consisted of 10 mM formic acid in acetonitrile (B) and 10 mM formic acid in water (A) (Tab. 6).

Time (min)	Flow-rate (µl/min)	%A	%B
0	400	100	0
1.5	400	100	0
2	400	85	15
3.5	450	70	30
5.5	450	0	100
7.5	450	0	100
7.6	500	100	0
10	450	100	0

Table 6

Mass Spectrometry

All data were acquired using electrospray ionization (ESI) on AB Sciex 5500 Q-Trap mass spectrometer operating in the positive mode. The instrument was controlled by the Analyst 2.1 software. Multiple reaction monitoring (MRM) mode was used to monitor both ZA and ibandronate (IS) (table 7-7a)

Zole-1	329,1	203,3
Zole-2	329,1	135,1
IS-1	376,5	250,7
IS-2	376,5	114,2

Table 7: Multiple reaction monitoring (MRM) mode used to monitor both zoledronic acid and ibandronate (IS).

Alen-1	348.2	289.5
Alen-2	348.2	163.5
Pami(IS)-1	334.1	275.2
Pami(IS)-2	334.1	149.4

Table 7a: Multiple reaction monitoring (MRM) mode used to monitor both alendronic acid and pamidronate (IS).

Sample pre-treatment and extraction of zoledronic acid from human bone

Prior to extraction, bone samples (20 mg) were placed into 1 mL of 0.2 M H_3PO_4 (lysis buffer). The particulate was centrifuged at 16,500 x *g* for 10 min at room temperature (RT). The supernatant was carefully removed with a pipette and placed into a 10 mL tube. The remaining pellet was resuspended in 1 mL of 0.2 M H_3PO_4, vortexed and centrifuged again. The supernatant was removed and combined with the supernatant previously collected. Before loading the samples into a solid phase extraction (SPE) column, 9 mL buffer phosphate, pH 9.0, were added to each sample. The SAX SPE cartridges were preconditioned with 1 mL methanol and 1 mL buffer phosphate pH 9.0/0.2 M H_3PO_4 (2:1). The columns were then washed with 1 mL buffer phosphate pH 9.0, 1 mL of water and 2 mL of methanol. Immediately, 2.0 M TMS-DAM in ether (0.2 mL) and, then, methanol (0.75 mL) were directly added to the column and the reaction was allowed to proceed for 60 min at RT. The resulting ZA tetramethyl phosphonate was eluted with methanol (1mL), concentrated under nitrogen and reconstituted in 1:1 CH_3OH:H_2O (0.1 mL).

RESULTS

6.1. Method development

Currently, the estimation of bisphosphonate concentrations in skeleton relies on the measurement of drug in plasma and urine after derivatization [Veldboer, K *et al* 2011]. To the best of our knowledge, a method to detect and quantify ZA from murine bone has been recently proposed [Center for Drug Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, 2001.], but still there is no LC-MS method to quantify ZA from human skeleton, in particular from BRONJ sequestrations. These data could be correlated to the risk of BRONJ development as well as to its severity. Nonetheless, the development of new suitable techniques to quantify bisphosphonates from blood and urine by mass spectrometry-based methods has been challenging due to the high polarity of these compounds. ZA has to be derivatized to a less polar compound before it can be retained on a reverse phase LC column and ionized effectively [European Medicines Agency, 2009]. Recent studies have demonstrated the success of derivatizing ZA in urine and plasma with trimethylsilyl diazomethane (TMS-DAM) to produce ZA tetramethyl phosphonate [Brianne *et al* 2013]. The ZA tetramethyl phosphonate is less polar than its parent compound and can be used for LC-MS/MS based methods. Moreover, this approach does not allow a highly reliable determination nor the exact bisphosphonate localization within the skeleton.

In order to determine the skeletal uptake, studies have focused on the administration of fluorescent or radiolabeled derivatives of bisphosphonates to animal [.M.R. Allen 2008; D. Wen *et al* 2011; H.M. Weiss *et a*l 2008; J. Lin *et al* 2008], showing that bisphosphonate uptake and release per unit calcium were similar in oral and appendicular bones, but lower than those in axial bones. Therefore, hydroxyapatite-bound bisphosphonate released by acid decalcification was the highest in oral, relative to axial and appendicular bones.

However, some limitations arouse since it is unknown if the fluorescent or radiolabeled derivatives have the same behavior within the skeleton of the unlabeled bisphosphonates. In addition, the

development of a mass spectrometry-based analytical assay would be preferable over a radioassay, due to safety conditions required for handling radiolabeled compounds.

This is the first method able to quantify successfully ZA from the human bone BRONJ sequestrations, using a protocol modified from a previous report on mice [Center for Drug Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, 2001]. Trimethylsilyl diazomethane (TMS-DAM) was used to derivatize ZA prior to analysis by LC-MS/MS.

6.2 Validation

Calibration and linearity

Results obtained from the 10 calibration curves showed linear trends, with average precision for each concentration in the range of 93-103% (table 8)

Concentration range 1-150 ng/mg		
	Equations	*r*
1	y=0,0085x+0,0071	0,9998
2	y=0,0085x+0,0156	0,9996
3	y=0,0088x+0,0075	0,9996
4	y=0,0081x+0,0177	0,9996
5	y=0,008x+0,0307	0,9987
6	y=0,0079x+0,0237	0,9999
7	y=0,0088x+0,0037	0,9999
8	y=0,0081x+0,0193	0,9998
9	y=0,0092x+0,005	0,9999
10	y=0,0083x+0,028	0,9999

Table. 8: linearity.

Accuracy

Variations and deviations of accuracy ≤ 10% were observed. The highest limit of the calibration range, corresponding to the upper limit of quantification and precisions, was associated to deviations of the accuracy of ± 15%, as required [Veldboer K *et al* 2011, Center for Drug

Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, 2001]. Table 9 summarizes the values of this parameter for distinct concentrations obtained in 10 series consisting of bone sample blanks added with the following ZA concentrations: 1-2.5-5-10-20-50-100 ng/mg.

Theoretical Con. ng/mg	1	2,5	5	10	20	50	100
Measured Conc. ng/mg	0,83-1,11	2,3-2,8	4,7-5,3	8,5-11	19,7-21,6	48,6-51,3	96,6-101,9
Accuracy % min-max	99,2-100,6	87,1–103,3	96,9 – 101,5	85,0-109,7	98,3-107,8	97,2-102,6	97-101,9

Table 9

Selectivity

The analysis of six batches of blank samples showed no interfering peak in the MRM traces for ZA and IS (ibandronate) in human bone samples. Blank responses could not be distinguished from the detector noise (signal to noise ratio < 3) for both ZA and IS, and were all below 15% of the ZA LLOQ. A threshold of 20% is required [19] and the regular signal of IS is below 0.2%. The absence of any interference in these experiments is a proof of the high selectivity of the assay.

Six individual samples were processed to test the selectivity of the assay. These samples were processed as double blanks (without ZA and IS) and after spiking with 1 ng/mL ZA (QC-low), 5 ng/mL (QC-medium) and 10 ng/mL (QC-high), and after adding the IS (100 ng/mg). The spiked samples were also used to assess the inter-batch variation of the matrix effect.

Repeatability

Ten samples of blank bone samples spiked with ZA were measured. For each concentration, the average of the values processed by the instrument, the standard deviation and the coefficient of percentage variation were calculated

N=10	Mean measured conc. – ng/mg	Standard deviation	CV%
1 ng/mg	1,0	1,1334	113,3

2,5 ng/mg	2,57	0,0884	3,44
5 ng/mg	4,6	0,2179	4,73
10 ng/mg	10	0,8770	8,77
20 ng/mg	20,2	0,6129	3,03
50 ng/mg	50,0	1,1449	2,29
100 ng/mg	99,9	1,4058	1,41
150 ng/mg	150,1	0,8512	0,57

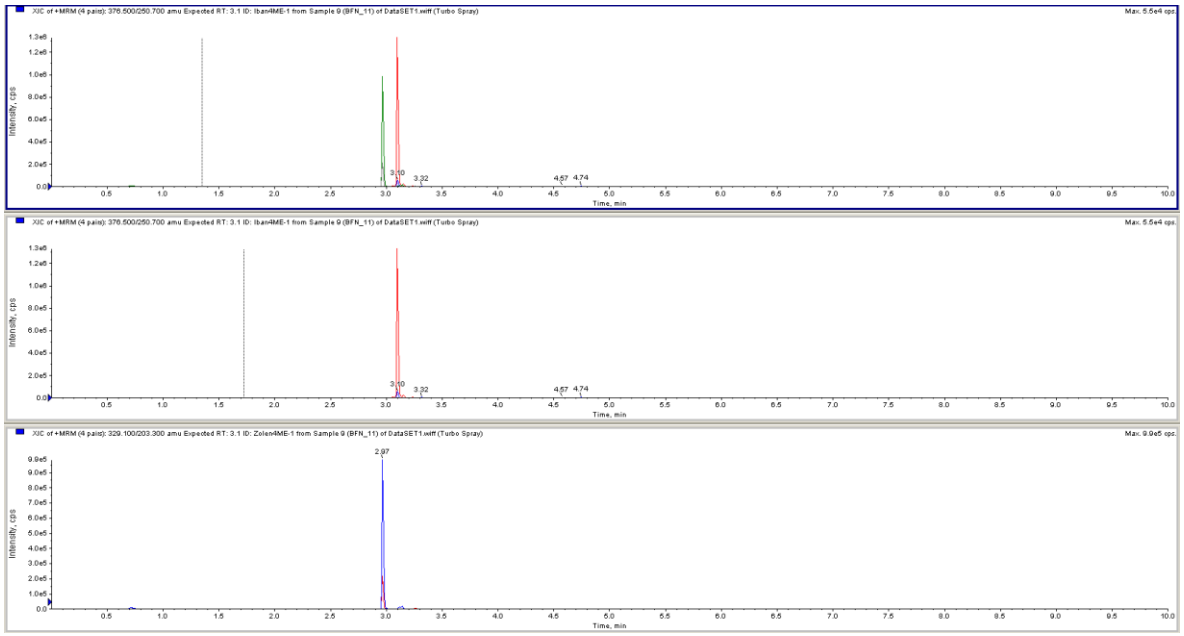
Table 10.

11.5 Lower limit of quantification and detection (LLOQ and LLOD)

The lower limits have been calculated using the concentrations of ZA added to the blank. This was repeated for each of the 10 calibration curves. A series of calculations considering the average factor of the ratio ZA/IS area were performed (table. 11). LLOQ value was 3.4 ng/mL, while LLOD value 1 ng/mg.

ZA concentrations in "blank" bone	
N	Average F.
1	0.49
2	0.46
3	0.325
4	0.98
5	0
6	0.47
7	0
8	0.66
9	0
10	0.75
Avarage	**0.413**
Standard deviation	**0.3378**
LOD (3*standard deviation)	**1.0**
LOQ (10* standard deviation)	**3.4**

Table 11



LC-MS/MS chromatograms of bone samples from patient A (female, 62 years old) showing peak for zoledronic acid and ibandronate (internal standard)

11.6 Recovery

The recovery experiments showed a decline in values after extraction below 10% for the three QC concentrations of ZA. The recovery of the extraction for the IS was 89 ± 15% (n = 6). Ion suppression was below 13% for all QC concentrations of ZA, and below 15 ± 6% for the IS (n = 6). This slight decrease of ZA and IS during the procedures of extraction and ionization of compounds, as well as the low inter-batch variability of the matrix effect contributed to the method validation [Veldboer K *et al* 2011, Center for Drug Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, 2001].

Patient samples

The developed assay was able to determine ZA levels in bone sequestrations from two oncological patients Noteworthy, the method was able to discriminate the drug content in samples from patient receiving a long lasting therapy (patient A, 33 infusions of ZA) respect to patient with a shorter treatment (patient B, 9 infusions of ZA). Indeed, we found 9.4 ng/ng ZA in patient A and 1 ng/ng

ZA in patient B. Interestingly, both of them suspended the treatment 4 and 5 months before the bone sample collection, respectively. Although this suspension of the therapy, the drug was still bound to the bone tissues of patients and, therefore, detectable, as suggested by previous literature, which used indirect methods of analyses, i.e. the ZA urinary metabolite [European Medicines Agency, 2009].

6.3. Patients'characteristics

Starting from April 2008 until Semptember 2016, we collected, from study and control groups, 71 fragments of necrotic bone from a total amount of 50 patients at the ASST Santi Paolo e Carlo - San Paolo Hospital and 85 fragments of necrotic bone from 67 patients from the Dental Unit of the Papa Giovanni XXIII Hospital of Bergamo.

We analyzed the quantity of zolendronate and alendronate, for which we have set a dosage sampling method, in fragments of necrotic bone belonging to study groups and the absence of these drugs in the control groups.

6.4 Study group

The study group from the ASST Santi Paolo e Carlo - San Paolo Hospital and from Papa Giovanni XXIII Hospital is formed by 133 necrotic bone fragments from 98 patients.

- **Study group's characteristics**

Sex distribution

The entire study group included 133 sequestra, from 98 patients of which 61 women (62%) and 37 men (38%).

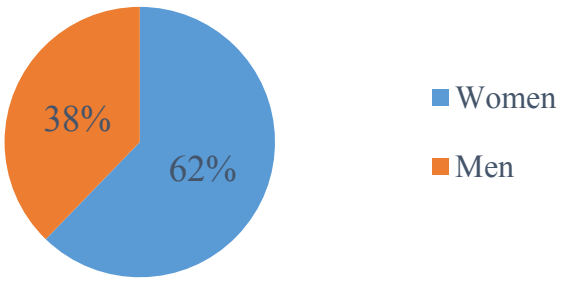


Figure 13: subdivision of the study group

The study group from ASST Santi Paolo e Carlo- San Paolo Hospital of Milan included 57 sequestra from 40 patients of which the 25% (10 patients) were belonging to the male population while 75% were belonging to the female population (30 patients). For what concerns zolendronate, the group included 15 female patients (62%) and 9 male patients (38%); for alendronate, 15 patients were women (94%) and 1 was a man (6%).

The study group from Papa Giovanni XXIII Hospital of Bergamo, included 75 sequestra from 58 patients of which 53% (31 patients) were women and 47% (27 patients) were men. In particular, 14 female patients (39%) and 22 male patients (61%) had taken zolendronate while 17 female patients (77%) and 5 male patients (23%) had taken alendronate.

Distribution by age

The mean age for patients was 69 years ± 11.9 (Standard Deviation)..

In particular, the mean age of the group from ASST Santi Paolo e Carlo- San Paolo Hospital was 72 years old ± 10.5 while the mean age of the group from Bergamo was 67 years old ± 12.5. We could not collect the general data of one patient from Bergamo.

Distribution of the patients in therapy with zolendronate and alendronate.

The study group was composed by 85 osseous sequestra from 60 patients (61%) that were taking zolendronate (mean age 67 years old ± 11.48) and 47 sequestra from 38 patients (39%) taking alendronate (mean age 71 years old ± 12.62).

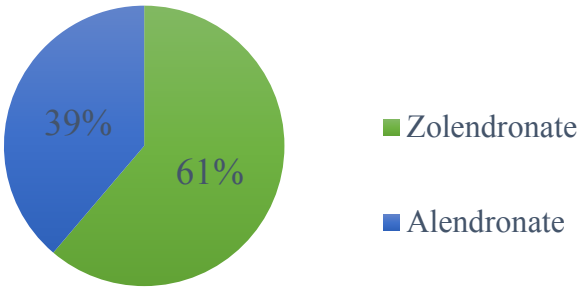


Figure 14: Subdivision of the study group by drug

From ASST Santi Paolo e Carlo- San Paolo Hospital, from 40 patients, 24 had taken zolendronate (60%) and 16 alendronate (40%). The study group from Bergamo was composed by 36 patients taking zolendronate (62%) and 22 patients taking alendronate (38%) summing up to a total of 58 patients.

For what concerns zolendronate, the mean age was around 67.86 years ± 11.48. In particular, the mean age was around 69 years ± 10.45 in Milan and 67±12,24 in Bergamo.

For alendronate, the mean age of the patients was around 71 years ± 12.62. In particular, 76 ± 8 .9 years in Milan and 66.95 ± 13 in Bergamo.

Distribution by pathology

The pathology causing the administration of zolendronate was, in 42 cases (70%) an osseous secondarism from a carcinoma, in 16 cases (27%) multiple myeloma, in 1 case (15) osteoporosis and in 1 case (1%) plasmacytoma.

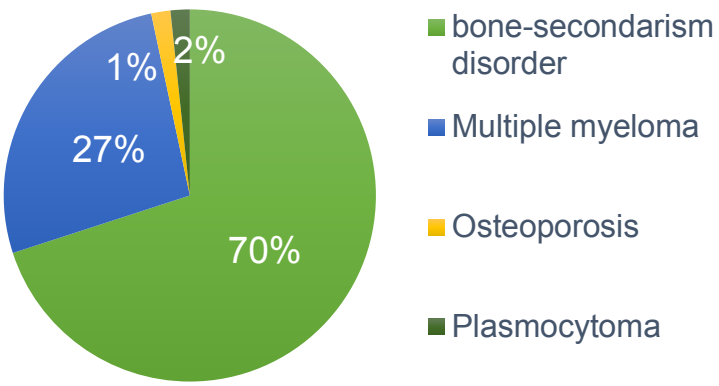


Figure 15: Subdivision of patients administered with zolendronate by pathology.

Bone secondarisms derived from prostate cancer in 12 cases, breast cancer in 21 cases, renal carcinoma in 4 cases, prostate and renal carcinoma in 1 case, gastric, pancreas, lung and neuroendocrine tumor in 1 case.

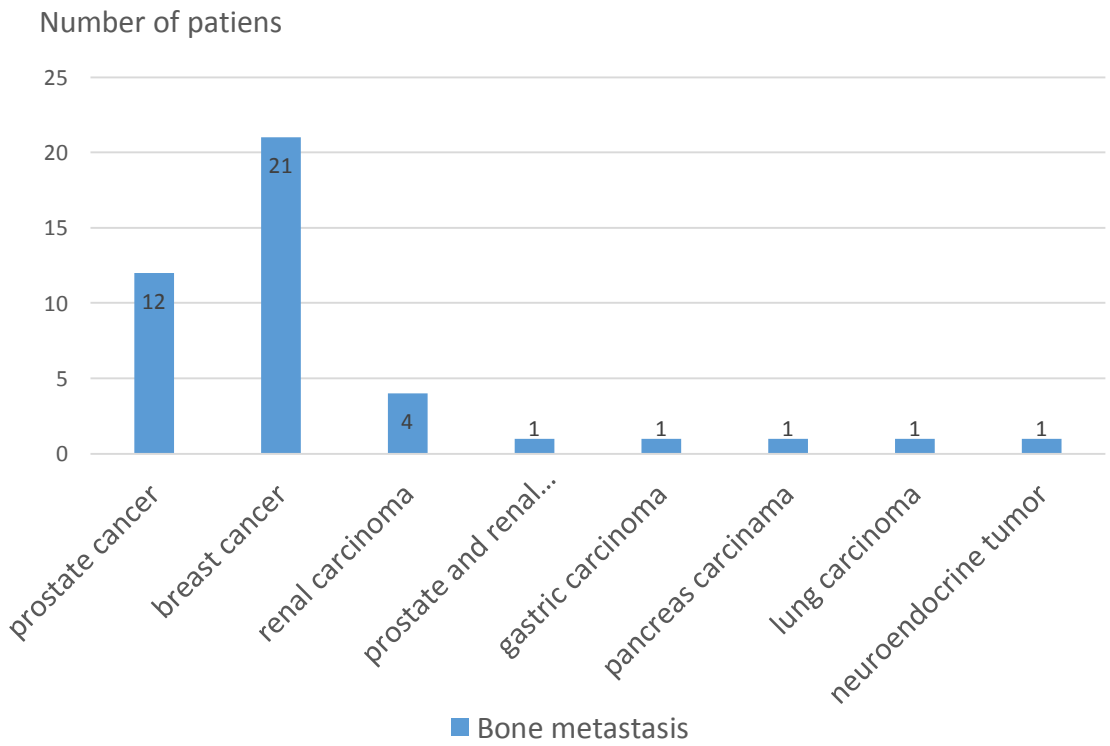


Figure 16 Distribution of the primary carcinomas that caused bone metastasis in patients treated with zolendronate.

In Milan, the base pathology that caused the administration of the drug was multiple myeloma in 8 cases (35%), bone metastasis due to carcinoma in 15 cases (65%). Bone metastases from breast cancer were seen in 10 patients (69%), from prostate cancer in 3 cases (19%), from pancreatic cancer in 1 case (6%) and from neuroendocrine cancer in 1 case (6%).

In Bergamo, the base pathology causing the administration of the drug was multiple myeloma in 8 cases (22%), bone metastasis from carcinoma in 27 cases (75%) and in 1 case (3%) plasmacytoma. In particular, bone secondarisms derived from prostate cancer in 9 cases, breast cancer in 11 cases (61%), gastric cancer in 1 case (5%), renal cancer in 4 cases (22%), lung cancer in 1 case (6%) and from an association of renal and prostate cancer in 1 case (6%).

For what concern alendronate, the base pathology was osteoporosis in 34 patients (89%), rheumatoid arthritis in 3 patients (8%) and bone secondarisms from breast cancer in 1 patient (3%).

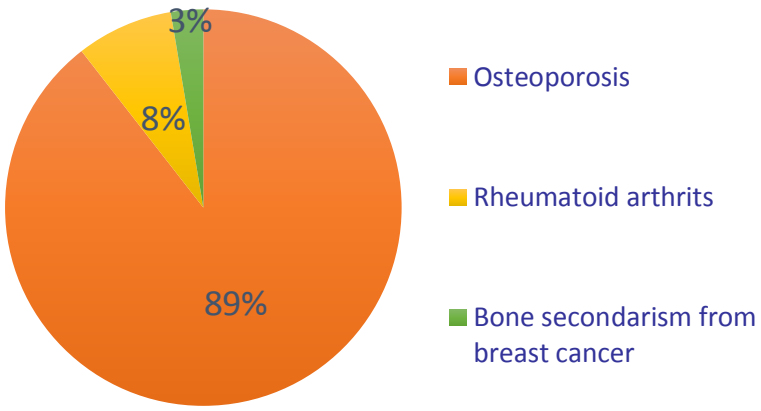


Figure 17: Patients taking alendronate by pathology.

For what concerns Milan, the base pathology for the 16 patients was osteoporosis (100%).

In Bergamo, alendronate was administered for osteoporosis in 18 cases (80%), rheumatoid arthritis in 3 cases (15%) and osseous metastasis from breast cancer in 1 case (5%).

Dosage of the therapy with bisphosphonates.

- Zolendronate: 60 patients were administered with this drug, 24 from the study group of Milan and 36 from the study group of Bergamo. All patients were treated with IV zolendronic acid.

  Patients received a mean number of infusions of 17.5 ± 11.77.

  In Milan, the average dose of an infusion was equal to 20.64 ± 16.27. We have no data about 7 patients. For Bergamo, the mean dose of infusion was equal to 16.05 ± 8.74.

  In one case, zolendronate was administered with alendronate.

- Alendronate: 38 patients were administered with this drug, 16 in Milan and 22 in Bergamo.

  All patients were treated with oral alendronic acid.

  We could not collect the exact dose administered to patients.

  In some cases, an association of alendronate with ibandronate (2 patients) or risendronate (1 patient) was necessary.

Duration of therapy with bisphosphonates

For what concerns zolendronate, the average duration of therapy was around 2.17 years ± 2.35; 11 patients took the drug for less than 6 months, 16 patients for 1 year, 15 patients for 2 years, 6 patients for 3 years, 1 patient for 4 years, 1 patient for 5 years, 2 patients for 6 years, 1 patient for 9 years, 1 patient for 10 years and 1 patient for 12 years.

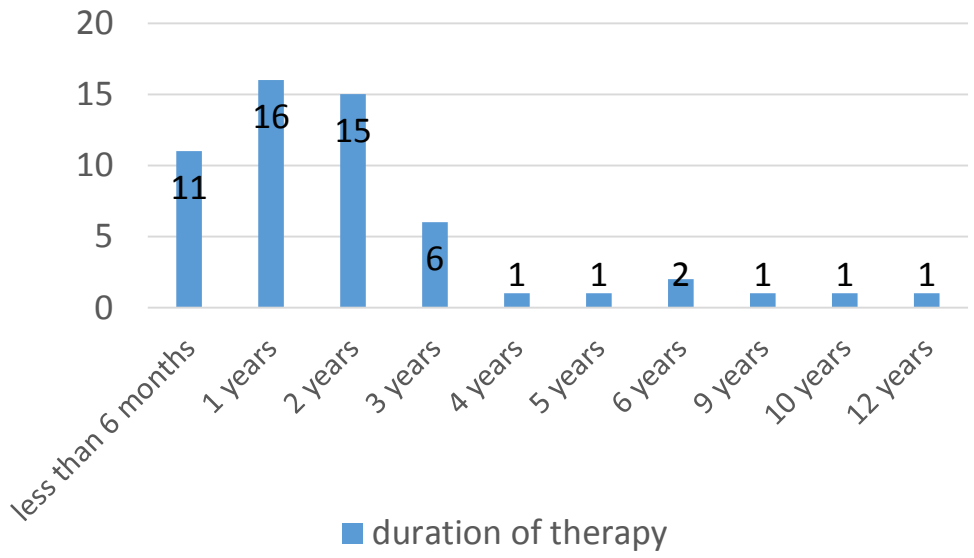


Figure 18: Duration of zolendronate therapy

In Milan, 3 patients took it for less than 1 year, 6 for 1 year, 7 for 2 years, 1 for 5 years, 2 for 6 years, 1 for 10 years and, at last, 1 for 12 years. The mean duration of therapy resulted to be 2.88 years ± 3.1. We could not collect any data for zolendronate in 3 patients.

In Bergamo, 9 patients took the drug for less than 1 year, 10 patients for 1 year, 8 patients for 2 years, 6 patients for 3 years, 1 patient for 4 years and 1 patient for 9 years. The average duration of therapy was 1.75 years ± 1.60.

Aledronate was taken for a mean duration of 8.5 years ± 5.18: 2 patients took it for 1 year, 4 for 3 years, 4

for 4 years, 3 for 5 years, 1 for 6 years, 1 for 7 years, 3 for 8 years, 1 for 9 years, 4 for 10 years, 1 for 12 years, 2 for 13 years, 2 for 14 years, 1 for 15 years, 2 for 16 years, 1 for 18 years and 1 for 20 years.

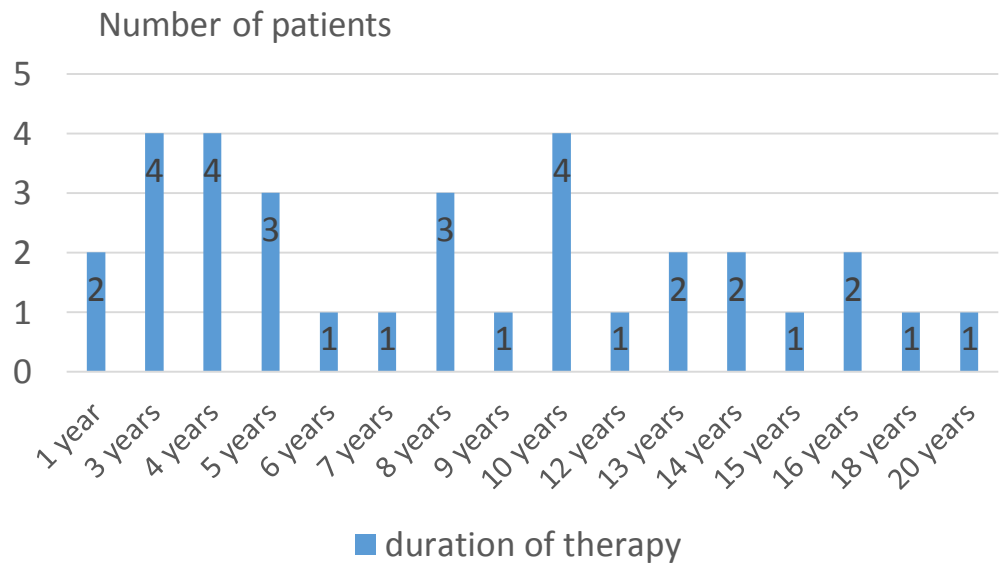


Figure 19: Duration of alendronate consumption

About the duration of consumption of the drug in Milan, we could collet data on the years of therapy from 14 patients out of 16 at the moment of surgery: 4 patients took the drug for less than 5 years, 5 for less than 10 years, 2 for less than 15 years and 2 patients for less than 20 years. The mean duration of therapy was 9 years ± 5.4 years.

In Bergamo: 2 patients were taking the drug since 1 year, 1 patient since 3 years, 3 patients since 4 years, 3 patients since 5 years, 1 patient since 7 years, 3 patients since 10 years, 2 since 13 years, 2 since 14 years and 2 since 16 years. The mean duration of therapy was 8.15 years ± 5.05. We could not collect data from 3 patients.

Interruption of therapy with bisphosphonates

About zolendronate, 18 patients were still in treatment at the moment of surgery. From the remaining 61 patients, 31 cases had interrupted therapy less than 6 months before, 12 cases less than 1 year before, 2 cases had interrupted the treatment 1 year before, 4 cases 3 years before, 1 case 5 years before, 1 case 10 years before and 3 cases 18 years before. The mean interruption, in months, was 21.78 ± 48.05 months.

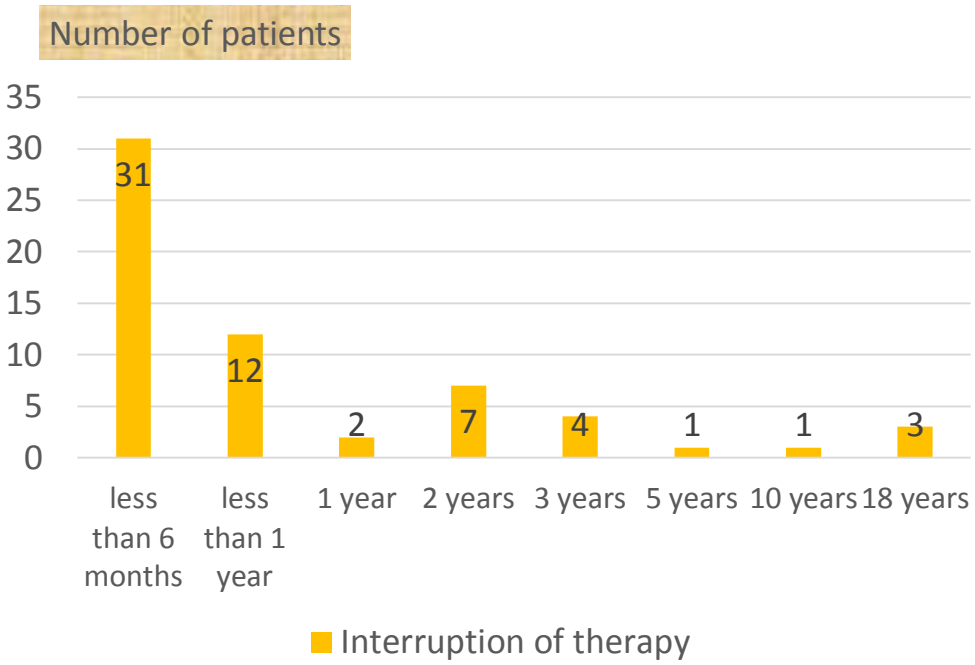


Figure 20: Interruption of therapy with zolendronate

	Patient had assumed zolendronate
Patients in therapy	18
Patients had interrupted the	61

Figure 21: Distribution of patients in treatment and patients that have interrupted the therapy with zolendronate.

In Milan, 5 patients were still in treatment at the moment of surgery while 27 patients had interrupted therapy: 12 patients less than 6 months before, 6 patients less than 1 year before, 5 patients 1 year before, 1 patient 2 years before and 3 patients 8 years before. The mean duration of the interruption results to be 31.25 months ± 69.82 months. Data about 5 patients are missing.

In Bergamo, 13 patients were still in treatment at the moment of surgery while the remaining 34 patients had interrupted it: 19 less than 6 months before, 6 less than 1 year before, 1 case 1 year before, 2 cases 2 years before, 4 cases 3 years before, 1 case 5 years before, 1 case 10 years before. The mean duration of the interruption is 16.2 months ± 16.21 months. There were no data available for 1 patient.

For what concerns alendronate, 19 patients were still in treatment at the moment of surgery, while the remaining 22 had interrupted it: less than 6 months before in 12 cases, less than a year before in 5 cases, 1 year before in 2 cases, 3 years before in 1 case and 12 years before in 2 cases. The mean duration of interruption was 21.3 months ± 40.47.

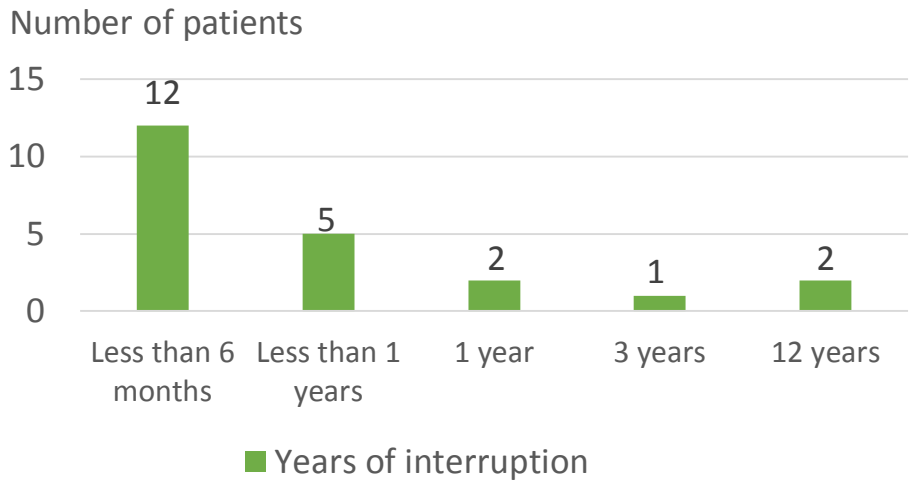


Figure 22: Distribution of the years of interruption of alendronate

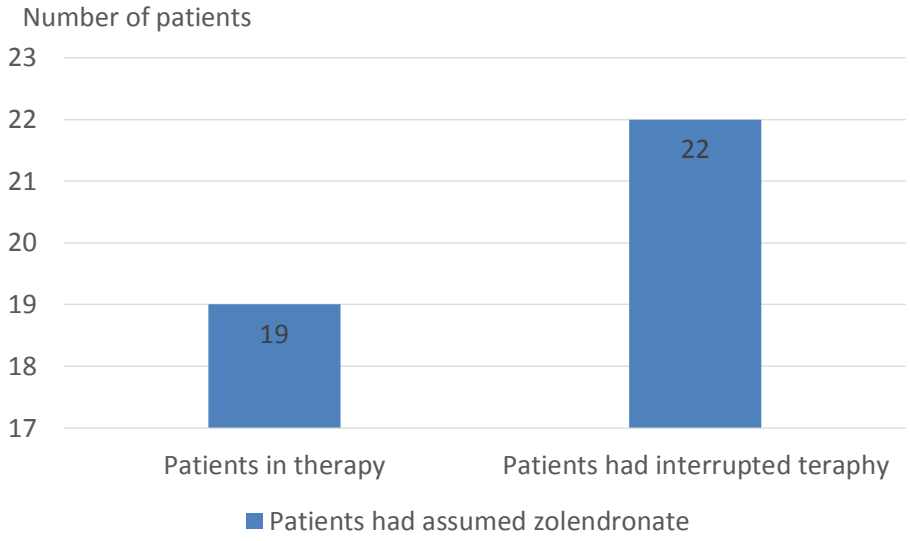


Figure 23: Distribution of patients in treatment that have interrupted treatment with alendronate.

In Milan, 4 patients were still in treatment at the moment of surgery, while 14 had interrupted it; 7 of them less than 6 months before, 5 less than 1 year before, 2 since 1 year before. The mean duration of interruption was 7.78 months ± 3.63. We could not collect data from 2 patients.

In Bergamo, at the moment of surgery, 16 patients were still in treatment with alendronate while 8 had interrupted it: 1 since 2 months before, 4 since 3 months before, 1 since a year before and 2 since 12 years before. The mean duration of interruption was 42.25 months ± 63.83. We could not collect data from 3 patients..

6.5 Control group

The control group from ASST Santi Paolo e Carlo- San Paolo Hospital and from Papa Giovanni XXIII Hospital was composed by 23 bone sequestra from 18 patients. The control group of Milan was composed by 13 sequestra from 9 patients, while the control group from Bergamo was composed by 10 sequestra from 9 patients.

- **Control group's characteristics**

Administered drugs

Drugs causing osteonecrosis in these patients were denosumab (5 cases), risedronate (4 cases), clodronate (3 cases), ibandronate (2 cases) pamidronate (1 case), ibandronate/clodronate (1 case) and ibandronate/risedronate (1 case).

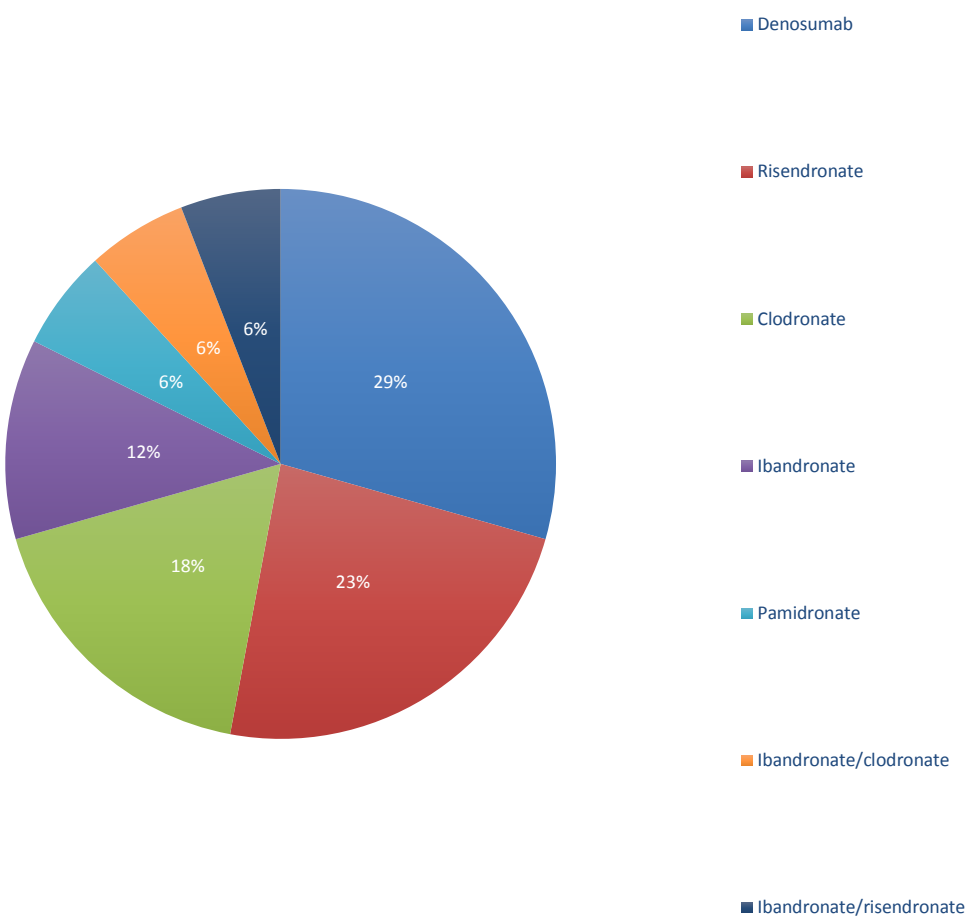


Figure 24: Subdivision of the control group from San Paolo Hospital and Papa Giovanni XXIII Hospital by drugs.

In particular, from the control group in Milan, patients in treatment with denosumab were 4, 1 was in treatment with ibandronate, 1 with ibandronate in association with clodronate, 1 with ibandronate in association with risedronate, while 2 patients underwent radiant therapy.

The control group from Bergamo was composed by 3 patients in treatment with clodronate, 4 in treatment with risendronate, 1 with denosumab, 1 with ibandronate and 1 with pamidronate.

Distribution by sex

From 18 patients, 14 were women, while 4 were men.

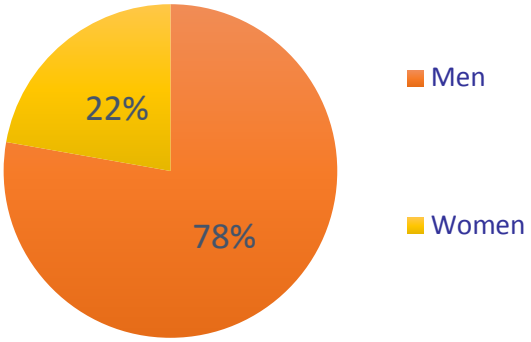


Figure 25: Distribution of the control group by sex

The control group from Milan was composed by 6 female patients and 3 male patients, while the control group from Bergamo was composed by 8 female patients and 1 male patient.

Age

The mean age was 73 years old ± 9,63.

In Milan, the mean age was 71 years old ± 10,4 while in Bergamo was 75 ± 8,8..

Base pathologies

Pathologies that caused the administration of drugs in the control group were osteoporosis in 12 cases, rheumatoid arthritis in 1 case and bone secondarisms in 4 cases: from prostatic cancer (2 cases), renal cancer (1 case) and breast cancer (1 case). The remaining 2 patients underwent radiation therapy due to tongue carcinoma (1 case) and sub-mandible lymphonode metastasis from a squamous cell cancer of unknown origin (1 case).

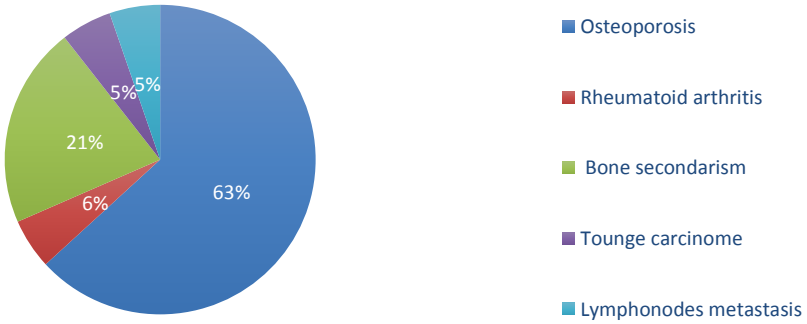


Figure 26: Subdivision of patients based on the pathology

In Milan, 4 patients suffering from osteoporosis were in treatment with ibandronate, or ibandronate in association with clodronate, or with denosumab, or with ibadronate in association with risendronate. Patients with metastasis from renal and breast cancer were trated with denosumab. The patient with tongue carcinoma and with sub-mandible lymphonode metastasis from squamous cell cancer of unknown origin underwent head and neck radiation therapy.

In Bergamo, 8 patients were suffering from osteoporosis and were in treatment with clodronate (2 cases) risedronate (4 cases), ibadronate (1 case) and pamidronate (1 case); 1 patient was in treatment with clodronate for rheumatoid arthritis and 1 patient was taking denosumab for osseous metastasis from prostatic cancer.

6.6 Bone sequestra's characteristics

A total of 154 bone samples has been analyzed from patients that developed osteonecrosis, 86 of which were treated with zolendronate, 47 sequestra from patients treated with alendronate and 23 fragments from patients that were treated with ibandronate, denosumab, clodronate, risendronate, pamindronate or radiotherapy.

From the total of samples, necrosis appeared to involve the jaw in 68% of cases (104 sequestra), while it involved the maxilla in 32% of cases (50 sequestra).

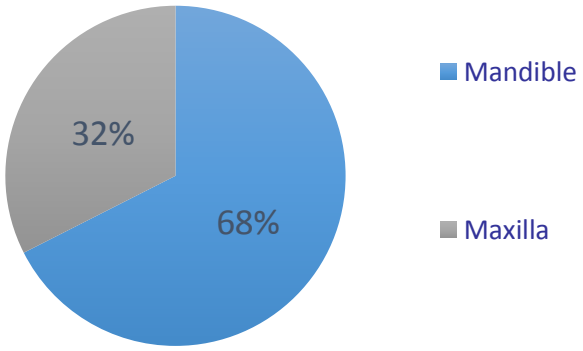


Figure 27: Distribution of osteonecrosis' localization

We experienced 10 cases of spontaneous exfoliation of the sequestra, though we could not be sure that this phenomenon did not occur in a greater number of events.

Osteonecrosis developed following extraction in 48 cases (31%), following not specified surgery in 7 cases (5%), following abscess in 9 cases (6%), alveolitis in 3 cases (2%), following the insertion of an implant in 5 cases (3%), periimplantitis in 3 cases (2%), parodontal disease in 10 cases (6%), maxillary fracture in 1 case

(1%), traumatism caused by prosthesis in 10 cases (6%) and unknown origin in 58 cases (38%).

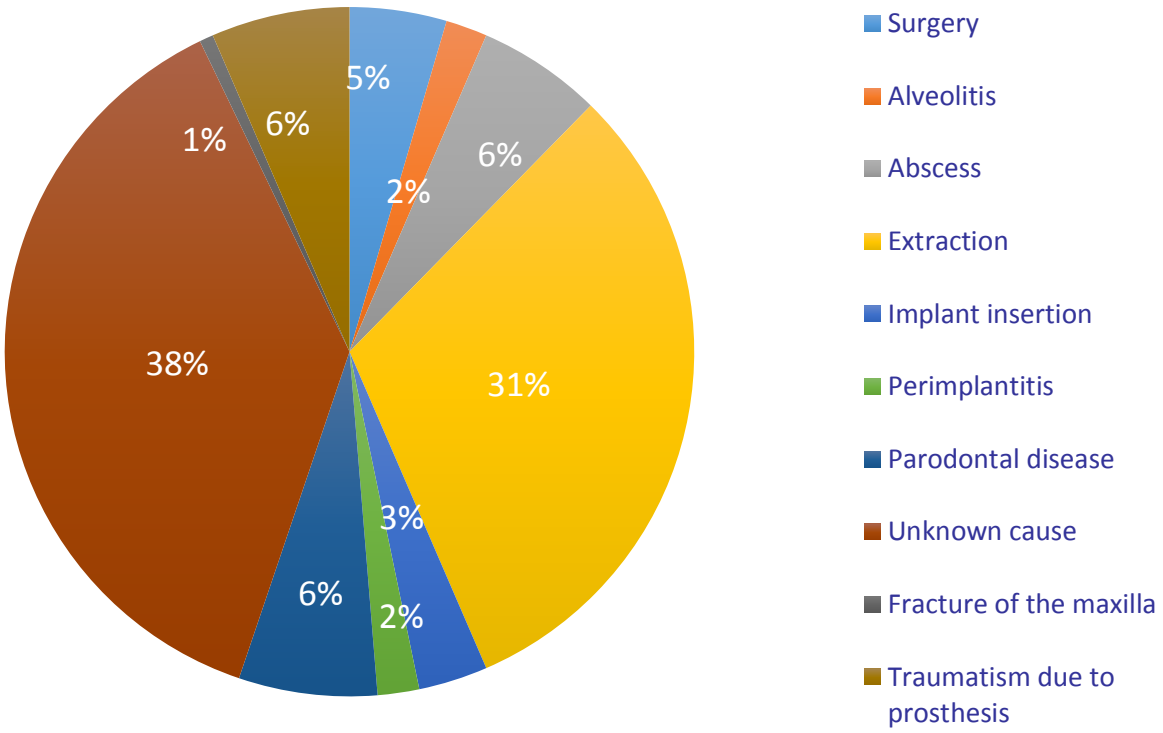


Figure 28: Distribution by cause of onset of bone necrosis

Here are listed the results divided by each center.

- *ASST Santi Paolo e Carlo –San Paolo Hospital*: for what concerns San Paolo Hospital, we analized 37 sequestra from patients in treatment with zolendronate, 20 from patients in treatment with alendronate and 13 from patients that were in treatment with other drugs specific for the bone tissue (ibandronate, denosumab, clodronate, risendronate and radiotherapy).

Osteonecrosis developed in 48 cases in the inferior maxilla (68%) and in 22 cases (32%) in the superior maxilla.

In 3 cases, the sequestrum exfoliated spontaneously while, in the remaining cases, a surgical intervention was needed.

Osteonecrosis developed in 9 cases following a dental abscess, in 1 case after implant insertion, in 1 case after an episode of periimplantitis, in 5 cases following parodontal disease, in 19 cases after dental extraction, in 3 cases after traumatism caused by prosthesis and in 32 cases after unknown cause.

- *Papa Giovanni XXIII Hospital:* for what concerns the Papa Giovanni XXIII Hospital, we analized 48

osseous fragments from patients in treatment with zolendronate, 27 fragments from patients in treatment alendronate and 10 fragments from patients in treatment with other drugs specific for the bone tissue.

Necrosis affected in 56 cases (66%) the inferior maxilla and in 28 cases (33%) the superior maxilla; in 1 case (1%) we didn't have a sure localization of the area affected by osteonecrosis.

In 13 cases, healthy bone was removed and analyzed, from patients that had already developed osteonecrosis, during the surgery for sequestrectomy.

In only 7 cases, the sequestra exfoliated spontaneously, while in the remaining cases a surgical intervention was needed in order to remove the necrotic bone.

The cause of osteonecrosis was identified in 3 cases following alveolitis, in 29 cases following the extraction of dental elements, in 7 cases following not specified surgical operations, in 4 cases after implants insertion, in 2 cases following periimplantitis, in 5 cases after periodontal disease, in 7 cases after prosthesis traumatism, in 26 cases following unidentified phenomena and in 1 case after maxillary fracture.

6.7 Zolendronate and alendronate's levels in the samples

We determined the concentration of zolendronate and alendronate in the bone sequestra from the study group.

Due to technical reasons (correct conservations, developing and validation of the method), we could not analyze 29 samples (15 of zolendronate and 14 of alendronate).

In particular, for what concerns the study group from the San Paolo Hospital of Milan, we could not examine 11 fragments of necrotic bone from patients that were in treatment with zolendronate and 4 fragments from patients in treatment with alendronate.

From the Papa Giovanni XXIII Hospital of Bergamo, it was not possible to analyze 4 fragments of necrotic bone from patients in treatment with zolendronate and 10 fragments from patients in treatment with alendronate.

Patient	**Sex**	**Age**	**Centre of origin**	**Drug**	**Pathology**	**Quantity (ng)**	**Quantity (ng/mg)**
1	M	69	MI	zolendronate	prostate cancer	127,9	6,73
2	F	70	MI	zolendronate	breast cancer	NEGATIV	0

3	F	68	MI	zolendronate	breast cancer	2,01	0,1
4	M	48	MI	zolendronate	multiple myeloma	221,9	11,68
5	F	62	MI	zolendronate	breast cancer	80,9	10,11
6	F	65	MI	zolendronate	multiple myeloma	189,4	9,97
7	M	66	MI	zolendronate	multiple myeloma	117,2	6,17
8	M	69	MI	zolendronate	pancreatic cancer	77,8	4,86
9	F	51	MI	zolendronate	breast cancer	732,28	33,29
10	F	49	MI	zolendronate	breast cancer	517,02	24,62
11	F	51	MI	zolendronate	breast cancer	9,1	1,82
12	M	66	MI	zolendronate	multiple myeloma	1309,384	63,304
13	F	76	MI	zolendronate	multiple myeloma	502,358	31,39
14	F	76	MI	zolendronate	multiple myeloma	508,418	22,1
15	F	82	MI	zolendronate	breast cancer	809,475	40,47
16	M	70	MI	zolendronate	multiple myeloma	288,974	14,45
17	M	76	MI	zolendronate	multiple myeloma	266,448	13,32
18	F	82	MI	zolendronate	breast cancer	879,458	43,97
19	M	76	MI	zolendronate	multiple myeloma	510,668	25,53
20	M	76	MI	zolendronate	multiple myeloma	466,004	23,3
21	M	76	MI	zolendronate	prostate cancer	314,522	15,72
22	F	76	MI	zolendronate	breast cancer	238,9	9,556
23	F	79	MI	zolendronate	breast cancer	63,75	3,18
24	F	72	MI	zolendronate	breast cancer	54,7	2,88
25	F	53	MI	zolendronate	neuroendocrine tumor	102,1	6,38125

26	M	71	MI	zolendronate	multiple myeloma	117,2	6,168421053
27	F	70	MI	alendronate	osteoporosis	268,59	12,79
28	F	65	MI	alendronate	osteoporosis	1221,89	64,31
29	F	80	MI	alendronate	osteoporosis	3667,4	166,7
30	M	78	MI	alendronate	osteoporosis	1185,8	53,9
31	F	90	MI	alendronate	osteoporosis	270,6	12,3
32	F	80	MI	alendronate	osteoporosis	706,2	47,08
33	F	76	MI	alendronate	osteoporosis	4896,1	222,55
34	F	67	MI	alendronate	osteoporosis	3132	156,6
35	F	88	MI	alendronate	osteoporosis	404,14	36,74
36	F	77	MI	alendronate	osteoporosis	208,74	9,94
37	F	89	MI	alendronate	osteoporosis	1234,8	137,2
38	F	70	MI	alendronate	osteoporosis	1328,24	60,37
39	F	91	MI	alendronate	osteoporosis	423,17	38,47
40	F	81	MI	alendronate	osteoporosis	326,82	15,56
41	F	86	MI	alendronate	osteoporosis	1468,9	91,8
42	F	64	MI	alendronate	osteoporosis	1596,3	69,4
43	M	72	BG	zolendronate	multiple myeloma	36,2	1,91
44	M	83	BG	zolendronate	prostate cancer	189,9	9,99
45	M	83	BG	zolendronate	prostate cancer	1	2
46	F	62	BG	zolendronate	breast cancer	174,6	9,19
47	M	72	BG	zolendronate	multiple myeloma	58,9	3,1
48	M	38	BG	zolendronate	multiple myeloma	224,4	8,98

49	M	73	BG	zolendronate	prostate cancer	52,9	2,65
50	M	68	BG	zolendronate	renal cancer	54,7	2,88
51	M	49	BG	zolendronate	multiple myeloma	21,4	1,02
52	F	62	BG	zolendronate	multiple myeloma	34,9	1,66
53	M	76	BG	zolendronate	renal and prostate cancer	45,2	2,38
54	M	80	BG	zolendronate	lung cancer	52,9	2,78
55	F	73	BG	zolendronate	renal cancer	56,6	2,83
56	M	70	BG	zolendronate	renal cancer	23,1	1,22
57	M	70	BG	zolendronate	renal cancer	36,7	1,84
58	F	65	BG	zolendronate	breast cancer	43	1,79
59	F	65	BG	zolendronate	breast cancer	85,1	4,48
60	F	55	BG	zolendronate	renal cancer	8,2	0,43
61	F	55	BG	zolendronate	renal cancer	65,1	2,71
62	M	84	BG	zolendronate	prostate cancer	102,1	6,38
63	M	84	BG	zolendronate	prostate cancer	117,1	5,58
64	M	39	BG	zolendronate	breast cancer	110,3	5,01
65	M	39	BG	zolendronate	breast cancer	49,8	1,99
66	F	65	BG	zolendronate	breast cancer	59,9	18,15
67	F	53	BG	zolendronate	breast cancer	88,22	4,01
68	M	38	BG	zolendronate	multiple myeloma	135,72	7,54
69	M	39	BG	zolendronate	breast cancer	39,1	2,3
70	M	59	BG	zolendronate	plasmacytoma	22,4	1,12
71	M	78	BG	zolendronate	multiple myeloma	368,87	33,53

72	M	73	BG	zolendronate	prostate cancer	278,74	13,27
73	F	64	BG	zolendronate	gastric cancer	985,4	109,48
74	M	74	BG	zolendronate	multiple myeloma	235,2	14,7
75	F	66	BG	zolendronate	breast cancer	121,7	7,17
76	F	49	BG	zolendronate	breast cancer	39	6,5
77	M	74	BG	zolendronate	multiple myeloma	<0	
78	M	85	BG	zolendronate	multiple myeloma	121,4	8,67
79	M	85	BG	zolendronate	multiple myeloma	114,5	8,17
80	F	50	BG	zolendronate	breast cancer	1185,8	53,9
81	F	57	BG	zolendronate	breast cancer	269,06	12,23
82	M	75	BG	zolendronate	prostate cancer	1656,3	82,81
83	F		BG	zolendronate	breast cancer	2215,6	100,7
84	M	77	BG	zolendronate	prostate cancer	215,3	10,25
85	M	77	BG	zolendronate	prostate cancer	206	9,36
86	M	75	BG	zolendronate	prostate cancer	<0	
87	M	61	BG	alendronate	osteoporosis	2787	126
88	F	45	BG	alendronate	breast cancer	5,32	0,28
89	F	81	BG	alendronate	osteoporosis	89,32	6,38
90	F	38	BG	alendronate	osteoporosis	69,79	9,97
91	M	72	BG	alendronate	rheumatoid arthritis	447,8	22,39
92	F	68	BG	alendronate	osteoporosis	230,3	46,06
93	M	64	BG	alendronate	osteoporosis		< 0
94	F	55	BG	alendronate	osteoporosis	404,14	36,74
95	F	70	BG	alendronate	osteoporosis	208,74	9,94

96	F	81	BG	alendronate	osteoporosis	1234,8	137,2
97	F	72	BG	alendronate	rheumatoid arthritis	2536,8	126,84
98	F	70	BG	alendronate	osteoporosis	1828,42	166,22
99	M	76	BG	alendronate	osteoporosis	1334,72	41,71
100	F	77	BG	alendronate	osteoporosis	2536,8	126,84
101	F	65	BG	alendronate	osteoporosis	1828,42	166,22
102	F	59	BG	alendronate	osteoporosis	1334,72	41,71
103	F	63	BG	alendronate	osteoporosis	4896,1	222,55

Table 12: Results obtained from the analysis of the osseous sequestra from patients in treatment with zolendronate and alendronate.

The average dose of zolendronate was 14,96 ng/mg ± 22,14.

The average dose of alendronate was 77,58 ng/mg ± 66,48.

- *Zolendronate*

Dose and therapy

About zolendronate, no correlation could be observed between years of therapy and quantity of zolendronate in the bone sequestrum. For this analysis, we had to limit the samples to those for which we had data and that had not interrupted the therapy at the moment of the sampling (14 patients, mean age 74 years old ± 9.84).

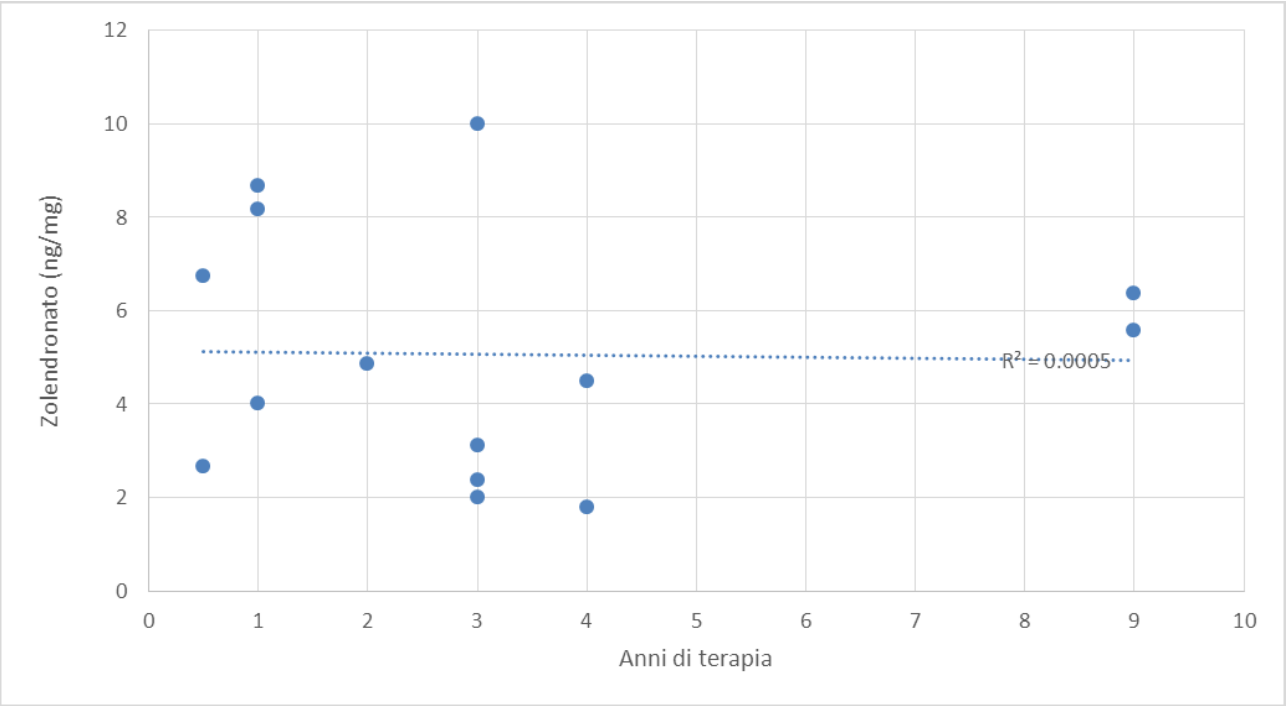


Figure 29: Correlation between years of therapy and quantity of zolendronate

Dose and number of infusions administered

In accordance with previous data, no correlation was highlighted between the number of administrations of zoledronate and quantity of zolendronate in the removed bone sequestrum. For this analysis, we had to limit the number of samples to those belonging to patients for which we had data at the moment of sampling and that had not yet interrupted the treatment (13 patientis, mean age 74 years old ± 9.73).

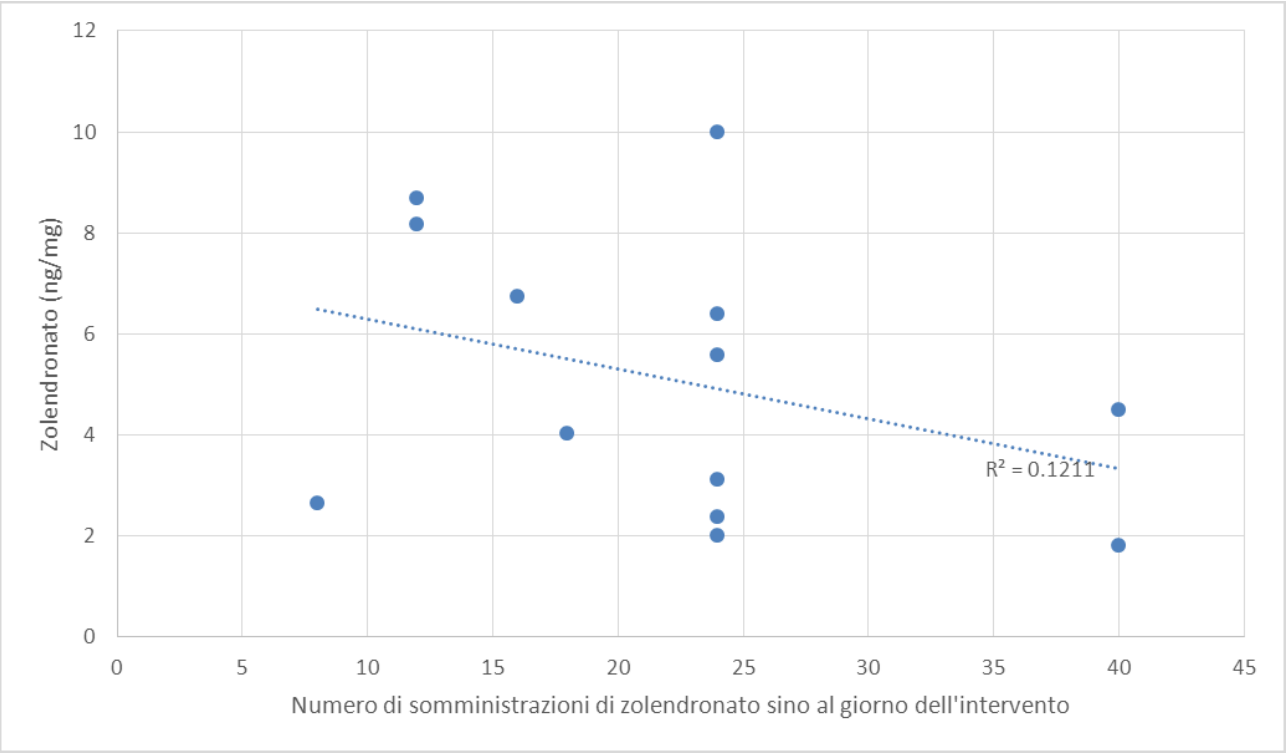


Figure 30: Correlation between number of administrations and quantity of zolendronate.

Dose and interruption of the therapy

In this case, it was possible to highlight a certain trend of reduction of the drug with the passing of the months of interruption. For this analysis we had to limit the number of samples to those for which we had available data (47 patients, mean age 64 years old ±13.36).

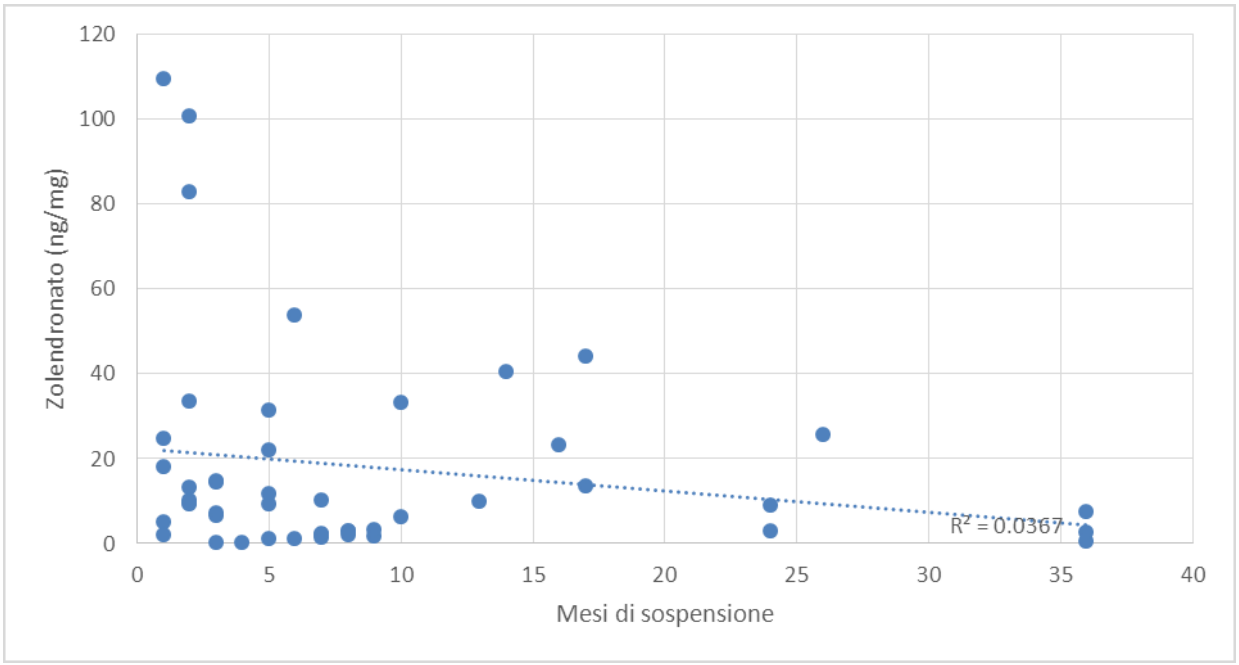


Figure 31: Correlation between months passed from the interruption of the therapy and quantity of zolendronate

- *Alendronate*

Dose and duration of the therapy As for zolendronate, alendronate as well was not highlighted with a correlation between years of therapy and quantity of alendronate in the bone sequestrum (Fig. 32). For this analysis, we had to limit the sampling to those for which we had available data and for those patients that had not interrupted the therapy at the moment of sampling (15 patients, mean age 69 years old ± 13.28).

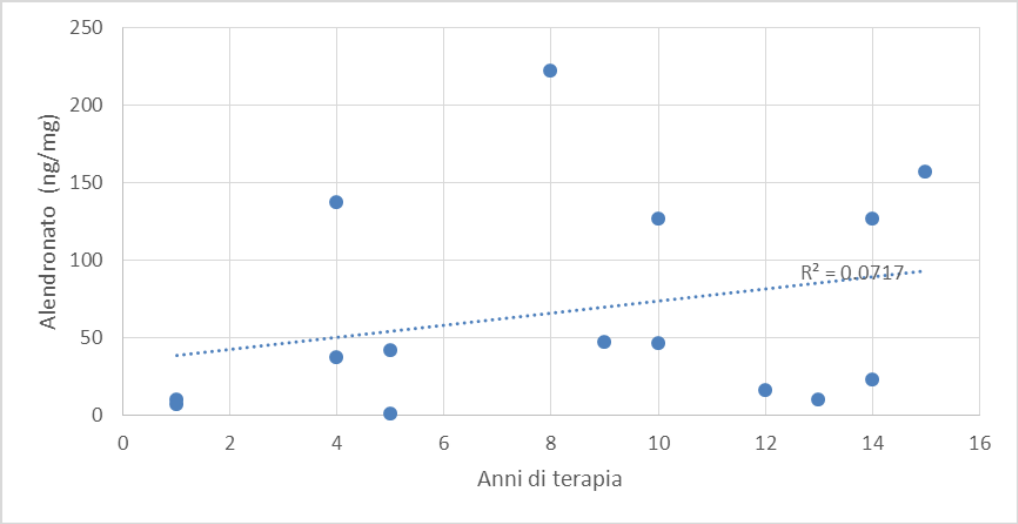


Figure 32: Correlation between years of therapy and quantity of alendronate

Dose and interruption of therapy

In this case, it was possible to highlight a certain trend of reduction of the drug with the passing of months of interruption of therapy (fig 33). For this analysis we had to limit the number of samples to those belonging to patients for which we had available data (13 patients, mean age 74 years old± 11.51).

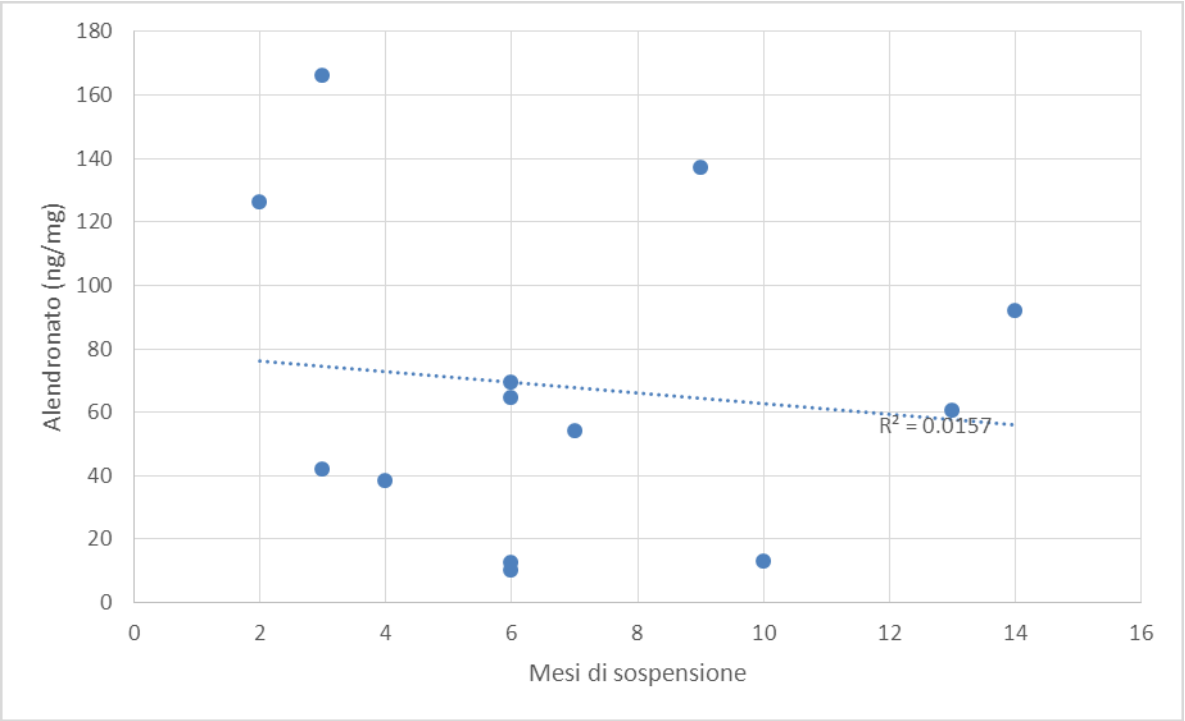


Figure 33: Correlation between months of interruption and quantity of alendronate

DISCUSSION

7. Discussion

Bisphosphonates are drugs that work on osseous reabsorption. Their efficacy varies according to the type of the drug, way of administration and dose.

In particular, zolendronate is indicated in 2015 AIOM (Italian Association of Medical Oncology) guidelines for treatment of bone metastasis of several carcinomas, as in breast cancer, lung cancer and renal cancer. The optimal duration of treatment has not been set yet, but the benefits of zolendronate reported in several studies are obtained after a period of treatment of 2 years [Saad F. *et al*, 2004; Hirsh V *et al*, 2004]. The standard dose is 4 mg in infusion of 15 minutes every 3-4 weeks [Wong *et al*. 2004]. The annual dose corresponds to 48 mg. The dose of zolendronate has to be adjusted to the conditions of the patients and to renal functionality.

Alendronate, on the other side, is the gold standard drug in treating osteoporosis and is administered orally in 70 mg pills, ingested weekly. The annual dose is equal to 3640 mg and usually this drug is taken for several years. Doses are dependent on oral bioavailability of alendronate which is quite low: 0.7% of the administered dose. It is estimated that an oral dose of 10 mg equals to a systemic exposure to ~70 µg of IV alendronate [Cocquyt, MD *et al*, 1999].

The bio-distribution of bisphosphonates, regarding the bone, is limited. It is not clear the way used by the drug to pass from the circulatory system to the bone and viceversa, even though it is generally assumed that the drug could pass in the extracellular compartment of the bone thanks to paracellular transport and bonding the available hydoxylapatite [Cremers *et al,* 2011]. We have not precise data about the quantity of drug absorbed in human bone. Pre-clinical data on dogs and rats, however, may give some indications. Weiss *et al.* administered infusions of 0.15 mg/kg of radioactive zolendronate to rats for 16 consecutive days and tested the distribution of the drug after 5 minutes, 4 and 24 hours after the first infusion, 24 hours after the eighth infusion and 1, 16, 31, 128 and 240 days after the sixteenth infusion. It has been noted that 5 minutes after infusion, the highest concentration is in plasma but decreases rapidly. After 4 hours, the highest concentration was in calcified tissues showing, opposite to non-calcified tissues, a continuous absorption of zolendronate until 24 hours after the administration of the dose. No signs of saturation could be noted and the quantity of zolendronate was 6-7 times higher 24 hours after the eighth administration and 10-12 times higher 24 hours after the sixteenth administration compared to single dose. The quantity of zolendronate decreased very slowly in the observation time of 240 days. Sixteen days after the last dose, the quantity of drug in plasma and non-calcified tissues decreased below 0.4 nmol/g, while the quantity in bone

remained over 10 nmol/g 240 days after the last dose. In this study, the distribution of zolendronate in dogs was investigated 96 hours after a single IV administration. It has been noted that the exposure was greater in the bone of the axial skeleton compared to the bones of the appendicular skeleton and of the skull. Moreover, the absorption of the drug in the compact bone is lower compared to trabecular bone and only a very small quantity of drug could be detected in the central cavity of compact bones [Weiss *et al*, 2008].

Bisphosphonates seem to bond in particular to the trabecular component of bones [Porras *et al,* 1999]. Moreover, it has been observed that bisphosphonates are able to bond better to bones with a high degree of bone remodeling [Porras *et al,* 1999] and that saturation can not be reached, even after several administrations [Porras *et al*,1999; Weiss *et al*, 2008]. Bisphosphonates are then slowly eliminated with urine, taking advantage of the "tissutal storage" of the osseous tissue; 60% of the administered dose of zolendronate, it has been esteemed, is detained by the bone [De Luca *et al,* 2013].

Purpose of the study was to evaluate the effective presence of drug in bones undergoing osteonecrosis and the possible correlation between years of therapy, months of interruption of the therapy and quantity of bisphosphonates detained by the bone.

In collaboration with the Dental Unit of the Papa Giovanni XXIII Hospital of Bergamo, we have collected the osseous sequestra, surgically removed or spontaneously exfoliated, from patients in therapy with bone anti-reabsorption drugs like zolendronate, alendronate, risendronate, clodronate, ibandronate and denosumab and samples from patients undergoing radiotherapy to the head and neck district. We examined two different drugs: zolendronate and alendronate and we have collected a detailed medical history of all patients. The remaining osseous sequestra (from radiotherapy, denosumab and other bisphosphonates) were considered as a control group and were analyzed in order to exclude the presence of the two drugs examined in the study.

This is the first study able to directly detect and quantify the ZA within the bone sequestrations of patients receiving ZA therapy. The method was based on the high resolution and sensitivity of LC-MS/MS, with a simple SPE purification procedure required for the sample preparation.

High performance was demonstrated in terms of precision, sensitivity and accuracy, making the proposed method useful to detect and quantify other classes of bisphosphonates from human bone tissues. The method showed to be a valuable tool for clinical studies on BRONJ patients, to better clarify the pathophysiological role of ZA bone concentration in the development of

BRONJ and related prognosis.

The analytical method was accurate and reproducible, in accordance with previous literature [Hunter *et al,* 2011; Raccor *et al*, 2015]. Mean dosage of zolendronate was 14.96 ng/mg ± 22.14 while for alendronate it was 77.58 ng/mg ± 66.48. These quantities were in agreement with those expected from data presented in literature [Weiss *et al*, 2008; Cocquyt, MD *et al*, 1999].

For what concerns zolendronate, we have evaluated the correlation between years of therapy and number of administrations in patients still in treatment compared to quantity of bisphosphonates and correlation of months of interruption in patients no longer in treatment and quantity of bisphosphonates. For what concerns the years of treatment and the number of administrations, no increase of bisphosphonates related to years of administration could be noted. A further fact examined was the amount of months of interruption: in this case, as suspected, a decrease of the quantity of drug in the bone sequestrum was noted with the increasing of the months of interruption.

For what concerns alendronate, in a similar way, we evaluated the correlation between years of therapy and months of interruption with the quantity of drug stored in the osseous sequestrum. In this case, we observed a relative trend with a slight increase of the quantity of alendronate with the passing of the years of therapy; in a similar way, with the passing of the years of interruption we observed a decreasing of the quantity of bisphosphonates.

Nevertheless, the obtained results were not substantial in order to demonstrate a correlation statistically significant. This fact could be explained, at least partially, with the fact that the dosage was assessed on necrotic bone that could have lost part of its content of drug. In fact, the dose-dependent pre-clinical storage [Weiss *et al*, 2008] could be failing from the moment the necrotic bone becomes non-vital, not vascularized, not able to undergo remodeling and partially demineralized; all these factors could contribute to the premature releasing of the drug from the bone tissue [Hunter *et al,* 2011]. Moreover, osteonecrosis caused by bisphosphonates is often associated with important infective patterns creating a very acid setting. The acid environment is known to divide the bone-bisphosphonate binding [Otto *et al*, 2010].

It is necessary to expand the patient sample of this study in order to improve the results here presented. In fact, it was possible to demonstrate that the variable studied (concentration detected in the bone) demonstrated very low measures, detected in ng). Any possible and

eventual variation, therefore, is in a very small range of values and would be hardly measurable with the analytical methods available, unless not measured on expanded samplings. A second aspect that could help to clarify the obtained data, would be the possibility to collect the temporal data about the onset of the osteonecrosis in patients, so linking the total dose to the exact moment of onset of the osteonecrosis (when the bone has lost its capacity of remodeling and potentially the capacity of storing the drug) with the concentration then detected in the sequestrum.

CONCLUSIONS

8 Conclusions

Purpose of the study was to evaluate the effective presence of drug in bones undergoing osteonecrosis and the possible correlation between years of therapy, months of interruption of the therapy and quantity of bisphosphonates detained by the bone.

Bisphosphonates are among the 20 most often prescribed drugs, thanks to their ability of preventing pathological fractures in patients suffering from osteoporosis, for the treatment of malignat hypercalcaemia and Paget's disease, to contrast tumoral pathologies like multiple myeloma and metastatic diffusion from breast, prostate, kidney tumors and other types of cancer. Despite these facts, and association between administration of bisphosphonates and the onset of osteonecrosis has been ascertained.

Considering the wide use of these drugs and the impossibility of preventing osteonecrosis in 100% of cases, it is necessary to determine how long the risk of such a side effect remains after the interruption of therapy with bisphosphonates.

At present, literature is not able to provide this information due to the limited number of articles treating this topic and to the complexity of studying this side effect due to pharmacokinetics and pharmacodynamics properties of bisphosphonates and the characteristics of the necrotic bone.

Beyond the need of examining in depth these studies, it remains of essential importance to concentrate on possible prevention: patients starting treatment with bisphosphonates must be cleared of all possible infective foci and must be educated to a correct oral hygene in order to avoid potential aggressive therapies. For all those patients that have already started the treatment, it is important to carry out preventive protocols, like the one suggested by the Unit of Oral Pathology of the University of Milan [Lodi *et al,* 2010] in order to prevent the onset of osteonecrosis after oral surgery.

In closing, since our data were still limited, it is compulsory to increase the amount of samples, in order to carry out a more complete, substantial and statistically significant study, and to collect more detailed information from the clinical history of the patients and from the onset of osteonecrosis.

We believe that more studies would be needed in order to clarify the effective permanence of

bisphosphonates in the osseous context.

9. References

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