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DOCTORAL DISSERTATION

"Impacts on the growing and adult skeleton of different genetically-achieved RANKL activity levels, consequences on the response to zoledronic acid"

Submitted in partial fulfillment of the requirements for the Ph.D. degree in *Molecular and cellular biology*

JORGE WILLIAM VARGAS FRANCO.

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***AREA** : BIOLOGY – MÉDICINE – DENTISTRY- HEALT*
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By
«JORGE WILLIAM VARGAS FRANCO

« *Impacts on the growing and adult skeleton of different genetically-achieved RANKL activity levels, consequences on the response to zoledronic acid*»$*

Thèse présentée et soutenue à «Nantes », le « 10 decembre 2019»
Unité de recherche : INSERM UMR1238, Sarcomes osseuxe et remodelage des tissus calcifiés
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UNIVERSITE BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSIDAD DE ANTIOQUIA

UNIVERSITÉ DE NANTES

THESE DE DOCTORAT

L'UNIVERSITE DE NANTES ET L'UNIVERSITE D'ANTIOQUIA

ECOLE DOCTORALE N° 605
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THESES JURY:

- Françoise Rédini
- Claudine Blin
- Uriel Zapata Múnera
- Mónica Tatiana Parada Sánchez
- Diego Alejandro Velásquez Puerta

"If, before every action,
we were to begin by weighing up the consequences,
thinking about them in earnest,
first the immediate consequences,
then the probable,
then the possible,
then the imaginable ones,
we should never move
beyond the point
where our first thought brought us
to a halt."

José Saramago
BLINDNESS

ACKNOWLEDGMENTS

Making science should be more than staying immersed for long time in the laboratory`s room scrutinizing molecules, looking for explanations, forgetting ourselves, our environment and the world, staying away like hermits. As a contribution to the well-being of humanity, science should be, by its principle of serving life, characterized by the desire to share, to deliver, to appreciate and to enjoy the knowledge in relationship and in action with others without selfishness.

After four years dedicated to my research work, true to my principles, I can calmly say that I tried to do it. I enjoyed and lived science sharing with others. I found wonderful people like Dr. Frédéric Lézot who made of his tutorial work a real-life commitment. I have no words to thank his support, knowledge, teaching and his experience shared with me throughout this time. Frédéric, I want you to know how much I value your unconditional support: “Mon professeur; je te remercie beaucoup”. We still have many more remarkable experiences to share.

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My greatest affections belong to my family. Although they are not interested in my subject of study, they care for what it represents to me. They are my father, my

mother, my brothers, my sister, my little brother Diego, and in a special way my beloved niece María Paula. I owe my family the great support and help they have given me during my health problems this past year, making it a little less harsh. I couldn't forget my cousin Cristina and her special husband Jost; they always supported me and represented in Europe all my beloved family.

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This academic process does not end, it just begins!

IMPACTS ON THE GROWING AND ADULT SKELETON OF DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS, CONSEQUENCES ON THE RESPONSE TO ZOLEDRONIC ACID

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GLOSSARY

- **APRIL:** A-proliferation inducing ligand.
- **AFF**: Atypical femoral fracture.
- **BAFF:** B cell-activating factor family.
- **BPs:** Bisphosphonates.
- **BRONJ:** Bisphosphonates related osteonecrosis of the jaws.
- **BLCs:** Bone lining cells.
- **BRC:** Bone remodeling compartment.
- **BV:** Bone volume (mm^3). Corresponds to the volume of the region segmented as bone (1).
- **BV/TV:** Fraction (%) bone volume/total volume. Corresponds to the ratio of the segmented bone volume to the total volume of the region of interest (1).
- **DKK-1:** Dickkopf-related protein 1.
- **EDTA:** Ethylene-diamine-tetraacetic acid.
- **IGF:** Insulin-like growth factor.
- **IL:** Inteleukine.
- **FGFs:** Fibroblast growth factors.
- **IL-34:** Interleukine 34**.**
- **LIGHT:** Lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells.
- **LGR4:** Leucine-rich repeat-containing G-protein-coupled Receptor 4.

- **LOX:** lysyl oxidase.
- **LPL:** Lipoprotein related protein.
- **LPS:** Lipopolysaccharides.
- **M-CSF:** Macrophage colony-stimulating factor.
- **Micro-CT:** Micro-computed tomography.
- **MITF:** Microphthalmia transcription factor.
- **MRONJ:** Medical related osteonecrosis of the jaws.
- **NGF:** Nerve growth factor.
- **N-BPs:** Nitrogen bisphosphonates.
- **NF- κB:** Nuclear Factor κB.
- **NON-N-BPs:** Non-nitrogen bisphosphonates.
- **OBs:** Osteoblast cells.
- **OCs:** Osteoclasts cells.
- **ODs:** Osteolytic diseases.
- **ONJ:** Osteonecrosis of the jaw.
- **OPG:** Osteoprotegerin, a soluble decoy receptor for RANKL **(**TNFSF11B).
- **OPG^{KO}:** Osteoprotegerin deficient mice (-/-), null mutant for OPG (ko).
- **PCR:** Polymerase Chain Reaction.
- **$RANK^{Tg}$:** Transgenic mice with overexpression of the receptor activator of the nuclear factor-kB ligand.
- **RANK:** Receptor activator of nuclear factor-kB (TNFSF11A).
- **RANKL:** Receptor activator of nuclear factor-kB Ligand (TNFSF11).

- **SOFAT:** Secreted osteoclastogenic factor of activated T cells.
- **SOST**: Secreted proteins Sclerostin.
- **TAK:** TGF-β-activated kinase 1-binding protein (TAB2 and TAK1).
- **TGF:** Transforming growth factor.
- **TRAF:** TNF receptor-associated factors.
- **TRAP:** Tartrate-resistant acid phosphatase.
- **TGF-β:** Transforming growth factor-beta.
- **TNF:** Tumor necrosis factor.
- **TNFRSF:** Tumor necrosis factor receptor superfamily.
- **T.N:** Trabecular number (1/mm). The measure of the average number of trabeculae per unit length (1).
- **T.Sp**: Trabecular separation (mm). Mean distance between trabeculae, assessed using direct 3D methods (1).
- **T.Th:** Trabecular thickness (mm). Mean thickness of trabeculae, assessed using direct 3D methods (1).
- **Wnt:** Wingless-type.
- **ZOL:** Zoledronic acid.

ABSTRACT

Bone is a dynamic tissue with constant adaptative physiological changes in its homeostasis through life called bone modeling and remodeling. These processes enable to achieve a balance between bone formation and bone resorption at any particular age. These processes involve the activity of distinct types of cells namely chondroblasts, osteocytes, osteoblasts, bone lining cells and osteoclasts. The role of the osteoclasts has been deeply analyzed in endochondral ossification during development and growth, and in adulthood throughout bone remodeling. Osteoclast differentiation requires two essential factors named macrophage colony stimulating factor (M-CSF), acting on its receptor c-FMS, and receptor activator of nuclear factor-κB ligand (RANKL), acting on its main receptor RANK which binding is modulated by the decoy receptor osteoprotegerin (OPG). M-CSF is involved in the osteoclastogenesis process mainly by promoting the proliferation and survival of osteoclast precursors. RANKL functions as the primary factor driving differentiation of osteoclasts precursors into ostéoclasts, as well as in their maturation and activity. Any imbalances between bone formation and resorption secondary to osteoclasts alterations lead to pathologies that could appear either in childhood or adulthood. Those pathologies are known as osteopetrotic and osteolytic diseases. Osteopetrotic disease group is related to a greater bone apposition, resulting in a bone mass increased, mainly due to osteoclasts dysfunction or absence. The osteolytic disease group, on the contrary, is related to an increase in bone turnover associated to an increase in osteoclasts number or/and activity. Osteolytic diseases are so characterized by a negative balance between bone formation and resorption. Osteolytic diseases generate a low bone density and a deterioration of bone microarchitecture, leading towards a weak bone phenotype and a higher fracture risk. These pathologies have different etiologies and affect both children and adults. Osteolytic diseases affecting children include an inherited group of rare disorders and can be divided into two types, early and late, depending on the timing of the osteoporotic onset. Early-onset osteoporosis is related to genetic mutations and

appears soon after birth. Late-onset forms are related with hypercalcemic disorders (hyperparathyroidism, vitamin D-related causes, malignancy, medications, endocrine disorders, genetic disorders, and miscellaneous causes). In adults, osteolytic diseases include primary (or age-related) osteoporosis that is the most common form with two types. The first is associated with estrogen deficiency at menopause and the second related to long-term remodeling inefficiency. Secondary osteoporosis could appear in the context of endocrine, reproductive, gastrointestinal and nutritional disorders, following treatments with drugs like glucocorticoids and anti-convulsants, in the case of Paget disease, in the presence of malignant primary bone tumors or associated with bone metastases of other cancers as breast and prostate cancers.

The implication of RANKL and its receptors OPG and RANK during bone modeling and remodeling was recognized 20 years ago. RANKL activity has been related to physiological conditions during growth and adulthood but also to osteolytic diseases, including genetic osteolytic pathologies (early-onset osteolysis), age-related osteoporosis, tumors related osteolysis and other conditions marked by an increased bone resorption. In the craniofacial skeleton, the implication of RANKL/RANK/OPG triad in the homeostasis of dental and periodontal tissues as well in the genesis of different pathologies affecting this system has been related in numerous studies. However, to date there is still no complete demonstration of the specific role of RANKL/RANK/OPG system in the health and disease of the craniofacial skeleton.

Different drugs have been used to control osteolytic diseases. Among them, the bisphosphonates (BPs), a group of drugs that inhibit the osteoclasts function, has been recognized as one of the most effective osteolytic diseases treatment both in pediatric and adult. However, despite their high efficacy, several side effects have been related to their use in clinical and preclinical reports, affecting mainly the craniofacial skeleton. Medical Related Osteo-Necrosis of the Jaw (MRONJ)

(knowing also as bisphosphonates related osteonecrosis of the jaws–BRONJ) in adults and alterations in the tooth development and eruption in children have been clinically reported. Several preclinical assays in mice have confirmed the existence of these BPs-side effects in the craniofacial skeleton. Whatever until now, none of these studies was conclusive and many questions remained unanswered regarding N-BPs side effects. Rare studies have analyzed the potential relationship between the RANKL signaling activity level and the N-BPs response, and always in the sense of N-BPs impact onto expression level of elements in the RANKL signaling. Recognizing this information gap, the present research works aimed to describe the impact of RANKL activity level variations onto the craniofacial skeleton modeling and remodeling, to analyze if the RANKL signaling level activity may modify the response to N-BPs during growth and adulthood and if it may be linked to the appearance of BRONJ in adults. In order to accomplish this, analyses of morphometric and mineral parameters of young and adult transgenic mice models with different OPG/RANK genotypes *(*$Opg^{+/+}$\$Rank^{Tg-}$*,* $Opg^{+/+}$\$Rank^{Tg+}$*,* $Opg^{+/-}$\$Rank^{Tg-}$*,* $Opg^{+/-}$\$Rank^{Tg+}$*,* $Opg^{-/-}$\$Rank^{Tg-}$ *and* $Opg^{-/-}$\$Rank^{Tg+}$) corresponding to different RANKL signaling activity levels were realized, in presence or not of zoledronic acid (ZOL), the most potent known N-BP. The wild type (*$Opg^{+/+}$\$Rank^{Tg-}$) mouse group was considered as the control group in all the analyses. The skeletal phenotypes of young and adult mice associated to the different genotypes were so comparatively analyzed emphasizing on craniofacial structure, structural dimensions, mineral parameters, and dental and periodontal status. The protocol of ZOL administration was designed according to the pharmacokinetics data of ZOL and mimicking the clinical protocol administered in pediatric and adult patients. To analyze the effects during growth, mouse pups were randomly divided into treated and none treated groups one day after birth. In the treated group each pup received 4 subcutaneous injections of 50 µg/kg of ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basel, Switzerland) at day 1, 3, 5 and 7 after birth. In none treated group each pup received four injections of sterile normal saline solution. In order to analyze the consequences of ZOL treatment at the end of growth, mice were sacrificed at one month and a half. In order to analyze

the long-term consequences of ZOL treatment during growth, a group of twelve mice (two for each genotype) was maintained and followed until ten months of age. To analyze de effects of N-BPs on the skeleton of adult mice, 22 two-months-old mice of different RANKL signaling activity levels (genotypes) were chosen aleatory and treated with ZOL. Each mouse received two subcutaneous injections per week of 2 µg/kg of ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basel, Switzerland), during eight weeks. In none treated group each mouse received the same number of injections with a sterile saline solution. The consequences of ZOL treatment in all groups of mice were followed by micro-CT analysis at different periods according to the group of age.

Concerning the analyses during growth, obtained results validated the hypothesis that the RANKL signaling activity level in the bone microenvironment has an impact on the response to ZOL. Indeed, the present study establishes that in mice the RANKL signaling activity level is a major modulator of the effects and side-effects of bisphosphonates on the growing skeleton. However, the modulatory actions are dependent on the ways in which this level of activity is increased. A decrease in OPG expression is beneficial to the skeletal phenotype observed at the end of growth, while RANK overexpression deteriorates it. Far removed from pediatric treatment, in adults, the skeletal phenotypes initially observed at the end of growth for the different levels of RANKL signaling activity were maintained, although significant improvement was associated only with reductions in OPG expression. However, further studies will be necessary to understand the underlying molecular mechanisms, which will help decipher the variability in the effects of N-BPs reported in the human population during growth.

On the other side, the analysis of RANKL signaling activity level consequences on the adult craniofacial skeleton demonstrated the importance of the OPG level for the homeostasis not only concerning the alveolar bone, as previous reported, but also regarding the cortical and cancellous bones. The absence of OPG impacts

negatively the different skull structures, comparatively to the RANK overexpression. Concerning the ZOL effects in adult craniofacial skeleton, no density modification related to atraumatic necrotic areas in the craniofacial skeleton were observed, neither during the periodic scanners nor in the last ex-vivo scanner, meaning that the hypothesis of the spontaneous appearance of the BRONJ related with the RANKL signaling level was not confirmed. Moreover, the analysis of basal bones (cortical and cancellous) and alveolar bone showed similar parameters (no significant variations) to those observed in untreated groups, with only minor modifications due to the effects of ZOL. Although those results cannot be considered conclusive (mainly due to the small number of animals), they will have to be considered as an important point of reference for future studies, increasing the mice number and exploring the molecular events related to the RANKL signaling activity levels. Besides these not significant results, the decrease of the root resorption following the ZOL treatment, except in the *Opg*$^{-/-}$ mice, whatever *Rank*^{tg} status, is an important and significative result. Indeed, this validates the fact that the absence of OPG enables a rapid and important “rescue” of the bone resorption (osteoclast differentiation) following the end of the ZOL treatment with a resurgence of wrong effects of OPG deficiency.

This descriptive study, using different genetic models, has also demonstrated the implication of the RANKL signaling activity level in the homeostasis of the craniofacial skeleton. The relevance of the OPG in this triad was evidenced in this research, which enforces the previous analyses that have demonstrated the higher affinity of RANKL for OPG than for RANK, mainly link to the conformational difference between OPG and RANK. In this way, it is possible to consider that a fine regulation of OPG expression, increasing or decreasing its level, could help to reduce the risk of occurrence of different pathologies associated to the RANKL signaling activity level.

Add to the relevance of the OPG expression in the craniofacial morphology structures supported here, reproductive mouse models with severe osteopenic phenotypes have been proposed, named $Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$. In the future, the use of these models will help to better understand, at the molecular level, the etiology of the osteolytic diseases, from genetic origins like osteogenesis imperfecta and Paget disease, or from non-genetic origins like age-related osteoporosis, glucocorticoid-induced osteoporosis, primary bone tumors and skeletal metastases from multiple myeloma and other tumors. Moreover, concerning specifically the craniofacial system, these mouse models will be important tools to a better understanding of the etiology of dental diseases like periodontitis, osteonecrosis of the jaw and different causes of root resorption, like trauma, incorrect orthodontia force and periapical periodontitis. Finally, these models will be of strategic help in a near future to an in dept characterization of the antiresorptive drug response variabilities observed *in vivo*, like those to bisphosphonates, Rankl blocking antibody, ranelate strontium, corticosteroids and anabolic agents among others.

KEY WORDS:

Growing skeleton, Craniofacial skeleton, RANKL/RANK/OPG, N-BPs, Zoledronic acid, Growing skeleton

RÉSUMÉ

L'os est un tissu dynamique avec des modifications physiologiques continuelles adaptatives de son homéotasie au cours de la vie, appelées modelage et remodelage osseux. Ces processus permettent d'atteindre une balance entre la formation et la résorption osseuse à chaque âge. Ils impliquent l'activité de types cellulaires distincts nommés chondroblaste, ostéocyte, ostéoblaste, cellule osseuse bordante et ostéoclaste. Le rôle des ostéoclastes a été analysé en détail pour l'ossification endochondrale au cours du développement et de la croissance, et chez l'adulte au cours du remodelage.

La différenciation ostéoclastique requière deux facteurs essentiels nommés « macrophage colony stimulating factor » (M-CSF), qui agit via son récepteur c-FMS, et « receptor activator of nuclear factor-κB ligand » (RANKL), qui agit via son récepteur principal RANK auquel la liaison est modulée par le récepteur leurre « osteoprotegerin » (OPG). Le M-CSF est impliqué dans l'ostéoclastogenèse principalement en stimulant la prolifération et la survie des précurseurs ostéoclastiques. RANKL agit comme le facteur clef de la différenciation des précurseurs ostéoclastiques en ostéoclastes, ainsi que de leur maturation et activité. Toutes perturbations de la balance entre la formation et la résorption osseuse, secondaire aux altérations des ostéoclastes, mènent à des pathologies qui peuvent apparaître chez l'enfant comme chez l'adulte. Ces pathologies sont connues comme les maladies ostéopétrotiques et ostéolytiques. Le groupe des maladies ostéopétrotiques correspond à une apposition osseuse plus importante, aboutissant à une augmentation de la masse osseuse, principalement due à un dysfonctionnement ou une absence des ostéoclastes. Le groupe des maladies ostéolytiques, au contraire, correspond à un renouvellement osseux augmenté, associé à une augmentation du nombre et/ou de l'activité des ostéoclastes. Les maladies ostéolytiques sont caractérisées par une balance négative entre la formation et la résorption osseuse. Les maladies ostéolytiques génèrent une densité

osseuse basse et une détérioration de l'architecture osseuse, aboutissant à un phénotype osseux lègé et un risque élevé de survenue de fracture. Ces pathologies ont des étiologies différentes et affectent aussi bien les enfants que les adultes. Les maladies ostéolytiques affectant les enfants comprennent un groupe de désordres rares héritables et peuvent être divisées en deux types, précoce et tardif, selon l'âge de survenue des signes ostéoporotiques. L'ostéoporose précoce est associée aux mutations génétiques et survient tôt après la naissance. L'ostéoporose tardive est associée aux désordres hypercalcémiques (hyperparathyroïdisme, causes liées à la vitamine D, malignité, traitements, désordres endocrinologiques, désordres génétiques, et autres causes).

Chez l'adulte, les maladies ostéolytiques inclues l'ostéoporose primaire (ou liée à l'âge) qui est la forme la plus commune avec deux types. Le premier est associé à une déficience en œstrogène à la ménopause et le second à une inefficacité prolongée du remodelage. L'ostéoporose secondaire apparaît pour sa part dans le contexte de désordres d'ordres endocrinologique, reproductif, gastrointestinal ou nutritionnel, à la suite de traitements avec des drogues comme les glucocorticoïdes et les anti-convulsants, dans le cas de la maladie de Paget, en présence de tumeurs osseuses primitives malignes ou associé aux métastases osseuses d'autres cancers comme ceux de la prostate et du sein.

L'implication de RANKL et ses récepteurs OPG et RANK durant le modelage et le remodelage osseux a été mis en évidence il y a vingt ans. L'activité physiologique de RANKL a été caractérisée durant la croissance et chez l'adulte ainsi que son implication dans les maladies ostéolytiques, incluant les pathologies ostéolytiques génétiques (ostéolyse précoce), l'ostéoporose liée à l'âge, les ostéolyses associées aux tumeurs et les autres conditions caractérisées par une augmentation de la résorption osseuse. Dans le squelette craniofacial, l'implication de la triade RANKL/RANK/OPG dans l'homéostasie des tissus dentaires et parodontaux ainsi que la genèse de différentes pathologies affectant ce système a été rapporté dans

de nombreuses études. Cependant, à ce jour il n'y a toujours pas de démonstration complète du rôle spécifique du système RANKL/RANK/OPG dans le squelette craniofacial sain comme pathologique. Différentes drogues ont été utilisées pour contrôler les maladies ostéolytiques. Parmi elles, les bisphophonates (BPs), un groupe de drogues qui inhibent la fonction ostéoclastique, ont été reconnues comme l'un des plus efficace traitement pour les maladies ostéolytiques en pédiatrie comme chez l'adulte. Cependant, au-delà de leur haute efficacité, plusieurs effets secondaires ont été rapportés lors de leur utilisations cliniques et précliniques, affectant principalement le squelette craniofacial. L'ostéonécrose de la mâchoire liée à la médication (aussi connue sous le nom d'ostéonécrose de la mâchoire liée aux bisphosphonates : BRONJ en Anglais) chez l'adulte et les altérations du développement dentaire et de l'éruption chez les enfants ont ainsi été rapportées en clinique. Plusieurs essais précliniques chez la souris ont confirmé l'existence de ces effets secondaires des BPs au niveau du squelette craniofacial. Quoi qu'il en soit, jusqu'à présent, aucune de ces études n'a été concluante et de nombreuses questions restent sans réponse concernant les effets secondaire des N-BPs. De rares études se sont intéressées à la relation potentielle entre le niveau d'activité de la signalisation RANKL et la réponse aux N-BPs, et toujours dans le sens de l'impact des N-BPs sur le niveau d'expression des membres de la signalisation RANKL. Prenant en compte cette lacune, le travail de recherche présenté ici avait pour objectifs la description de l'impact des variations du niveau d'activité de la signalisation RANKL sur le modelage et le remodelage du squelette craniofacial, l'analyse de la capacité du niveau d'activité de la signalisation RANKL à modifier la réponse aux N-BPs pendant la croissance et chez l'adulte, et la recherche d'un potentiel lien entre ce niveau et la survenue de BRONJ chez l'adulte. Dans ce cadre, des analyses des paramètres morphométriques et minéraux ont été réalisés sur des modèles de souris transgéniques, souris jeunes et adultes, correspondant à différents génotypes d'OPG et de RANK (*$Opg^{+/+}$\$Rank^{Tg-}$, $Opg^{+/+}$\$Rank^{Tg+}$, $Opg^{+/-}$\$Rank^{Tg-}$, $Opg^{+/-}$\$Rank^{Tg+}$, $Opg^{-/-}$\$Rank^{Tg-}$ and $Opg^{-/-}$\$Rank^{Tg+}$*) induisant différents niveaux d'activité de la signalisation RANKL, et cela en présence ou absence de

traitement à l'acide zolédronique (ZOL), le plus puissant des N-BPs. Le groupe de souris dites "sauvages" (*Opg*$^{+/+}$*Rank*$^{Tg-}$) a été considéré comme le groupe contrôle dans toutes les analyses. Les phénotypes squelettiques des souris jeunes et adultes des différents génotypes ont ainsi été analysés comparativement se concentrant sur la structure craniofaciale, les dimensions structurelles, les paramètres minéraux, et le statut dentaire et parodontal. Le protocole d'administration du ZOL a été choisi suivant les données pharmacocinétique du ZOL et en copiant les protocoles cliniques administrés aux patients pédiatriques et adultes. Afin d'analyser les effets pendant la croissance, les souriceaux ont été divisés de manière aléatoire en un groupe traité et un groupe non-traité un jour après leur naissance. Dans le groupe traité, chaque souriceau a reçu quatre injections sous-cutanées de ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basel, Switzerland) à la dose de 50 µg/kg au jours 1, 3, 5 et 7 après la naissance. Dans le groupe non-traité chaque souriceau a reçu quatre injections d'une solution saline isotonique suivant le même échéancier. Afin d'analyser les conséquences du traitement avec le ZOL à la fin de la croissance, le souris furent sacrifiées à un mois et demi. Afin d'analyser les conséquences à long terme du traitement au ZOL pendant la croissance, un groupe de douze souris (deux de chaque génotype) a été maintenu et suivi jusqu'à l'âge de dix mois. Afin d'analyser les effets des N-BPs sur le squelette des souris adultes, vingt-deux souris âgées de deux mois présentant les différents génotypes ont été choisies aléatoirement et traitées avec le ZOL. Chaque souris a reçu deux injections en sous-cutanée par semaine de ZOL à la dose de 2 µg/kg pendant huit semaines. Dans le groupe non-traité, chaque souris a reçu le même nombre d'injections d'une solution saline isotonique. Les conséquences du traitement au ZOL dans chaque groupe de souris ont été suivies par analyse micro-CT à différentes périodes suivant le groupe d'âge.

Concernant les analyses des conséquences pendant la croissance, les résultats obtenus ont validé l'hypothèse selon laquelle le niveau d'activité de la signalisation RANKL dans le microenvironnement osseux a un impact sur la réponse au ZOL. En

effet, L'étude présentée établie que chez la souris le niveau d'activité de la signalisation RANKL est un modulateur majeur des effets et effets secondaires des bisphosphonates sur le squelette en croissance. Cependant, les modulations sont dépendantes des voies suivant lesquelles le niveau d'activité a été augmenté. Une diminution de l'expression d'OPG est bénéfique pour le phénotype squelettique observé à la fin de la croissance alors que la surexpression de RANK le détériore. A distance du traitement pédiatrique, chez l'adulte, les phénotypes squelettiques initialement observés à la fin de la croissance pour les différents niveaux d'activité de la signalisation RANKL sont maintenus, bien qu'une amélioration significative soit associée à la réduction d'expression d'OPG. Cependant, des études complémentaires seront nécessaires pour comprendre les mécanismes moléculaires sous-jacents, ce qui aidera à décrypter la variabilité des effets des N-BPs rapportés dans la population humaine au cours de la croissance.

D'autre part, l'analyse des conséquences du niveau d'activité de la signalisation RANKL sur le squelette craniofacial adulte a démontré l'importance du niveau d'OPG pour l'homéostasie non seulement de l'os alvéolaire, comme précédemment décrit, mais aussi des os cortical et spongieux. L'absence d'OPG a un impact négatif sur les différentes structures du crâne comparativement à la surexpression de RANK. Concernant l'effet du ZOL sur le squelette craniofacial adulte, aucune modification de densité en relation avec une aire nécrotique atraumatique n'a été observée au niveau du squelette craniofacial, que cela soit sur les scanners du suivi périodique ou sur le scanner terminal, signifiant que l'hypothèse d'apparition spontanée de la BRONJ en relation avec le niveau de la signalisation RANKL n'est pas confirmé. De plus l'analyse des os de la structure basale (cortical et spongieux) et de l'os alvéolaire a montré des paramètres similaires (pas de variations significatives) à ceux observés dans le groupe non-traité, avec simplement des modifications mineures induites par le ZOL. Bien que ces résultats ne puissent être considérés comme concluant (principalement lié au petit nombre d'animaux), ils devront être pris en compte comme point de référence important pour les études

futures, en augmentant le nombre de souris et en explorant les événements moléculaires relatifs aux niveaux d'activité de la signalisation RANKL. Au-delà de ces résultats non significatifs, la diminution des résorptions radiculaires suivant le traitement avec le ZOL, excepté chez la souris *Opg*$^{-/-}$ quel que soit leur statut *Rank*^{tg} est un résultat important et significatif. En effet, cela valide le fait que l'absence d'OPG permet une récupération rapide et importante de la résorption osseuse (différenciation ostéoclastique) suivant la fin du traitement au ZOL avec la résurgence des mauvais effets de l'absence d'OPG.

Cette étude descriptive, utilisant différents modèles génétiques, a aussi démontrée l'implication du niveau d'activité de la signalisation RANKL dans l'homéostasie du squelette craniofacial. L'importance de l'OPG dans cette triade a été mise en évidence par ces travaux de recherche, renforçant les analyses précédentes qui avaient montré la plus grande affinité de RANKL pour l'OPG par rapport à RANK, principalement liée aux différences conformationnelles entre OPG et RANK. Ainsi, il est possible de considérer qu'une fine régulation de l'expression d'OPG, augmentant ou diminuant son niveau, pourrait aider à réduire le risque de survenue de plusieurs pathologies associées au niveau d'activité de la signalisation RANKL.
En plus de l'importance de l'expression d'OPG pour la morphologie des structures craniofaciales établie ici, des modèles murins reproductifs avec des phénotypes ostéopéniques sévères ont été mis en avant, nommés *Opg*$^{-/-}$*Rank*$^{tg-}$ et *Opg*$^{-/-}$*Rank*$^{tg+}$. Dans le futur, l'utilisation de ces modèles aidera à mieux comprendre, au niveau moléculaire, l'étiologie des maladies ostéolytiques, d'origines génétiques comme l'ostéogenèse imparfaite et la maladie de Paget, ou d'origines non-génétiques comme l'ostéoporose liée à l'âge, l'ostéoporose induite par les glucocorticoïdes, les tumeurs osseuses primitives et les métastases squelettiques des myélomes multiples et autres tumeurs. De plus, concernant spécifiquement le système craniofacial, ces modèles murins seront des outils importants pour une meilleure compréhension de l'étiologie des maladies dentaires telles que la parodontite, l'ostéonécrose de la mâchoire et des différentes causes de résorptions radiculaires

comme les traumatismes, les forces orthodontiques incorrectes et la parodontite périapicale. Finalement, ces modèles seront d'une aide stratégique dans un futur proche pour une caractérisation en détail des variabilités de réponse aux drogues antirésorptives observées in vivo, comme celles aux bisphosphonates, aux anticorps bloquant RANKL, au strontium ranelate, aux corticostéroïdes et agents anaboliques entre autres.

RESUMEN

El hueso es un tejido dinámico, sometido a constantes cambios fisiológicos adaptativos para mantener su homeostasis presentes a lo largo de la vida, estos procesos se denominan modelado y remodelado óseo. Con ellos se busca, tanto en la niñez como en la edad adulta, lograr un equilibrio entre la formación ósea y la resorción ósea. Para el adecuado desarrollo de estos procesos se requiere la participacion de distintos tipos de células como los condroblastos, los osteocitos, los osteoblastos, las células de revestimiento óseo y osteoclastos. El papel de los osteoclastos ha sido analizado ampliamente tanto en la osificación endocondral, durante el desarrollo y el crecimiento, y en la edad adulta durante la remodelación ósea. El proceso de diferenciación osteoclastica requiere dos factores esenciales llamados factor estimulante de colonias de macrófagos (M-CSF), que actúa sobre su receptor c-FMS, y el ligando activador del receptor del factor nuclear-κB (RANKL), que actúa sobre su receptor principal RANK y cuya unión es modulada por la osteoprotegerina (OPG), actuando como un receptor señuelo, para controlar su actividad. El M-CSF está involucrado en el proceso de osteoclastogénesis principalmente promoviendo la proliferación y supervivencia de los precursores osteoclásticos. Por su parte RANKL funciona como el factor principal que impulsa la diferenciación de los precursores osteoclásticos en osteoclastos, favoreciendo también su maduración y actividad.

Desbalances entre la formación de hueso y la reabsorción, secundaria a alteraciones en la actividad de los osteoclastos, conducen a patologías osteoliticas que pueden aparecer en la infancia o la edad adulta y que se han conocido como enfermedades osteopetróticas y/o enfermedades osteolíticas. Las enfermedades del tipo osteopetrótico están relacionadas con una mayor aposición ósea debido a la disfunción o ausencia de osteoclastos, dando como resultado un aumento de la masa ósea. Por el contrario, las enfermedades del tipo osteolíticas, se deben a un aumento en el recambio óseo y están asociadas a un aumento en el número o

actividad de los osteoclastos, se caracterizan por un equilibrio negativo entre la formación y resorción ósea. Estas enfermedades osteoliticas generan una baja densidad y un deterioro de la microarquitectura ósea, lo que conduce a un fenotipo óseo débil con un mayor riesgo de fractura. La etiologîa de estas patologías es diversa y se considera que pueden afectar tanto a niños como a adultos. Dentro de las osteolisis que afectan a los niños se incluyen un grupo trastornos raros de tipo hereditario, que se dividen en temprano y tardío según el periodo de inicio. La osteoporosis de inicio temprano está relacionada con mutaciones genéticas y aparecen poco después del nacimiento. Las formas de inicio tardío están relacionadas con trastornos hipercalcémicos (hiperparatiroidismo, causas relacionadas con la vitamina D, malignidad, medicamentos, trastornos endocrinos, trastornos genéticos y causas diversas). En los adultos, las enfermedades osteolíticas incluyen osteoporosis primaria (o relacionada con la edad) que es la forma más común y que ha sido asociada con dos tipos diferentes: el primero está asociado con la deficiencia de estrógenos en la menopausia y el segundo relacionado con la ineficiencia de remodelación a largo plazo. La osteoporosis secundaria en los adultos se ha relacionado con transtornos endocrinos, reproductivos, gastrointestinales y nutricionales, posterior a tratamientos con medicamentos como glucocorticoides y anticonvulsivos, en el caso de la enfermedad de Paget, en presencia de tumores óseos primarios malignos o asociados con metástasis óseas de otros cánceres como los de mama y próstata.

Las implicaciones del RANKL y sus receptores OPG y RANK durante el proceso de modelado y remodelado óseo han sido reconocidas desde hace 20 años. Su papel han sido asociado tanto en aspectos fisiologicos del desarrollo normal del hueso como con patologias óseas durante el crecimiento y en la edad adulta. Dentro de estas patologías se incluyen las patologias óseas de tipo hereditario (de aparicion temprana), las enfermedades osteolíticas relacionadas con la edad, las asociadas a tumores y otras condiciones caracterizadas por un incremento en la reabsorcion osea. Varios estudios han relacionado la triada RANKL/RANK/OPG en el sistema

craniofacial, no solo en la homeostasis de los tejidos dentales y periodontales, sino tambien en el origen de diferentes patologías que afectan este sistema. Sin embargo, hasta la fecha no se ha demostrado un papel especifico del sistema RANKL/RANK/OPG ni en la salud ni en procesos patolôgicos que afecten el esqueleto craneofacial.

Varios grupos de fármacos han sido indicados para tratar y controlar las enfermedades de tipo osteolítico, entre estos se encuentran los bisfosfonatos (BPs). Este grupo de medicamentos que inhiben la funcion osteoclástica, han sido reconocidos como uno de los grupos farmacológicos mas efectivos para el tratamiento de las enfermedades osteolîticas tanto en niños como en adultos. Sin embargo a pesar de su eficacia demostrada, diferentes informes clínicos y preclínicos han asociado su uso con la aparición de diferentes efectos secundarios que afectan principalmente al esqueleto craneofacial. En adultos la Osteonecrosis de los Maxilares asociada a Medicamentos (MRONJ) (conocida también como Osteonecrosis de los maxilares asociada a bifosfonatos-BRONJ) ha sido el efecto adverso mas reportado, en tanto que en niños se han identificado alteraciones en el desarrollo y en la erupción dental. Ensayos preclínicos en ratones han confirmado la existencia de los efectos secundarios de BPs en el esqueleto craneofacial, sin embargo ninguno ha sido concluyente y, por el contrario, muchos cuestionamientos se han generado con respecto a los efectos secundarios de N-BPs. Algunos de estos estudios han analizado la posible relación entre el nivel de actividad de señalización RANKL y la respuesta de los N-BP, teniendo como referente siempre el efecto de los N-BP en el nivel de expresión de los elementos en la señalización de RANKL. Considerando esta falta de información, el presente trabajo de investigación tuvo como objetivo describir el impacto de las variaciones del nivel de actividad RANKL en el modelado y remodelado del esqueleto craneofacial, evaluando si la actividad del nivel de señalización RANKL puede modificar la respuesta a los N-BP tanto durante el crecimiento como en la edad adulta, y si adicionalmente puede estar relacionado con la aparición de BRONJ en adultos.

Para lograr esto, se hizo un análisis de los parámetros morfométricos y minerales de modelos de ratones transgénicos, jóvenes y adultos, con diferentes genotipos OPG/RANK *(Opg$^{+/+}$\Rank^{Tg-}, Opg$^{+/+}$\Rank^{Tg+}, Opg$^{+/-}$\Rank^{Tg-}, Opg$^{+/-}$\Rank^{Tg+}, Opg$^{-/-}$\Rank^{Tg-} and Opg$^{-/-}$\Rank^{Tg+}*), que fenotipicamente corresponden a diferentes niveles de señalizacion RANKL, en presencia o no de uno de los mas potentes N-BP conocidos, el ácido zoledronico (ZOL). El tipo genotipo salvaje (*Opg$^{+/+}$\Rank^{Tg-}*) fue considerado como el control de comparacion para todos los grupos.

Los fenotipos esqueléticos de los diferente genotipos se analizaron de manera comparativa haciendo enfasis en las dimensiones y la estructura craneofacial, los parámetros minerales y el estado dental y periodontal. El protocolo de administración de ZOL se estableció de acuerdo a las características farmacocineticas del ZOL y simulando el protocolo clínico administrado en pacientes pediátricos y adultos. Para analizar los efectos durante el crecimiento, las crías de ratón se dividieron aleatoriamente un día después del nacimiento en grupos tratados y no tratados. En el grupo experimental, cada cria de ratón recibió 4 inyecciones subcutáneas de 50 µg/kg de ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basilea, Suiza) los días 1, 3, 5 y 7 después del nacimiento. En el grupo control cada cachorro recibió cuatro inyecciones de solución salina estéril. Para evaluar las consecuencias del tratamiento con ZOL al final del crecimiento se sacrificaron los ratones al mes y medio, mientras que para analizar las consecuencias a largo plazo se mantuvo un grupo de doce ratones (dos para cada genotipo) y se hizo un seguimiento hasta los diez meses de edad. Para analizar los efectos de los N-BP en el esqueleto de ratones adultos, 22 ratones de dos meses de edad con diferentes niveles de actividad de señalización de RANKL (genotipos) fueron elegidos aleatoriamente y tratados con ZOL. Cada ratón recibió dos inyecciones subcutáneas por semana de 2 µg/kg de ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basilea, Suiza), durante ocho semanas. En el grupo control, cada ratón recibió el mismo número de inyecciones con una solución salina estéril. El análisis de los efectos del tratamiento con ZOL en todos los grupos de ratones se realizó mediante

análisis con microtomografia computarizada (micro-CT) en diferentes períodos según el grupo de edad.

En relación a los análisis durante el crecimiento, los resultados obtenidos validaron la hipótesis confirmando que el nivel de actividad de señalización de RANKL en el microambiente óseo tiene un impacto en la respuesta a ZOL. De hecho, el presente estudio establece que, en ratones, el nivel de actividad de señalización de RANKL es un modulador importante de los efectos tanto principales como secundarios de los N-BPs en el esqueleto en crecimiento. Sin embargo, este efecto modulador esta relacionado con la forma en que se incrementa el nivel de actividad RANKL. Una disminución en la expresión de OPG es beneficiosa para el fenotipo esquelético observado al final del crecimiento, mientras que la sobreexpresión de RANK lo deteriora. Para el grupo de ratones adultos que recibieron el tratamiento después del nacimiento, los fenotipos esqueléticos inicialmente observados al final del crecimiento para los diferentes niveles de actividad de señalización de RANKL se conservaron, aunque una relativa mejoria se observo en ratones en los que había una reducción en la expresión de OPG. Serán necesarios estudios complementarios para comprender los mecanismos moleculares subyacentes, que ayudarán a descifrar la variabilidad en los efectos de los N-BP reportados en la población humana durante el crecimiento.

Por otra parte, el análisis de las consecuencias del nivel de actividad de señalización RANKL en el esqueleto craneofacial del ratón adulto demostró la importancia del nivel de OPG en la homeostasis no solo con respecto al hueso alveolar, como anteriormente había sido reportado, sino también en relación a los huesos corticales y esponjosos. La ausencia de OPG afecta negativamente las diferentes estructuras del cráneo, en comparación con la sobreexpresión del RANK. Con respecto a los efectos de ZOL en el esqueleto craneofacial adulto, no se observaron modificaciones en la densidad del hueso relacionadas con áreas necróticas atraumáticas en el esqueleto craneofacial, ni durante los escáneres de seguimiento

ni en el último escáner ex vivo, lo que significa que la hipótesis de la aparición espontánea del BRONJ en relacion al nivel de señalización RANKL no se confirmó. Adicionalmente, el análisis de los huesos basales (cortical y esponjoso) y el hueso alveolar mostraron parámetros similares (sin variaciones significativas) a los observados en los grupos no tratados, con modificaciones poco significativas asociadas a los efectos del ZOL. Aunque esos resultados no pueden considerarse concluyentes (principalmente debido al pequeño número de animales) deberán considerarse como un punto de referencia importante para futuros estudios, aumentando el número de ratones y explorando los eventos moleculares relacionados con los niveles de actividad de señalización de RANKL. Además de estos resultados poco significativos, la disminución de la resorción de la raíz después del tratamiento con ZOL, excepto en los ratones *Opg*$^{-/-}$, independiente del estado de *Rank*^{tg}, es un resultado importante y significativo. Especificamente, este resultado valida el hecho de que la ausencia de OPG permite un "rescate" rápido e importante de la resorción ósea (diferenciación osteoclastica) después del final del tratamiento con ZOL con un resurgimiento de los efectos deletéreos asociados a la deficiencia de OPG.

Este estudio descriptivo, utilizando diferentes modelos genéticos, tambien ha demostrado la implicación del nivel de actividad de señalización de RANKL en la homeostasis del esqueleto craneofacial. La relevancia de la OPG en esta tríada se evidenció en esta investigación, reforzando los análisis anteriores que han demostrado la mayor afinidad de RANKL por el receptor señuelo OPG que por el RANK, principalmente relacionado con la diferencia conformacional desde el punto de vista estructural entre OPG y RANK. De esta manera, es posible considerar que una fina regulación de la expresión de OPG, aumentando o disminuyendo su nivel, podría ayudar a reducir el riesgo de aparición de diferentes patologías asociadas al nivel de actividad de señalización de RANKL.

Además de haber demostrado la importancia de la expresión de OPG en la homeostasis de las estructuras de la morfología craneofacial, este estudio también ha propuesto modelos de ratones reproducibles con fenotipos osteopénicos graves, denominados *Opg$^{-/-}$Rank^{tg-} and Opg$^{-/-}$Rank^{tg+}.* En estudios futuros, el uso de estos modelos ayudará a comprender mejor, a nivel molecular, la etiología de las enfermedades osteolíticas de orígen genético como la osteogénesis imperfecta y la enfermedad de Paget, o de orígenes no genéticos como la osteoporosis relacionada con la edad o inducida por los glucocorticoides, por tumores óseos primarios y relacionada con metástasis esqueléticas de diferentes tumores como el mieloma multiple, entre otros. Además, en relación específicamente con el sistema craneofacial, estos modelos de ratón osteopenicos serán herramientas importantes para una mejor comprensión de la etiología de las enfermedades dentales como la periodontitis, la osteonecrosis de los maxilares y las diferentes causas de la reabsorción radicular, como el trauma, los movimientos ortodóncicos incorrectos y la periodontitis apical. Finalmente, estos modelos serán de ayuda estratégica para una caracterización profunda de las variabilidades en la respuesta a fármacos antirresortivos observadas en vivo, como las de los bifosfonatos, el anticuerpo monoclonal RANKL, el ranelato de estroncio, los corticosteroides y los agentes anabólicos, entre otros.

IMPACTS ON THE GROWING AND ADULT SKELETON OF DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS, CONSEQUENCES ON THE RESPONSE TO ZOLEDRONIC ACID

1. GENERAL INTRODUCTION

Bone is a dynamic tissue with constant physiological changes in its homeostasis through life, e.g., bone modeling and remodeling (2,3). These processes enable to achieve a relative balance between bone formation and bone resorption at any particular ages (2,3). Bone modeling corresponds to bone formation and resorption as a biological process during growth (2–4). Bone remodeling is related to the response to changes in mechanical loading, altered serum calcium levels and secondary to a wide range of paracrine and endocrine factors in adulthood that leads to both bone formation and resorption (3,5,6). These processes involve the activity of distinct types of cells namely chondroblasts, osteocytes, osteoclasts (OCs), osteoblasts (OBs) and bone lining cells (BLCs)(3,7–10). The role of OCs has been deeply analyzed in endochondral ossification during development and growth, and in adulthood throughout bone remodeling (11–13). Any imbalances between bone formation and resorption secondary to OCs alterations lead to pathologies that could appear both in childhood and adulthood (14–16).

OCs differentiation requires two essential factors named macrophage colony stimulating factor (M-CSF), acting on its receptor c-FMS, and receptor activator of nuclear factor-κB ligand (RANKL), acting on its main receptor RANK which binding is modulated by the decoy receptor osteoprotegerin (OPG) (17–19). M-CSF is involved in the osteoclastogenesis process, mainly by promoting the proliferation and survival of osteoclast precursors. RANKL functions as the primary factor driving differentiation of OCs precursors into OCs, as well as in their function, maturation and activity (13,19).

The implication of RANKL and its receptors OPG and RANK during bone modeling and remodeling was recognized 20 years ago (20–22). RANKL activity has been related to physiological conditions during growth and adulthood (19,23–25), but also to physio-pathological situations like osteolytic diseases (ODs), including genetic osteolytic pathologies (early-onset osteolysis), age-related osteoporosis, tumors related osteolysis and any other conditions marked by an increased bone resorption (14,16,19,22,24,26,27). In the craniofacial skeleton, the implication of RANKL/RANK/OPG triad in the homeostasis of dental and periodontal tissues as well in the genesis of different pathologies affecting this system has been related in numerous studies (28–33). However, to date there is still no complete demonstration of the specific role of RANKL/RANK/OPG system in the health and disease of the craniofacial skeleton.

Different drugs have been used to control ODs (34–37) Among them, the bisphosphonates (BPs), a group of drugs that inhibit the OCs function, has been recognized as one of the most effective ODs treatment both in pediatric and adult (34,38–41). However, despite their high efficacy, several side effects, have been related to their use in clinical and preclinical reports, affecting mainly the craniofacial skeleton (39,42). Medical Related Osteo-Necrosis of the Jaw (MRONJ) (knowing also as bisphosphonates related osteonecrosis of the jaws–BRONJ) in adults and alterations in the tooth development and eruption in children has been clinically reported (39,42–49). Several preclinical assays in mice have confirmed the existence of the BPs-side effects in the craniofacial skeleton. Whatever until now, none of these studies is conclusive and many questions remain unanswered to N-BPs treatment. (50–54). Rare studies have analyzed the potential relationship between the RANKL signaling activity level and the N-BPs response, and always in the sense of N-BPs impact onto expression level of elements in the RANKL signaling. Recognizing this information gap, the present dissertation pretends to describe the impact of RANKL activity level variations onto the craniofacial skeleton modeling and remodeling, and to analyze if the RANKL signaling level of activity

could modify the response to N-BPs during growth and adulthood. In order to accomplish this, an analysis of morphometric and mineral parameters of young and adult transgenic mice model with different OPG/RANK genotypes (*Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/+}$*Rank*$^{Tg+}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg+}$, *Opg*$^{-/-}$*Rank*$^{Tg-}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$) corresponding to different RANKL signaling activity levels was realized, in presence or not of the most potent known N-BP, named zoledronic acid (ZOL). The *wild-type* (*Opg*$^{+/+}$*Rank*$^{Tg-}$) mouse group was considered as the control group in all the analyses.

In the present work, the first part is an introduction to bone physiology and pathologies followed by a review of the state of the art concerning BPs, their pharmacology aspects and their side effects on the skeleton[1]. Then, the results of the analyzes of the RANKL activity level impact during growth and in adulthood in the presence or not of ZOL will be present. A descriptive and experimental assay comparing the skeletal phenotypes of young and adult mice, emphasizing on craniofacial structure, structural dimensions, mineral parameters, and dental and periodontal status of mice with different RANKL activity levels genetically determined will be present in the presence or absence of ZOL[2].

[1] This topic was revised and issued in a review article which is available in the journal of cellular physiology, volume 233 (8), pages 5676-5715 (315).

[2] Results published in Biochemical Pharmacology, volume 168 (April), pages 133-148 (317).

2. THEORETICAL FRAMEWORK

The hypothesis that OBs influence the OCs formation was formulated in the 1980s (55,56) At the ends of the 1990s, the implication of the RANKL/RANK/OPG system in bone homeostasis was reported by Suda et al., who in 1999 revealed that osteoclastogenesis could be induced by RANKL through its receptor RANK, while OPG acted as a soluble molecular decoy receptor (20,56,57). First, a brief update description of the bone biology, beginning with the identification of the main cells, cytokines and chemokines related to the RANKL system and after that, an analytical review of bone modeling and remodeling will be discussed. Finally, the pathologies related to the imbalance of the RANKL/OPG/RANK bone homeostasis is going to be described in order to understand better the current implication of this triad in bone homeostasis.

2.1 BONE: ESSENTIAL CONCEPTS

Bone -a specialized connective tissue- has several functions which include support for locomotion, protection for vital organs, calcium and phosphate reserve supplement to maintain mineral homeostasis, and endocrine function to regulate energy expense (6,9). In order to maintain its functions, bone is continuously renewed by the modeling and remodeling process (2). These processes involve different kinds of specialized cells that interacted each other through the action of several cytokines and chemokines (58–60). In this way, the cell activity could be stimulated or inhibited by signaling emanating from other cells (59,61). A significant number of signaling factors have been identified during bone modeling and remodeling, as actors of much cell's interactions (2,25). The different cells and signaling factors involved in bone modeling and remodeling are described in the following paragraphs.

2.1.1 Cells involved in bone homeostasis. There are at least five types of bone cells involved in bone modeling and remodeling: the bone lining cells, the chondroblasts, the osteocytes, the osteoblasts, and the osteoclasts (2). Each of those cells has specific characteristics.

2.1.1.1 Bone lining cells: (BLCs). BLCs are considered as quiescent fully mature osteoblasts (3) which cover inactive (non-remodeling) bone surfaces and form a thin monolayer of active cells (9,62). In certain physiological or pathological situations, these cells can be reactivated. Indeed, BLCs may be involved in the propagation of the activation signal of bone resorption which starts bone remodeling process (3,58)

2.1.1.2 Chondroblasts. Those cells are essential during bone modeling and central elements of the endochondral ossification process (3,8,60). Endochondral ossification ends by the replacement of the embryonic cartilaginous skeleton by bone during the organogenesis and is central for the long bones growth at the epiphysial growth plates site until the adult height (8,60). Chondroblasts produce components of the cartilage extracellular matrix as the collagen type II and the proteoglycan aggrecan. Once the mineralized cartilage has been formed as a template for bone formation, the chondroblasts undergo hypertrophic differentiation, produce a mineralizing type X collagen matrix and undergo apoptosis (8,60). Cartilage matrix envelops rare chondroblasts that become chondrocytes in the cartilage that is maintained like articular cartilage (2,8,60).

2.1.1.3 Osteoblasts (OBs). The OBs are the central cells related to bone formation. They are located mainly in the stroma of the bone marrow and at the surfaces of all bone compartments (63,64). The OBs share a common progenitor cell with the chondroblasts, the adipocytes, the myoblasts known as the bone marrow mesenchymal stem cells (MSCs) (63,64). Two main transcription factors Sox9 and Runx2, are required to differentiate mesenchymal stem cells in OBs. This process is regulated by several hormones, growth factors and cytokines. Among them the transforming growth factor-beta (TGF-β) stimulates chemotaxis of the precursors to

the site of differentiation; the fibroblast growth factors (FGFs) activates the OBs to promote bone formation; bone morphogenetic proteins (BMPs) are involved in skeletal growth during embryogenesis and further in the fracture repair (3,63). The OBs activity is also regulated by several signaling pathways like Hedgehog (Hh) and Notch (61,63,65).

The OBs formation is controlled by the wingless (Wnt) signaling and its receptor Frizzled and co-receptors lipoprotein related protein (LPL) 5 or 6 (LPL5/6)(3,61). In the presence of Wnt, Frizzled binds to the co-receptors LPL5/6 and blocks the cytoplasmic degradation of the β-catenin by the destruction complex (3,61,66). In this way, β-catenin accumulates in the cytoplasm and then is translocated to the nucleus displacing transcriptional co-repressors and recruiting co-activators, leading to an increased expression of specific target genes involved in OBs differentiation. In the absence of Wnt/Frizzeled/LPL5-6 union, the destruction complex degrades β-catenin, and target gene expression is repressed, consequently, the OBs formation is reduced (61,66).

The principal function of the OBs is to secrete the type I collagen rich bone matrix and to regulate the matrix mineralization (3,63). Once this is done, the OBs stops their activity and some of them are gradually entrapped in the bone matrix and become osteocytes. Another important function of the OBs during bone modeling is to induce OCs differentiation by the production of RANKL and M-CSF (Fig.1) (61,67,68).

2.1.1.4 Osteocytes. Osteocytes, considered as terminally differentiated cells of the OBs lineage, are the most abundant bone cells, entrapped within the bone during skeletal maturation or previous cycles of bone remodeling (69,70). Osteocytes have the most critical role in the bone remodeling compartment (BRC) (69). Indeed, these cells can sense and respond to external forces, mainly determined by muscle activity (3). They are considered the principal source of cytokines, M-CSF, RANKL and they

also express OPG (Fig. 1). Osteocytes also express IFN-β, which may act as a negative regulator of OCs differentiation (69), SOST (secreted proteins Sclerostin) and DKK-1 (Dickkopf-related protein 1) the negative regulators of Wnt signaling that limits OB bone formation(3). Based on these facts, osteocytes are considered the central actors of the bone remodeling process (68,69).

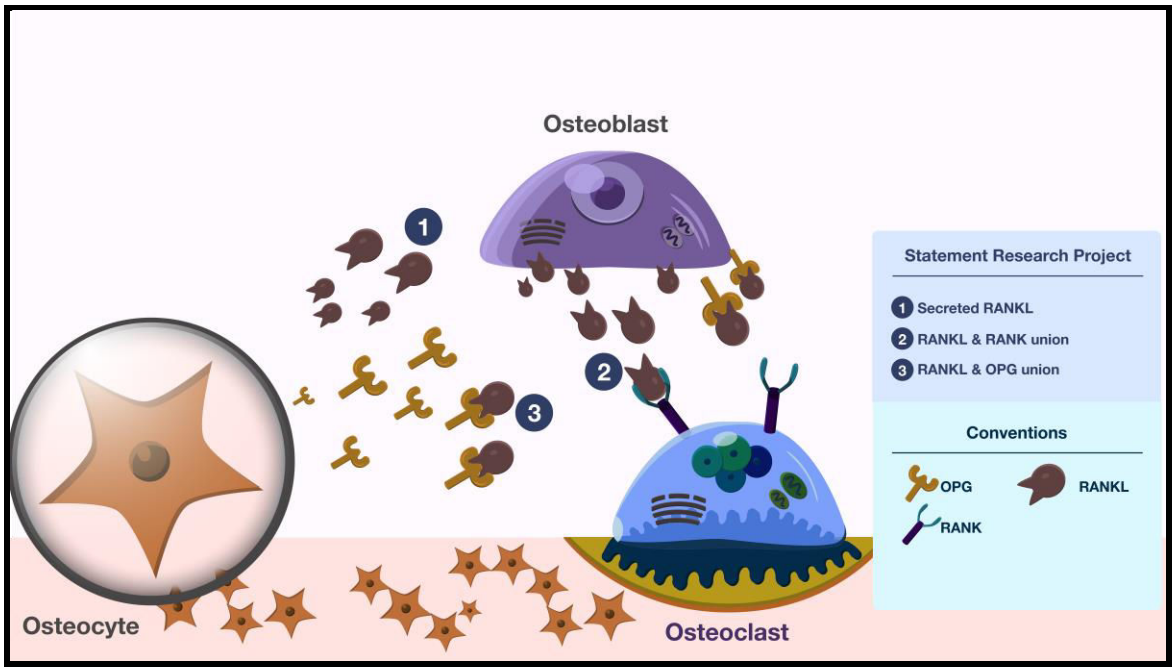

Figure 1. Main Cells involved in OCs function. Osteocytes are considered the main source of cytokines M-CSF, RANKL, and express OPG during bone remodeling while in bone modeling the main cells involved in this process are OBs (72,73)

2.1.1.5 Osteoclasts (OCs)

- **General aspects:** OCs, the bone-resorbing cells, are differentiated from mononuclear cells of the monocyte/macrophage lineage, found only on the surface of the calcified matrix. (19) (67). OCs differentiation is dependent on hematopoietic progenitor cells and signals from the microenvironment (11,67) (Fig. 2). Their recruitment and differentiation are mediated by hormones, growth factors, and cytokines (67). Different regulatory factors for the process of osteoclast differentiation include granulocyte/monocyte colony-stimulating factor (GM-CSF) and M-CSF. GM-CSF promotes the differentiation of common myeloid progenitors

into granulocyte/macrophage progenitors (GMP). Then, by stimulation of M-CSF, GMP further differentiates into cells of the monocyte/macrophage lineage, which are considered osteoclast precursors (67). M-CSF acts on hematopoietic stem cell system not only to maintain the survival of monocyte osteoclast precursors but also is required for the survival, motility and spreading of OCs (7,67) (Fig. 2). Under the influence of M-CSF, hematopoietic stem cells differentiate into macrophage colony-forming units (CFU-M), those are common precursor cells of macrophages and OCs (18). The change from CFU-M to mature OCs includes the cell-cell fusion of those precursors to give a specific characteristic to the OC mature which is the multinucleated stage (18). This process is mainly induced by RANKL and its downstream molecules (11,12,18,67).

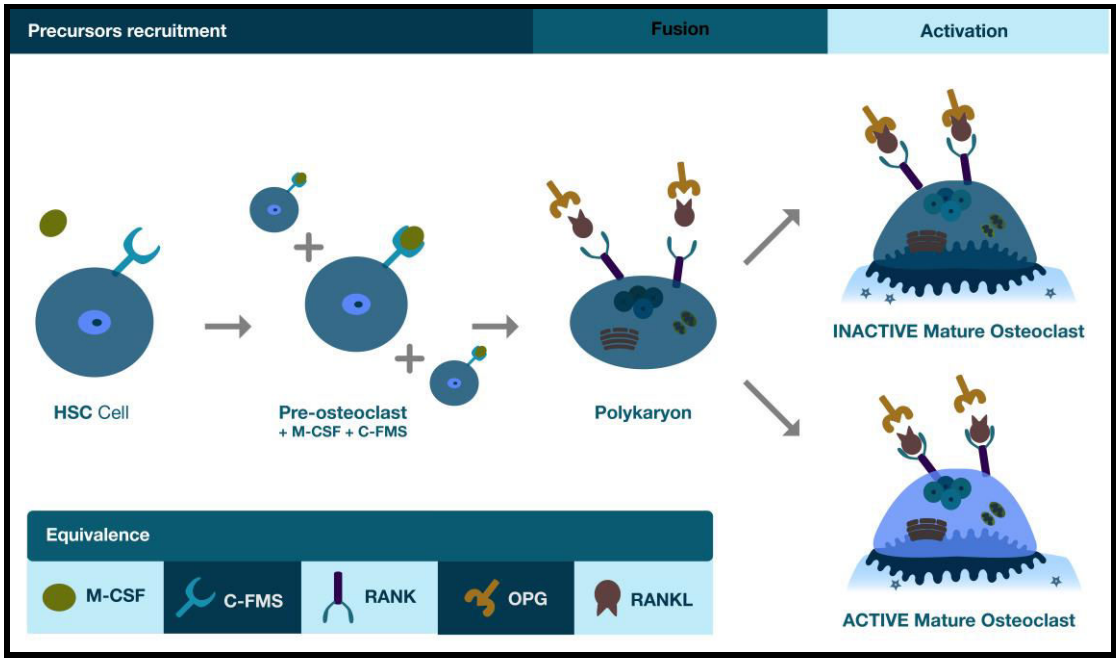

Figure 2. OCs differentiation is dependent on hematopoietic progenitor cells and signals. This process includes the macrophage colony stimulating factor (M-CSF), which acts on hematopoitic stem cell system to maintain the survival of osteoclast precursors monocytes (72)

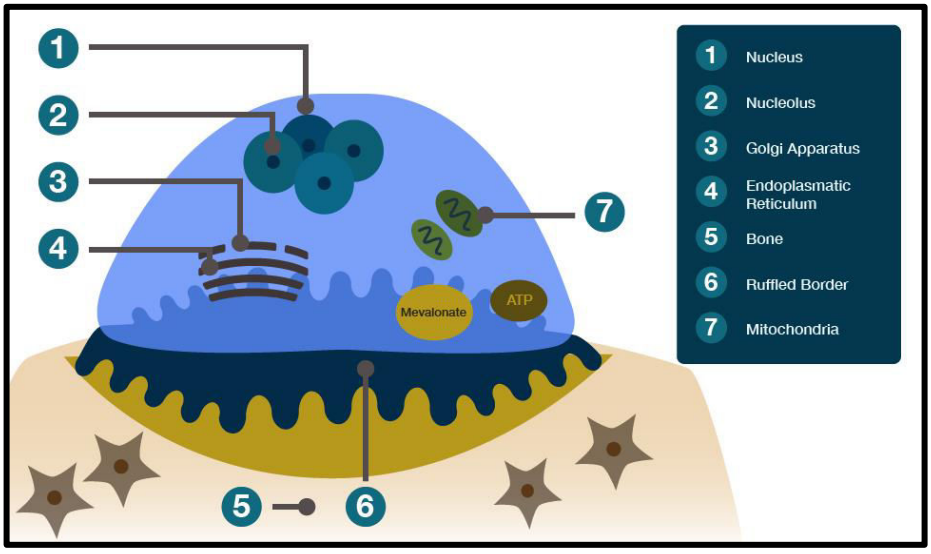

Figure 3. Main characteristics of mature OCs. Once the OCs is mature it contains the insulated compartment allowing the dissolution of bone mineral in the areas of bone resorption. OCs have specific characteristics. They are multinucleated cells, as result of the fusion of osteoclasts precursors, large size with a discoid sharpe, irregular edge and lighter areas (8).

Finally, the mature OCs have specific characteristics like the insulated compartment allowing the dissolution of bone mineral in the areas of bone resorption, multiples nucleus as result of the fusion of osteoclasts precursors, large size with a discoid shape, irregular edge and lighter areas (11) (Fig. 3).

OCs requires several important structural and functional characteristics to carry out its resorptive function, the most essential being the ruffled border and the insulated compartment (Fig 3). The ruffled border contains elements that degrade organic and inorganic bone components like the tartrate-resistant acid phosphatase (TRAP) that decalcified bone, the cathepsin K which fragment the bone matrix proteins and other lysosomal enzymes and cysteine proteases (9,67). Additionally, OCs can release protons H^+ ATPase by the ruffled border which create, in the insulated compartment, an acidic medium allowing the dissolution of bone mineral in the areas of resorption (11,67). In the OCs activity, the mevalonate pathway plays an essential role to achieve the final cell maturation (38,71) (Fig. 4). This pathway leads to the synthesis of cholesterol and other sterols such as isopentenyldiphosphate (IPP),

farnesyldiphosphate (FPP) and geranyl-geranyldiphosphate (GGPP), mediated by farnesylpyrophosphate synthase (FPPS) (Fig. 4) (71–73). FPP and GGPP are required for the post-translational modification (prenylation at a cysteine residue in C-terminal motifs) of small protein GTPases such as Ras, Rab, Rho and Rac proteins (71–74). These GTPases are needed for osteoclast function, including cell morphology adaptations, cytoskeleton arrangement, membrane ruffling, trafficking of vesicles and apoptosis (72,73,75) (Fig. 4). A review of the OCs differentiation process and their signaling pathways appears below.

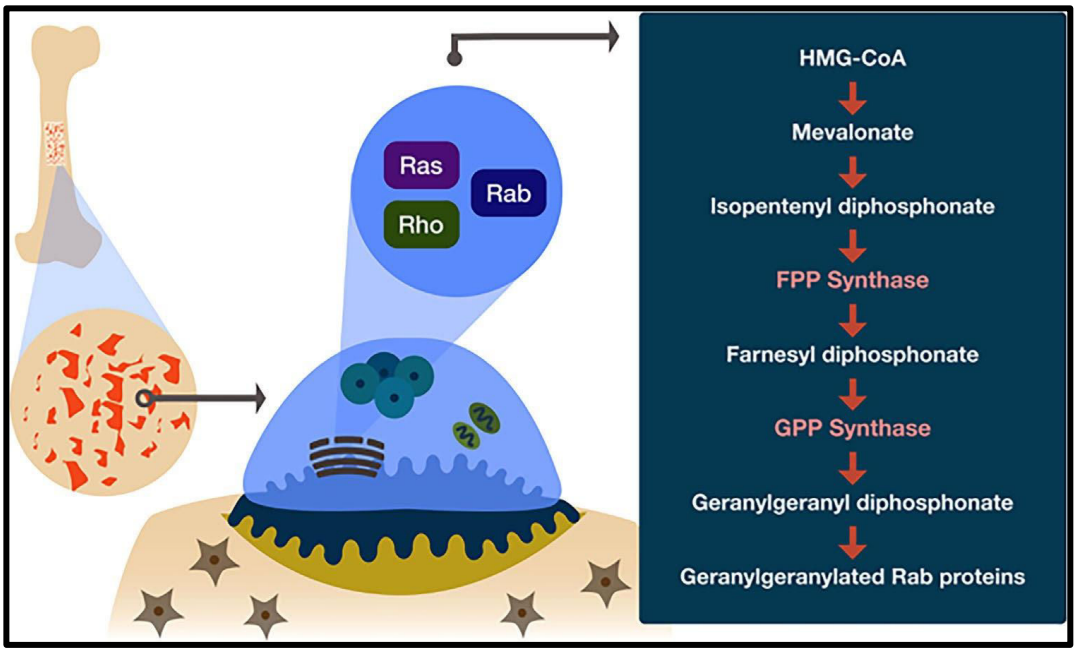

Figure 4. Mevalonate biosynthetic pathway (MBP). MBP plays and important role in many molecular sigmaling cascades within the osteoclast cell, regulating several processes related to GTPases proteins (e.g.Ras, Rab, Rho) such as the recruitment, differentiation, resorptive capacity, and/or apoptosis of osteoclasts

- **Cell signaling pathways involved in osteoclastogenesis:** here are two principal cytokines involved in OCs differentiation: the macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-*k*B ligand (RANKL)(11,17,67) The activity of those proteins is dependent on their receptors (C-FMS and RANK, respectively). However, other receptors and cytokines can modify and regulate their actions (67,76). Next, we will review the different receptors and cytokines involve and related to the OCs differentiation.

- **LIGANDS:**
  - **MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF)**
  - **INTERLEUKINE 34 (IL-34)**
- **RECEPTOR:**
  - **c-Fms (M-CSFR)**

The main factor responsible for the OCs differentiation is the M-CSF (also known as CSF-1). Through its receptor c-FMS, M-CSF promotes the proliferation and survival of OCs precursors. M-CSF is expressed by a variety of cells such as smooth muscle cells, vascular endothelial cells, hepatocytes, fibroblasts, T cells, bone marrow stromal cells, and osteoblasts (19,67). The main sources of M-CSF in the bone remodeling compartment (BRC) are bone marrow stromal cells (67). c-FMS receptor belongs to type III protein of the tyrosine kinase family (67). This receptor is characterized by a unique extracellular ligand-binding domain and a cytoplasmic tyrosine kinase domain (17,19). The binding of M-CSF to the receptor c-FMS results in auto and trans-phosphorylation of specific tyrosine residues in the c-FMS' cytoplasmic tail (77). This union attracts a signaling complex that implicates phosphorylated DNAX-activating protein 12 (DAP12) and the non-receptor tyrosine kinase, Syk (77). This activates ERK/growth-factor-receptor-bound protein 2, (Grb-2) and Akt/PI3K signaling that regulate several aspects of OCs activities, including the expression of RANK (77) (Fig. 5). The final result is the expression of the RANK receptor in the membrane of the OCs cells, what constitutes probably the most important characteristic of osteoclast differentiation (17,19,77) (Fig. 2 and 3).

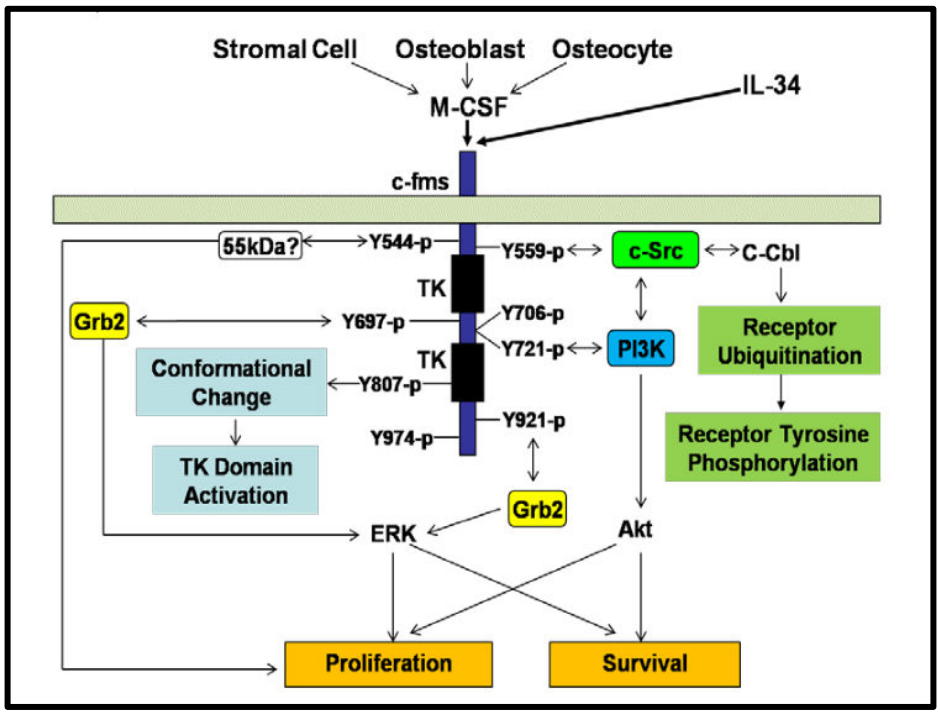

Figure 5. M-C SF and C-FMS Interaction. The binding of M-C SF to the receptor c-FMS results in the expression of RANK and the induction of OCs charateristics toward a functional resorbing cell. This process involves several intracellular signaling pathways that regulate several aspects of OCs activities, including the expression of RANK. Taking of Feng and Teitelbaum. In Osteoclasts: New Insights. Nature Publishing Group, 1 (1) 2013.

The c-FMS receptor serves another ligand named IL-34 (Fig. 5). This union is important as it increases the growth or survival of monocytes. IL-34 binding to c-FMS has a stronger but shorter-lived effect compared to M-CSF(67,78). In humans, IL-34 is abundant in the spleen and less expressed in other tissues like thymus, liver, small intestine, colon, prostate gland, lung, heart, brain, kidney, testes, and ovary (76,78). There are inconsistencies in its role in osteoclastogenesis. Some authors consider that IL-34 promotes the formation and differentiation of functional osteoclasts evidencing normal bone resorption capabilities, but with an important role in pathological conditions (76,78,79). However, other authors think that this cytokine is not important for the normal bone remodeling, and could be involved only in osteolytic pathological conditions.(67).

- **LIGAND:**
  - **Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL)**
- **RECEPTORS:**
  - **Receptor Activator of Nuclear Factor Kappa B (RANK)**
  - **Leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4)**

RANKL, which encoding gene located on human chromosome 13q14, is also named Tumor Necrotic Factor Superfamily member 11 (TNFS11), TNF-related activation-induced cytokine (TRANCE) or osteoprotegerin ligand (OPGL) (19,27,56). This ligand is a type II homotrimeric transmembrane protein, expressed as a membrane-bound or a secreted protein (19,27,67). The secreted protein is formed as a result of either proteolytic cleavage by matrix metalloproteinases (MMPs) or alternative splicing (19,27,56).

In bone, different types of cells express RANKL, the most important being osteoblasts and osteocytes. However, osteocytes have been shown to express a higher level of RANKL than OBs. This is the reason why osteocytes are considered the master cells in bone remodeling (68,69). RANKL is expressed on osteoblast progenitors, B cells, dendritic cells, and epithelial cells of the mammary gland. (67). This ligand is highly inducible and controlled by osteoactive factors including glucocorticoids, Vitamin D3 [1,25(OH)2D3], IL-1, TNF-a, TGF-b, Wnt ligands, and lipopolysaccharides (LPS) (22,27).

RANKL activity is mediated by its main receptor RANK, a member of the TNF-receptor superfamily (TNFR11A). RANK is also known as Tumor Necrosis Factor [TNF]-related Activation-induced Cytokine Receptor (TRANCE-R), osteoclast differentiation and activation receptor (ODAR), OPGLR or ODFR (19,67). The gene that encodes RANK (Tnfrsf11a) is located on chromosome 18q22 (27). RANK is a homo-trimerizing transmembrane protein type I of 616 amino acids. This receptor contains four extra-cellular cysteine-rich pseudo repeats, with a cytoplasmic tail of

about 383 amino acids containing three binding domains (I, II, III) (27,67). As a difference to c-FMS receptor, RANK has no intrinsic protein kinase activating capacity to mediate signaling. RANK trimerizes and transduces the signal via several adaptor molecules called TNF receptor-associated factors (TRAFs 2,3,5,6). Those are adaptor proteins that form complexes that activate Nuclear Factor κB (NF- κB), mitogen-activated protein kinases (MAPKs) and activator protein-1 (AP-1) signaling (80)(19)(67). TRAF2, 5 and 6 are the main TRAFs recruited by RANK, but only TRAF6 appears to sway RANK signaling (19,27). TRAF3, on the contrary, is not required for osteoclast formation and is associated with OCs activity reduction as we will discuss above (80).

The RANKL binding to RANK induces a specific intracellular activity (19,67,80). Indeed the RANK intracellular domain recruits TRAF6 to activate various signaling pathways in order to promote osteoclastogenesis (19,67,80). Then TRAF6 recruits TGF-β-activated kinase (TAK) 1-binding protein (TAB2 and TAK1), to induce the NF-κB and MAPK pathways. NF-κB induces c-Fos expression and MAPK pathway results in the activation of the Jun proteins (19,67). Both end products (c-Fos and Jun proteins) associate to form the AP-1 complex, which is the main regulator of the expression of NFATc1, the principal regulator of osteoclastogenesis (19,67). This osteoclast process has been associated with several co-stimulatory signaling pathways that stimulate the osteoclast formation, function and survival. These co-stimulatory pathways regulate the calcium signaling, necessary in the osteoclast activity, by activates calcineurin, which dephosphorylates NFATc1 and promoting its entry into the nucleus (19). The most significant co-stimulators are the Immunoglobulin-like receptors (IgLRs), Fc receptor common γ subunit (FcRγ), DNAX-activating protein (DAP) 12, Paired immunoglobulin-like receptor-A (PIR-A), osteoclast-associated receptor (OSCAR), Fcγ receptors (FcγRs) and Semaphorin (Sema) 6D and its receptor plexin-A1 (PlxnA1)(19,67,80,81).

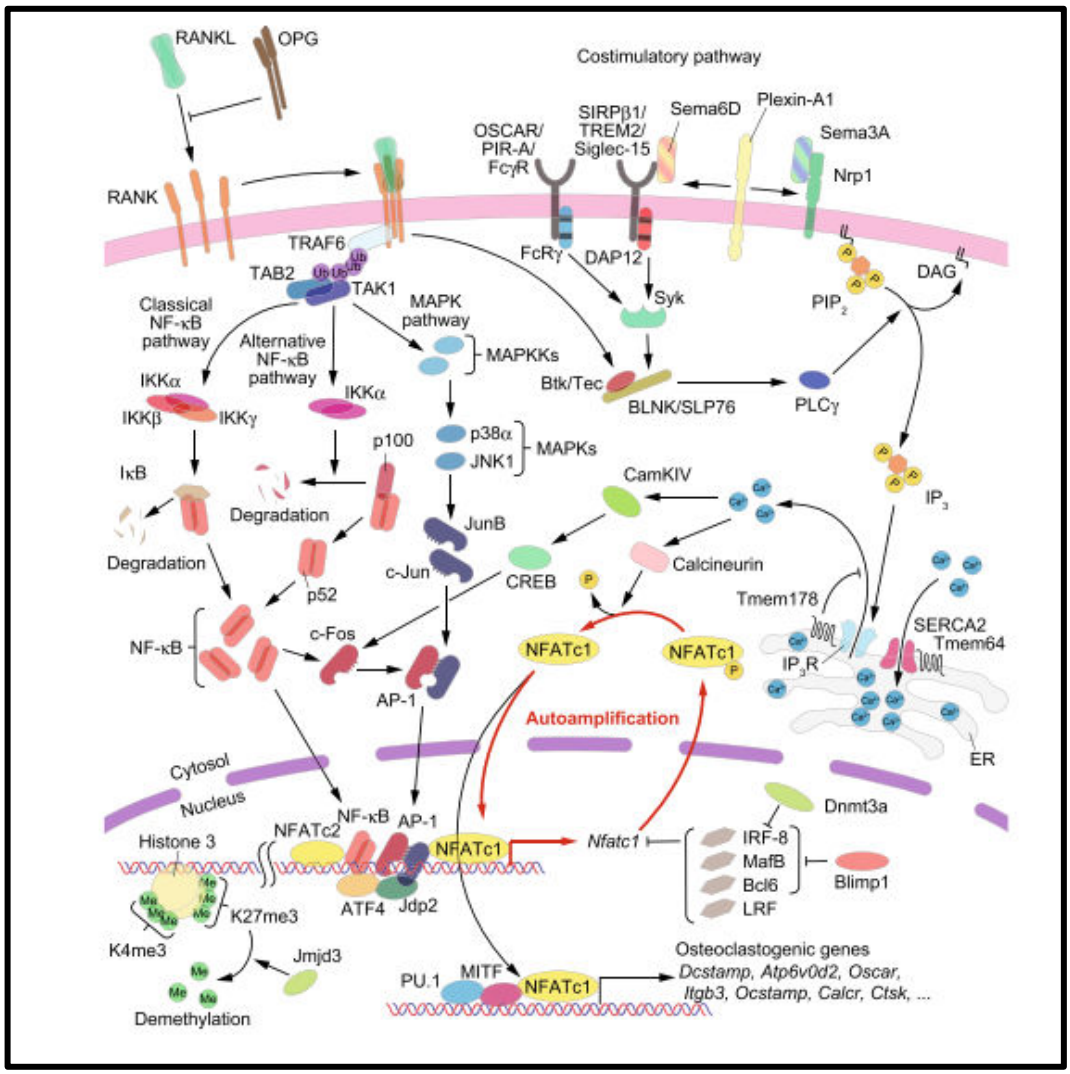

Figure 6. Signal cascade in the process of osteoclastogenesis. RANKL and its receptor RANK transduce a signal via the adaptor molecule TRAF6 recruits TAB2 and TAK1, which in turn activates the NF-kB pathway and MAPK pathway. NF-kB. Taken from: Recent advances in osteoclast biology, Ono T. Nakashima T. Histochemistry and Cell Biology 149 (4) 2018 (19)

The role of the adaptor molecule TRAF3 and the newer RANKL receptor LGR4 in the OCs activity deserve special attention. Related with TRAF3, when RANKL actives RANK on osteoclast precursors, this results in TRAF3 ubiquitination and lysosomal degradation, which is a basic process in the NF-κB activation. However, in absence of RANK/RANKL complex formation,TRAF3 is not ubiquitinated and the osteoclast formation is reduced due to the fact that TRAF3 inhibits NF-κB activation, having a negative regulatory role on osteoclastogenesis (19,67,80). On the contrary,

the recently recognized RANKL receptor, LGR4 (GPR48), is quite different from RANK and controls several developmental pathways through either potential classical G-protein signaling or via the potentiation of Wnt signaling (82). It has been demonstrated that the RANKL-LGR4 complex negatively regulates the osteoclast differentiation and the bone remodeling by blocking RANK–TRAF6 signaling, as well as by inhibition of NFATc1 (82,83). Moreover, it has been demonstrated that the receptor R-spondin 2 (Rspo2), activates the canonical Wnt/β-catenin signaling pathway, being a potent positive regulator of bone metabolism, OBs differentiation and maturation through their activation by LGR4 (82,83).

- **LIGANDS:**
  - Receptor Activator of Nuclear Factor-*K*B LIGAND (RANKL)
  - TNF-related apoptosis-inducing ligand (TRAIL).
- **RECEPTOR:**
  - Osteoprotegerin (OPG)

The osteoprotegerin (OPG), also known as the osteoclastogenesis inhibiting factor (OCIF) is a soluble receptor of the tumor necrosis superfamily factor (TNF), encoded by the gene Tnfrsf11B, located on chromosome 8q23-24 (21,22,84) OPG is synthesized as a 401 amino acid propeptide, that further lost a fragment of 21 amino acids, to obtain the mature protein of 380 amino acids length. The mature OPG is secreted as a soluble protein and acts as a soluble receptor for RANKL (21,22,56,84). This aspect constitutes the main difference with other TNFR receptors that are transmembrane proteins. Many human tissues express the OPG mRNA (e.g. bone, lung, heart, kidney, liver, intestine, stomach, brain, thyroid gland and spinal cord). In bone, the primary function of OPG is to inhibit osteoclast maturation and activation, as demonstrated both in vivo and in vitro (21,22,56,84).

The first activity of OPG is to serve as a decoy receptor for RANKL. Once the complex OPG-RANKL is achieving, the ligand is inactivated. According to the fact that RANK and OPG compete for the same ligand, several studies have compared their affinities for RANKL (19). The conclusion is that, due to the conformational difference with RANK in the hinge region, OPG may rotate more than RANK, it binds RANKL with a higher affinity (19).

TNF-related apoptosis-inducing ligand (TRAIL) has also been identified as a ligand for OPG (85). TRAIL, a member of the TNFSF family (TNFS10), exhibits an extensive tissue distribution including vascular smooth muscle cells and endothelial cells (85,86). The main action of TRAIL is to initiate cell death pathways through the induction of the release of apoptogenic factors from mitochondria (85). This activity, occurring through death receptors called DR4/ R1 and DR5/R2, has been associated with the protection against vascular calcification and cardiometabolic diseases (85). OPG is known to be released from vascular endothelial cells in response to inflammatory stimuli, as a mechanism against apoptosis. Consequently, TRAIL binding to OPG increases the risk of vascular injury, inflammation and atherosclerosis (85).

Table 1. Couples of receptors/ligands involves in osteoclast activity.

RECEPTORS	LIGANDS	ACTION ON THE OSTEOCLAST ACTIVITY	
c-FMS	Macrophage Colony-Stimulating Factor (M-CSF)	Promotes the proliferation and survival of OCs precursors by inducing the presence of RANK	
	Interleukine 34 (IL-34)	promotes the formation and differentiation of functional osteoclasts in vitro.	
RANK	Receptor Activator of Nuclear Factor-*k*b Ligand (RANKL)	Promotes osteoclastogenesis by recruits intracellular domain TRAF6 to activate several signaling pathways	In absences of RANKL, decrease the osteoclastogenesis by activating intracellular domain TRAF3
OPG	Receptor Activator of Nuclear Factor-*k*b Ligand (RANKL)	Decrease osteoclast activity by inactivating RANKL ligand	
	TNF-related apoptosis-inducing ligand (TRAIL)	increasing the risk of vascular injury, inflammation, and atherosclerosis by inhibiting the TRAIL activity.	
LGR4	Receptor Activator of Nuclear Factor-*k*b Ligand (RANKL)	Reduce the osteoclast activity by blocking TRAF6 and induce osteoblast differentiation by activating Wnt/β-catenin signaling pathway	

2.1.2 Bone modeling and remodeling.

2.1.2.1 General aspects. Bone remodeling is considered a continuous process that occurs to maintain bone homeostasis by control and repair (62,87). This process involves close communications between OBs and OCs, which evidence different functions. OCs resorb the old bone whereas OBs form new bone (11,84,87). Their interactions require at least three modes of communication: The first is the direct contact, enabling membrane-bound ligands and receptors to interact and further initiate intracellular signaling or gap junctions formations that allow the passage of water-soluble small molecules between the two cell types (58). The second mode is through the secretion of diffusible paracrine factors, such as growth factors, cytokines, chemokines, and other small molecules (58). The third mode is related to the liberation by OCs action of growth factors and other molecules included in bone matrix (58,64). These mechanisms of communication are related to the four stages of bone remodeling (Fig. 7) (58,67), presented in the following paragraphs.

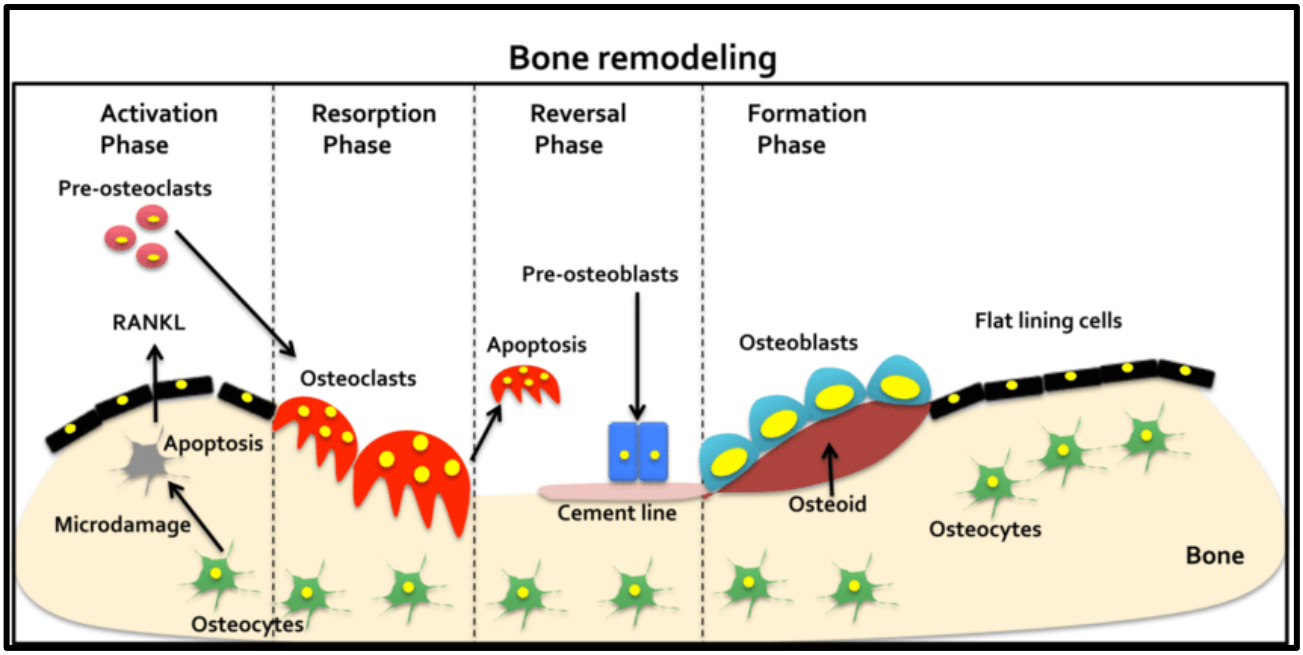

Figure 7. Bone remodeling stages. Bone remodeling involves four differente stages: Activation, resorption reversal and formation. Taken from: Recent advances in osteoclast biology, Ono T. Nakashima T. Histochemistry and Cell Biology 149 (4) 2018 (13)

- **Stage I: Activation:** The bone remodeling process begins with the recruitment of hematopoietic stem cells (HSCs) by chemotactic attraction, which drives them to the bone remodeling compartment (BRC) (22,88). Once in the BRC, HSCs are converted into myeloid progenitors through the action of granulocyte/macrophage colony-stimulating factor (GM-CSF). These granulocyte /macrophage progenitors sway eventually be transformed into OCs precursors by M-CSF action (67) or by IL-34 that both stimulate their common receptor c-FMS (67,78). The RANK expression is induced in these cells by M-CSF stimulation (67,78).

- **Stage II: Resorption:** This stage is considered as the proper bone remodeling stage. It corresponds to the OC differentiation and specific function acquisition and also to the migration of mesenchymal stem osteoprogenitor cells (67,89). OCs differentiation is reached through the activation of RANK. Once an OC precursor is activated, it converts into a cell with resorptive capability (19,67). OPG controls this process by competing with RANK for RANKL binding (19). By this way, it is possible to regulate the OCs maturation and activity. This is the reason why the RANKL/RANK/OPG signalization has consider a focus of interest in bone research. Its analysis allows a review of the mechanisms that alter the balance of activity and the regulation of bone formation and resorption (17,27,90).

Interestingly, this is not the only system able to modulate the OCs activity (19). Several other factors have been associated with an increase of OCs activity. These include the Interleukin 1 (IL-1) and the TNF-α, both considered stimulants of TRAF6, and associated with bone loss in postmenopausal osteoporosis and in rheumatoid arthritis (19,67,91). Other inductors of OCs activity include the A-proliferation inducing ligand (APRIL), the B cell-activating factor (BAFF), the nerve growth factor (NGF), the Insulin-like growth factors (IGF-1 and IGF-2), the lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), the transforming growth factor (TGF)-β, other

interleukins (IL-6, IL-11), the secreted osteoclastogenic factor of activated T cells (SOFAT) and ROS generated by lysyl oxydase (LOX) (19).

On the other side, different inhibitors, which reduce the OCs activity and therefore control the excessive bone resorption, include MafB, a bZIP motif-containing transcription factor, which inhibits the expression of microphthalmia transcription factor (MITF) and consequently reducing the activity of genes encoding factors that regulate OCs mediators as, cathepsin K, tartrate-resistant acid phosphatase and chloride channel 7 (9,19); LGR4 which competes with RANK for binding to RANKL and suppresses the canonical RANK signaling during osteoclast differentiation (82,92); Semaphorin 3A (receptor neuropilin-1) which inhibit the osteoclastogenesis (19,93). In addition to these factors, inhibitors of the OCs activity exist as the transcriptional repressor B lymphocyte-induced maturation protein (Blimp1), the Leukemia/lymphoma-related factor (LRF), the Interferon regulatory factor (IRF)-8, and the interferon-gamma (19,27,67).

- **Stages 3 and 4: Reversal and formation:** The final process of bone remodeling ends with stage 3 and 4. Stage 3, considering to the bone formation phase, is characterized by the OBs differentiation and function (osteoid synthesis) (9,67). The stage 4, considering the mineralization phase, is the terminal process during which the osteoid formation occurs to conclude the bone remodeling (67). Due to the fact that the relevance of these phases for the OCs activity is less significant, there will be no thorough review of these stages.

2.1.2.2 Bone remodeling disorders. Different bone disorders exist which can alter the modeling and remodeling process; they are known as osteopetrotic and osteolytic diseases (3,94). Osteopetrotic disease group (Albers–Schönberg diseases or marble bone diseases) is related to a greater bone apposition, resulting in a bone mass increased, mainly due to OCs dysfunction or absence (19). The osteolytic disease group (OD), on the contrary, is related to an increase in bone turnover associated to an increase in OCs number or/and activity (19). This work will focus on this second group.

ODs are characterized by a high bone turnover with a negative balance between bone formation and resorption (94). These pathophysiological conditions generate a low bone density and deterioration of bone microarchitecture, leading towards a weak bone phenotype and a higher fracture risk (39,48,94). ODs have different etiologies and affect both children and adults.

ODs affecting children include an inherited group of rare disorders. All these chronic, debilitating, severe, and life-threatening pathologies have all prevalence inferior to 1/5,000 births. Pediatric ODs can be divided into two types depending on the timing of the osteoporotic onset. Early-onset osteoporosis is related to genetic mutations and appears soon after birth. This group of diseases includes osteogenesis imperfecta syndromes (OI types I to XVI, OMIM #166200, 166210, 259420, 166220, 610967, 613982, 610682, 610915, 259440, 613848, 610968, 613849, 614856, 615066, 615220 and 616229) that are uncommon primary osteoporosis caused by disorders of connective tissues (95,96), idiopathic juvenile osteoporosis (IJO, OMIM #259750) a disease related to rheumatoid juvenile arthritis (97), osteoporosis pseudoglioma syndrome (OPPG, OMIM # 259770)(98), neurofibromatosis (mainly NF type I and Noonan syndrome, OMIM #162200, 601321) (99), Gaucher disease (all types, OMIM #230800, 230900, 231000 and 231005) (97), Hajdu-Cheney syndrome (OMIM #102500) (100)(101), familial idiopathic hyperphosphatasemia also known as Paget disease of bone 5–juvenile onset (PDB5, OMIM #239000) (16),

early-onset Paget disease or Paget disease of Bone 2 (PDB2, OMIM #602080) (16), familial expansile osteolysis and expansile skeletal hyperphosphatasia (FEO and ESH, OMIM #174810) (16). Late-onset forms include osteoporosis related with hypercalcemic disorders (hyperparathyroidism, vitamin D-related causes, malignancy, medications, endocrine disorders, genetic disorders, and miscellaneous causes) (97,102) and heterotopic calcifications (fibrodysplasia ossificans progressive (FOP, OMIM #135100), arterial calcification generalized of infancy (CAGI I and II, OMIM #208000 and 614473) and spondyloenchondrodysplasia (SPENCD OMIM #607944) (46,97). Table 2 summarize the main osteolytic disorders, their etiology and considerations.

ODs affecting adults include primary (or age-related) osteoporosis that is the most common form with two types. The first is associated with estrogen deficiency at menopause and the second related to long-term remodeling inefficiency (94). Secondary osteoporosis could appear in the context of endocrine, reproductive, gastrointestinal and nutritional disorders (94,103), following treatments with drugs like glucocorticoids and anti-convulsants (103), in the case of Paget disease (27,39,94), or in the presence of malignant primary bone tumors associated with breast or prostate cancer or bone metastases (24,104–106) (Table 2).

TABLE 2: MAIN DISORDERS ASSOCIATED WITH OSTEOLYTIC ACTIVITY				
	TYPE	SUBTYPE	CAUSE (oc-ob)	CHARACTERÍSTICS
OSTEOPOROSIS Inherited or unknown etiology rare orphan diseases.	PRIMARY GENETIC EARLY ONSET FORMS Bone fragility, higher fracture risk, bone deformity and linear growth deficiency.	OSTEOGENESIS IMPERFECTA SYNDROME (TYPE 1 – 4)	Mutations in COL1A1 and COL1A2 genes (↓Ob)	Abnormal production of type I collagen with abnormal bone matrix and reduced bone strength
		OSTEOPOROSIS PSEUDOGLIOMA SYNDROME	Mutations in LRP5 gene (↓Ob)	Congenital or infancy-onset visual loss and severe juvenile osteoporosis.
		NEUROFIBROMATOSIS	Mutations in NF1, NF2 gene (↑ Oc, aberrant Activation TGF-β may induce RANKL)	Abnormalities including the nervous system, skin and bone
		GAUCHER DISEASE TYPE I	Mutations in GBA gene (acid b-glucocerebrosidase) (↑ Oc)	Osteopenia/osteoporosis, Erlenmeyer flask deformity, bone crises, osteonecrosis, lytic bone lesions, osteosclerosis, cortical thinning, acute osteomyelitis, and growth retardation.
		HAJDU-CHENEY SYNDROME	Mutations in NOTCH2 gene. (↓Ob)	Progressive focal bone destruction, acroosteolysis, severe osteoporosis with fractures and craniofacial dysmorphism
		FAMILIAL HYPERPHOS- PHATASEMIA	Mutations in TNFRSF11B gene (↓0pg, ↑Oc)	Increased bone turnover, skeletal deformity, bone expansion, bone pain, increased risk of pathological fractures.
		EARLY-ONSET PAGET DISEASE	Mutations in TNFRSF11A gene (↑ Oc)	Affects entire skeleton, widespread bone and joint pain. Skull growth unusually large and thick.
		FAMILIAL EXPANSILE OSTEOLYSIS	Mutations in TNFRSF11A gene (↑ Oc)	Generalized and focal skeletal abnormalities, bone pain at affected sites, tooth loss and progressive loss of hearing.
		EXPANSILE SKELETAL HYPERPHOSPHATASIA	Mutations in TNFRSF11A gene (↑ Oc)	Affects entire skeleton, progressive hyperostotic expansion of long bones and fingers, early onset deafness, premature tooth loss.
		IDIOPATHIC JUVENIL OSTEOPOROSIS	Unknown etiology	Affect previously healthy child, prepubertal onset. Very low bone formation rate and decreased cancellous bone volume.
	SECONDARY FORMS	HYPERCALCEMIC DISORDERS	Including vitamin D intoxication, malignancy-induced hypercalcemia, subcutaneous fat necrosis, idiopathic infantile hypercalcemia, neonatal severe primary hyperparathyroidism, immobilization hypercalcemia (↑ Oc)	Osteoporosis of cortical bone, such as the wrist, is mainly associated with primary hyperparathyroidism. Excess PTH also can result in subperiosteal resorption, leading to osteitis fibrosa cystica with bone cysts and brown tumors of the long bones.
		HETEROTOPIC CALCIFICATION	Fibrodysplasia ossificans progressive	Rare and disabling genetic condition of congenital skeletal malformations and progressive heterotopic ossification.
OSTEOPOROSIS AGE-RELATED	PRIMARY OSTEOPOROSIS	TYPE 1	Estrogen lack at menopause (↑ Oc)	Loss of trabecular bone at menopause
		TYPE 2	Long-term remodeling inefficiency: Dietary inadequacy/Activation of the parathyroid axis (↑ Oc)	Loss of cortical and trabecular bone in men and women, over 70 years old
SPECIFIC CLINICAL DISORDERS ASSOCIATED WITH OSTEOPOROSIS	SECONDARY OSTEOPOROSIS	ENDOCRINE AND REPRODUCTIVE PATHOLOGICAL DISORDERS	Primary hyperparathyroidism, Thyrotoxicosis, Glucocorticoid excess. Hypophosphatasia Hyperprolactinemia, (↑ Oc)	High levels of PTH enhance RANKL- OPG-induced osteoclast differentiation, mainly resulting in cortical bone resorption.
			Acromegaly (↑ Oc)	Increased bone turnover and impaired trabecular bone microarchitecture.
			Hypogonadism (↑ Oc)	Estrogen deficiency induces osteoblasts apoptosis and impairs WNT- beta catenin signaling, regulating osteoblastic activity. Androgen deficiency inhibits proliferation and differentiation of osteoblasts and promotes osteoclastogenic activity.
		GASTROINTESTINAL/ NUTRITIONAL	Vitamin D deficiency, Chronic liver disease, Malabsorption. (↑ Oc)	Reduced calcium and secondary hyperparathyroidism, which stimulates osteoclastogenesis, increases bone resorption and results in osteopenia and osteoporosis.
		PHARMACOLOGICAL	Glucocorticoids, Gonadotropin-releasing hormone agonists, Anticonvulsants, Excess thyroid hormone replacement (↑ Oc)	These drugs impair WNT- beta catenin signaling (bone formation) or enhance RANK-RANKL-OPG signaling (bone resorption).
		PAGET DISEASE	A combination of genetic, variations in SQSTM1, TNFRS11A and TNFRSF11B genes with infections with certain viruses. (↑ Oc)	Focal increases in bone turn over, mainly affecting bones in the axial skeleton
	SECONDARY TO MALIGNANT PRIMARY BONE TUMORS	EWING SARCOMA	Presence of an aberrant transcription factor with oncogenic properties, results of the transcription of the EWS-FLI1 fusion gene. (↑ Oc)	Rapid and extensive osteolysis.
		OSTEOSARCOMA	imperfections mesenchymal stem cell differentiation, and inconsistencies in the osteogenic development from the parent mesenchymal stem cell. (↑ Oc)	Rapid and extensive osteolysis.

Table 2. Main disorders associated with excessive osteolytic activity and their associated causes. including their relationship with Osteoblastic (Ob), or osteoclastic (Oc) cells activity (↑increase activity, ↓decrease activity).

2.1.2.3 Regulators of bone remodeling: The drugs used to control disorders related with bone modeling and remodeling make it possible to a certain extent to balance the physiological turnover of the affected bone in almost all patients, with partial recovery of bone formation in lytic lesion sites, pain relief and improvement in the quality of life (46,95,107). Three different types of drug are used to control turnover in the altered bone seen in ODs: anti-resorptive, anabolic and dual-action mechanism agents (40,108–111). Anti-resorptive agents act on osteoclasts, decreasing bone turnover and preventing bone loss; these include estrogens, calcitonin, vitamin D, bisphosphonates (BPs), selective estrogen receptor modulators (SERMs) and the RANKL blocking monoclonal antibody (Denosumab) (40,108–111). Anabolic agents stimulate osteoblasts to induce bone formation (40,108–112). These agents include recombinant parathyroid hormone (PTH 1–34-Teriparatide), growth hormone, insulin-like growth factor-I, prostaglandin agonists, statins (lovastatin), decoy receptor neutralizing Activin A (Sotatercept), neutralizing anti-DKK1 antibody (BHQ880) and neutralizing anti-sclerostin antibodies (Romosozumab and Blosozumab) (40,108–112). Dual-functional agents stimulate bone formation and decrease bone resorption (40) and include Strontium Ranelate and Bortezomib (Proteasome and NF-kB signaling pathway inhibitor) (40,113–115). BPs, due to their prolonged response, high efficiency and lower costs, are to date considered the first line of management for controlling different types of OD (40,108–112). These drugs are classified in hormones and nonhormonal agents, according to their origin. Below a brief review of these agents is going to be done.

- **Hormonal agents:** Several hormones have been implicated in the control of calcium and phosphate homeostasis: parathyroid hormone (PTH), Calcitonin, vitamin D (through its active metabolite 1,25-dihydroxy vitamin D (1,25(OH)2D) and fibroblast growth factor 23 (FGF23).

- **Parathyroid hormone (PTH):** Regulates calcium and phosphate flux across cellular membranes in bone and kidney, resulting in an increased calcemia and

decreased phosphatemia in the bone. PTH increases the OCs number and activity by inducing the RANKL expression (34,108).

- **Vitamin D (1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$)):** $1,25(OH)_2D_3$ is the most potent stimulant of intestinal calcium and phosphate transport. This metabolite can induce RANKL expression in osteoblasts and proteins such as osteocalcin, which may regulate the mineralization process (34,108).

- **Calcitonin:** Calcitonin is produced by the thyroid parafollicular C cells of in response to an increase in the serum calcium (37,102). This hormone is a weak inhibitor OCs activation and has opposed effects to PTH in the kidneys, promoting calcium and phosphate excretion through its action on bone and kidney (37,102). Calcitonin lowers calcemia and phosphatemia (37,116).

- **Fibroblast growth factor 23 (fgf23):** FGF23 is a hypophosphatemic hormone whose actions parallel those of PTH but with effects restricted to the regulation of renal Pi absorption and vitamin D biosynthesis. The role of Klotho, a membrane protein that serves as an essential cofactor in the transduction of FGF23 signaling, is associated with the inhibition of $1,25(OH)_2D$ production and phosphate reabsorption in bone homeostasis (7,37,116).

- **Secondary hormonal regulators of bone mineral homeostasis:** Estrogens and glucocorticoids can modulate the actions of PTH, FGF23, and vitamin D on bone mineral homeostasis. Compared with the main hormones, the physiologic impact of such in secondary regulation on bone mineral homeostasis is minor. Estrogens prevent the acceleration of bone loss during the postmenopausal period and increase the bone mineralization in postmenopausal women (37). Glucocorticoids alter the bone mineral homeostasis by antagonizing vitamin D-stimulated the intestinal calcium transport, stimulating the renal calcium excretion, and blocking the bone formation. Prolonged administration of glucocorticoids is a frequent cause of

osteoporosis in adults and can cause stunted skeletal development in children (37,117).

- **Non-hormonal agents:** There are several drugs used to modify the altered bone turnover seen in ODs (107). According to the biological mechanism involved, drugs can be classified as anti-resorptive, anabolic, or dual-action mechanism agents. Anti-resorptive agents act on osteoclasts decreasing bone turnover and preventing bone loss. These include selective estrogen receptor modulators (SERMs) and synthetic molecules as bisphosphonates (BPs) and RANKL blocking monoclonal antibody (Denosumab) (118). Anabolic agents, insulin-like growth factor-I, prostaglandin agonists, statins (lovastatin), decoy receptor neutralizing Activin A (Sotatercept), neutralizing anti-DKK1 antibody (BHQ880) and neutralizing anti-sclerostin antibodies (Romosozumab and Blosozumab), stimulate osteoblasts to induce bone formation (39,40,112,119,120). Dual-mechanism of action agents (e.g. strontium ranelate and Bortezomib (Proteasome and NF-kB signaling pathway inhibitor) stimulates the bone formation and decreases the bone resorption (40). These different drugs enable to balance in a certain extent the affected physiological bone turnover in most patients, with a partial recovery of the bone formation in the lytic lesion sites. Their effects result in patient's pain relief and an improvement in the quality of life (46,95,107). BPs, due to their prolonged response, high efficiency and less costs are to date considered the first line for the management of inherited, age-related and other osteolytic disorders (39,94,120).

2.1.2.4 Bone modeling and remodeling turnover markers*:* Bone turnover markers are substances present in blood and urine, which are produced or released during bone turnover. There are two types of enzymes or proteins secreted by osteoblasts or osteoclasts during the formation or destruction of type I collagen, the main protein of the bone matrix. Some of them are used as markers to analyze the OCs activity both in clinical as in preclinical research. Table 2 summarizes the most significant markers and their origin (6,7). The importance of these markers and their clinical implication will be discussed in the following paragraphs.

Table 3. Markers of bone turnover.

SERUM MARKERS OF BONE FORMATION	SERUM MARKERS OF BONE RESORPTION	URINARY MARKERS OF BONE RESORPTION
• Bone-specific alkaline phosphatase • Procollagen type I N-propeptide • Procollagen type I C propeptide • Osteocalcin (Bone Gla-protein)	• Aminoterminal cross-linking telopeptide of bone collagen (NTX) • Carboxyterminal cross-linking telopeptide of bone collagen (CTX) • Tartrate-resistant acid phosphatase	• Pyridinoline (PYR) • Free Lysyl-pyridinoline (deoxypyridinoline) • Tartrate-resistant acid phosphatase • Hydroxy-proline (not very specific)

2.2 BISPHOSPHONATES:

2.2.1 General aspects. Different drugs have been used in recent years to control osteolytic diseases (ODs) (35,39,46,95,110,118,119,121). Bisphosphonates (BPs), used successfully for over forty years, have been reported as the most effective drugs for such control (46)(121) (35,39,95,110,118,119) Despite their greater efficacy, aspects related to their side effects have yet to be fully understood (122–125). In the last decade, significant progress has been made in understanding their underlying mechanisms, with the promise of safer use of bisphosphonates, and the possibility of preventing the side effects in the near future (122–125). The following paragraphs are an updated review of the skeletal bisphosphonate-related side effects that paradoxically mainly affect the very tissues that these drugs have been designed to protect. After a short description of the different drugs used to control ODs, the focus will turn to nitrogenous bisphosphonates (N-BPs), their current applications and their skeletal side effects linked to pediatric and adult treatments. Current management of these side effects and actual recommendations for their prevention and management will also be presented, as will be the future directions emanating from current research.

2.2.2 N-BPs structures and mechanisms of action. BPs were first synthesized in the 1800s, but have been used in medicine only since the 1960s (38,126). BPs are stable analogs of a naturally-occurring pyrophosphate (PPi) compound, in which the central oxygen (P-O-P) is replaced by a carbon atom (P-C-P), plus two side chains (R1 and R2) (38,74,108). The presence of these side chains makes possible the synthesis of several compounds with different properties, but mainly a hydroxyl substitution at R1 enhances the affinity of BPs for calcium crystals, while the presence of a nitrogen atom (N) in R2 enhances their potency and determines their mechanism of action (74). Based on the presence of nitrogen in the R2 chain, there are two groups of BPs: non-nitrogenous (non N-BPs) and nitrogenous (N-BPs) (72,74).

Non N-BPs, known as the first generation, correspond to the first BPs synthesized and are characterized by the presence of a hydroxyl group in one of the side chains (38,74,108). Their mechanism of action is related to the non-selective inhibition of adenosine triphosphate, causing the apoptosis of osteoclastic cells. Non-N-BPs are the least efficient with moderate pharmacological potency (38,74,108). This group includes etidronate, clodronate and tiludronate (38,74,108).

The second group, N-BPs, appeared later, improving the pharmacological activity of BPs, and their stability and retention in bone tissue. Their main modification, a nitrogen added to the side chain, generated more powerful, effective and specific molecules (38,71). Their mechanism of action is related to their ability to alter osteoclast activity by inhibiting the mevalonate pathway (38,71) (Fig. 8). In osteoclasts, this pathway leads to the synthesis of cholesterol and other sterols such as isopentenyldiphosphate (IPP), farnesyldiphosphate (FPP) and geranyl-geranyldiphosphate (GGPP), mediated by farnesylpyrophosphate synthase (FPPS) (Figure 6) (71–73). FPP and GGPP are required for the post-translational modification (prenylation at a cysteine residue in C-terminal motifs) of small protein GTPases such as Ras, Rab, Rho and Rac proteins (71–74).

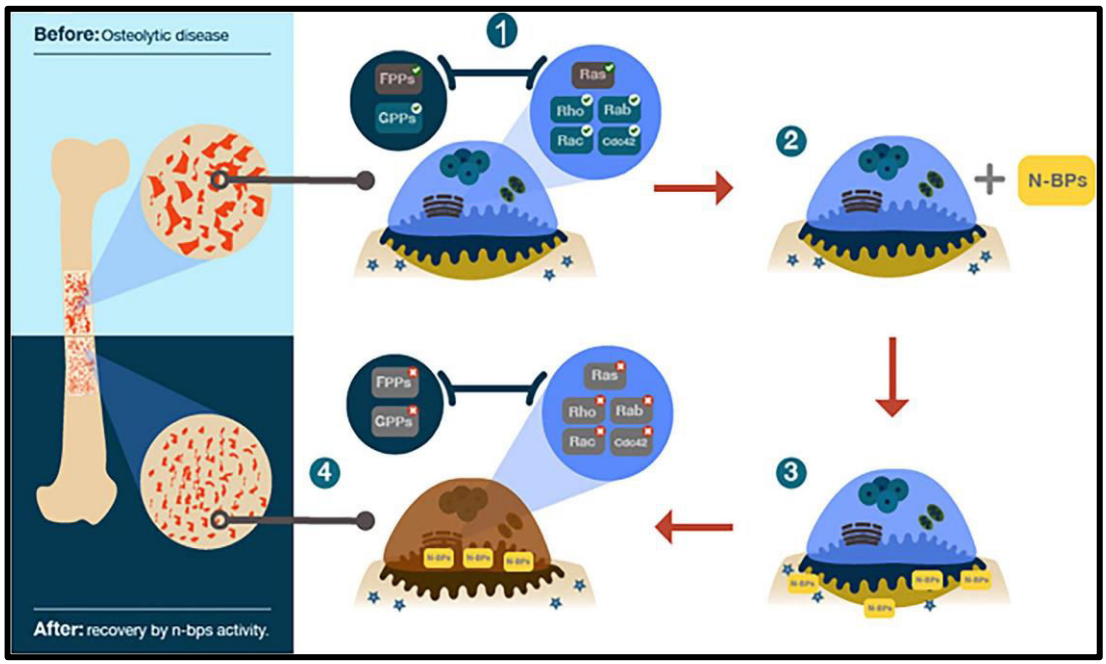

Figure 8. N-BP mechanism of action. 1: Increase osteoclast activity in osteolytic disease. 2: In order to regulate osteoclastic cell activity, N-BPs are administered. 3: N-BPs that have a great affinity for bone mainly concentrate in the sites of bone resorption. 4: N-BPs are captured and internalized by osteoclasts. N-BPs alter the protein GTPases prenylation by inhibiting FPPS and GPPS enzymes that affect many signaling cascades within the osteoclast. Bone remodeling is recovered.

These GTPases are needed for osteoclast function, including adaptations to cell morphology, cytoskeleton arrangement, membrane ruffling, trafficking of vesicles and apoptosis (Figure 8) (72,73,127). N-BPs have great affinity for bone, where they enter osteoclasts by means of pinocytosis and/or phagocytosis (39,73,74) or using butyrophilin 3A1 (128) or specific phosphate transporters such as SLC20 and/or SLC34 (129) in other cell types (128,129). Once inside the cell, N-BPs inhibit FPPS, causing the alteration of protein prenylation (71,73,74,127). Some N-BPs can also inhibit other enzymes in the mevalonate pathway, including GGPP synthase, IPP isomerase and squalene synthase (71). In this way, N-BPs affect the recruitment, differentiation, and resorptive capacity of osteoclast, and induce osteoclast apoptosis (38,71,73,74) (Figure 8). This group includes pamidronate, alendronate, ibandronate, risedronate, zoledronate, neridronate, olpadronate and minodronate (72,73,127) and, reported more recently, OX14 (130) (Table 4). There are different characteristics among the N-BPs, related to their chemical structure and their

potency, which ultimately define the clinical applications (44,71,73,74,131). Pamidronate and alendronate (alkylamino bisphosphonates) are 100 times more potent than first generation BPs. Ibandronate, which contains a tertiary N atom, exhibits greater potency than pamidronate or alendronate (44,71,73,74,130,131). Finally, zoledronate and minodronate, the last generation of N-BPs (third generation), have a heterocyclic N and the greatest potency of all N-BPs (44,71,73,74,131). These drugs are the most potent inhibitors of FPPS, have the highest affinity for hydroxyapatite, and exhibit the longest period of action (44,71,73,74,130,131). The new N-BP, OX14, is considered as potent as zoledronate and minodronate, but with a lower bone binding affinity (130). Table 4 summarizes the N-BPs, their brand names, doses and clinical indications.

TABLE 4: NITROGEN BISPHOSPHONATES						
GENERIC NAME	MAIN BRANDS NAME	LABORATORY	POTENCY (structure/activity)	DOSES AND ROUTE		INDICATIONS
				ADULTS	CHILDREN	
PAMIDRONATE	Aredia(R)	Novartis	100	30–90mg IV	1mg/kg/dose	Moderate or severe hypercalcemia with malignancy, with or without bone metastases. Osteolytic bone metastases of breast cancer and osteolytic lesions of multiple myeloma.
	Padium(R)	Furen Pharm				
	Pamidria(R)	Cipla				
ALENDRONATE	Fosamax(R) Fosamax Plus(R)	Merck	1000	5-, 10-, 35-, 40- and 70-mg tablets oral	Oral. Once/week 1 mg/kg d max 20 mg/d Per day: 5 – 10mg/d	Osteoporosis in women after menopause and men with osteoporosis; Paget disease
	Porosan(R)	Pharmathen				
	Lendrate(R)	Actavis				
	Binosto(R)	Lacer				
RISEDRONATE	Actonel(R)	Sanofi Aventis	5000	5-, 35-, 75- and 150-milligram tablets oral	once/week 15 mg <40 kg 30 mg >40 kg	Osteoporosis in postmenopausal women; and men with osteoporosis. Paget disease.
	Atelvia(R)	Warner Chilcott				
IBANDRONATE	Boniva(R) Boniva IV(R)	Genentech	10000	2.5-mg tablet once daily, 150-mg tablet once monthly. Oral 3 mg/3 mL single use IV	NA	To treat and prevent osteoporosis in women after menopause
	Bondronat(R) Bonviva(R)	Roche				
	Ibandrix(R)	Ginsberg				
ZOLEDRONATE	Zometa(R)	Novartis	20000	4 mg/5 mL single-dose	0.25 mg/kg every 3 months IV 0,066mg/kg/d	Hypercalcemia of malignancy; bone complications due to multiple myeloma and bone metastases from solid tumors.
	Reclast(R)	Novartis				
	Cytozol(R)	VHB life science Inc				
	Zolebenz(R)	McGlobe				
OLPADRONATE	Mosodium padrones(R)	Gador	1000	25mg		Back pain
NERIDRONATE	Nerixia(R) Attila (R)	Abiogen Pharma	100	25 – 100mg IV	2mg/kg	Osteitis deformans; Osteogenesis imperfecta Complex regional pain syndromes
	Neridronate(R)	Grunenthal				
MINODRONATE	Bonoteo Tablets(R)	Astellas Pharma	20000	1mg Tablets	NA	Osteoporosis
	Recalbon Tablets(R)	Ono Pharmaceutical				

Table 4. Main nitrogen bisphosphonates, brand names, doses and clinical application.

In addition to their main effect on osteoclast activity, some studies have suggested that N-BPs may also stimulate bone formation by osteoblast through the stimulation of the proliferation and the inhibition of apoptosis. Such effect, apparead to be dose-dependent (132,133). Indeed, beneficial effects on osteoblasts were reported at lower concentrations, while an inhibition of the cell differentiation was observed at high concentrations due to the repression suppressing of the osteoblastic production of vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANG-1) (133).

2.2.3 N-BPs main clinical applications. N-BPs are considered the most effective drugs for treating bone pathologies characterized by high bone turnover, and with a negative balance between bone formation and resorption (38,71). These pathophysiological conditions, known as osteolytic diseases (ODs), generate low bone density and deterioration of bone microarchitecture, leading to a weak bone phenotype and higher risk of fracture (39,48,94). ODs have different etiologies and can affect children and adults.

ODs affecting children include an inherited group of rare genetic disorders (prevalence inferior to 1/2,000 births) (134,135). Depending on the time of onset, these conditions are divided into early (appearing soon after birth) and late (appearing during childhood or adolescence) (16,94,95,97–102,105,136–139). To control pediatric ODs, N-BPs were first used in the 90s, with the application of pamidronate to treat osteogenesis imperfecta (OI) (44,140). Nowadays, N-BPs are considered the first line in the management of most pediatric ODs (44–46,139,141,142) and are also indicated for other conditions, such as the recovery of bone mass and the reduction of fracture rates in bone disorders associated with immobilization (43,44,46,140,141). The main N-BPs prescribed in pediatric patients are alendronate, pamidronate and zoledronate (43,44,46,140,141,143) the last one being specifically used in the treatment of pediatric bone malignancies (44,141) Despite their wide use in children, some questions remain unanswered: there are no standardized protocols for each condition (use and duration of therapy); their side

effects in axial-appendicular and craniofacial skeletal growth and their impact in adulthood have not been extensively evaluated (45,46,140,144); and the long-term safety of the drugs in childhood is unclear because of the pediatric physiological characteristics of bone remodeling (45,46,140,144).

In adults, the most common OD is age-related osteoporosis, associated with estrogen deficiency at menopause or long-term remodeling inefficiency (94,110,121,145). In both pathological conditions, N-BPs have been used for many years with excellent responses (39,40,72,110,113), maintaining bone density for at least 2 years after the withdrawal of the treatment (39,40,72,110,113). In postmenopausal women, N-BPs decrease the risk of fracture at vertebral and non-vertebral sites, including hip fractures (39,40,72,110,113); they also prevent heterotopic ossification after total hip replacement surgery and improve subsequent mobility (74,146), and prevent ectopic calcification and ossification after spinal cord injuries (38,74). N-BPs are also used in adults to control several factors associated with bone metastasis (breast, prostate and lung cancer and multiple myeloma) (147,148). N-BPs have been shown to increase the quality of life of patients by maintaining skeletal function and decreasing events such as bone pain, pathological fractures, spinal cord and/or nerve root compression, cancer-related hypercalcemia, and the need for orthopedic surgery to prevent or repair major structural damage (147,148). There are specific doses and protocols for N-BP use in adult ODs, although the ideal duration for N-BP treatments is unclear and selecting specific bisphosphonates is subject to the characteristics of the drugs, the evaluation performed of their response and the knowledge of potential undesirable effects (38,45,149). The main N-BPs prescribed in adults are zoledronate, risedronate, alendronate and ibandronate (35,39,40,121).

Other osteolytic disorders treated with N-BPs are secondary osteoporosis (97,103,150) and osteoporosis associated with primary bone tumors and bone metastases (105,106,147,148,151). Specifically in oncology, the N-BPs effect on

protein prenylation also constitutes an anti-tumor activity in different forms of human cancer such as myeloma, breast cancer, prostate cancer, pancreatic cancer, mesothelioma and sarcomas including bone sarcomas (38,126,148,152,153). The final effect is related to inhibition of integrin-mediated tumor cell adhesion to bone, inhibition of cancer cell migration and invasion, inhibition of cancer cell proliferation, induction of apoptosis at high concentrations, and some immuno-modulatory effects related to accumulation of mevalonate metabolites in tumor cells (97). Table 4 summarizes the main indications of different N-BPs.

2.2.4 N-BPs Skeletal side effects. The human skeleton is composed of bone, cartilage and teeth, and can be subdivided into axial, appendicular and craniofacial (154). The appendicular skeleton is mainly made up of long bones, which are necessary for locomotion, segmental bones are elements of the axial skeleton, and flat bones are the principal elements of the craniofacial skeleton (154). The development of the skeleton is initiated during the second half of gestation, and most elements have reached their definitive size by 16-18 years (154). Bones are formed through two different ossification processes: intramembranous, in the flat bones of the skull and jaw, and endochondral in the long bones (2,154). Those processes are controlled by genetic, epigenetic and environmental factors (2). Any perturbation to these processes can alter overall growth and lead to the appearance of dysmorphology of varying degrees and affecting all bone types (2,154).

2.2.4.1 N-BPs side effects on growing axial and appendicular skeletons. Although the effects of N-BPs on long bone development during childhood have been studied little, some authors have reported the importance of recognizing that N-BPs have special tropism for the region of the growth plate, where cartilage is being replaced, and could inhibit resorption and alter the elongation process (155,156). In several clinical studies, Rauch and collaborators described different findings in pediatric and adolescent patients treated with N-BPs (155–157). In one study evaluating longitudinal iliac bone samples from young OI patients treated with

pamidronate, they observed that pamidronate treatment had a marked effect on bone tissue during the first 2–4 years of treatment, but only small changes occur afterward. Also, they reported that pamidronate treatment was associated with calcified cartilage accumulation and delayed bone healing (155). Later, in a clinical observational study evaluating 23 young patients with OI who had received cyclic intravenous pamidronate treatment in at least 3 years, they found that bone mass and density at the distal radial metaphysis was generally elevated and when the treatment was discontinued the bone tissue added had a much lower density, creating zones of localized bone weakness (158). For this reason, they suggested continuing N-BP treatment for as long as growth continued (158). However, the benefits and risks associated with prolonged N-BP administration during growth were not clear or analyzed (158). In contradiction with these results, in a randomized placebo-controlled study with risedronate evaluating 26 children and adolescents with OI type I, the same authors reported that patients who received risedronate did not have radiographically detectable changes in the density of the long bone metaphysis and no change in trabecular volumetric bone mineral density (BMD) at the distal radial metaphysis (156). Comparing both studies, the authors concluded that these radiological observations were due to the fact that risedronate administered orally was weaker than the intravenously-administered pamidronate (156). They concluded that future studies are needed to evaluate the clinical benefits of oral risedronate in mildly-affected OI type I patients (156). Muderis et coll. (143), in a retrospective review of the radiographs of 35 children treated with cyclic intravenous pamidronate, and Kumar et coll. (125) in a retrospective evaluation of the short- and long-term side effects in 29 children with OI treated with zoledronate, reported sclerotic lines (zebra lines) in the metaphysis of long bones before closure of the epiphyseal growth plate (125,143). According to these authors, a temporal imbalance in bone turnover in primary and secondary spongiosas, areas of high metabolic activity, lead to an increase in osteoblastic function and significant bone mineralization radiologically translated into a radiolucent line parallel to the growth cartilage that with bone growth will travel to the diaphyseal area (125,143).

Histological analysis of these lines showed a decreased proportion of calcified cartilage with increasing distance from the growth plate (125,143).The intervals between the bands depended on the age of the patient, the rate of growth and the dosage regimen. The authors concluded that sclerotic lines did not have negative consequences for growth, but that N-BP treatments may decrease the speed of metaphyseal inwaisting by altering periosteal resorption, causing impaired bone modeling (125,143).

Preclinical studies using different animal models have reported specific effects on growing long bones (51–53,104,159,160). Evans et coll. using a mouse model of OI type III, evaluated the impact of alendronate on osteoclastic resorption of calcified cartilage septa at the base of the growth plate in the humerus and ulna (159). They observed that high dosages of alendronate increased overall growth plate height, particularly within the hypertrophic zone, which suggests a failure of vascular invasion-induced apoptosis in hypertrophic cells (159). According to the authors, these results indicated that high doses of alendronate inhibited long bone lengthening in mice through alteration of the resorption at the chondro-osseous junction of the growth plate (159). Smith et coll., using a rabbit model, examined the short- and long-term effects of zoledronate on physeal morphology and its effect on long-term longitudinal growth and final bone length in adulthood (160). They observed that in a growing animal model, zoledronate caused transient effects on cell morphology and retention of cartilaginous matrix, coinciding with a growth disturbance, and could generate a reduction in the final length of long bone at maturity (160). Battaglia et coll. analyzed the effect of high doses of zoldedronate (adapted from protocols used for osteosarcoma and Ewing sarcoma pediatric patients) on different mouse strains (104). They reported a transient inhibitory effect of zoledronate on bone length, with bone-growth arrest during treatment owing to an impressive increase in bone formation at the growth plate level (104), and observed that endochondral bone formation started again after the end of treatment and the osteoclasts and osteoblasts were still active at the growth plate (104). They

concluded that endochondral bone growth is transiently disturbed by high doses of zoledronate as also evidenced on the radiographs of oncopediatric patients (104).

Zhu et coll. (53), using the C57BL/6J mouse strain, studied in parallel the consequences of different N-BPs over a short period of therapy on the growth plate and the skeletal microarchitecture (53). They used human dose schemes and compared the effects of alendronate (100 µg/kg twice weekly), pamidronate (3 mg/kg), zoledronate (0.07 mg/kg) and clodronate (0.5 mg/kg to approximate the 1600-mg/d)(53). They found that the effects of pamidronate on trabecular microarchitecture were less beneficial than alendronate and zoledronate, and suggested that bisphosphonate administration, in short therapy, did not adversely affect skeletal growth (53). Nevertheless, longer-term studies are required to determine whether there is a progressive growth plate phenotype observed with chronic N-BP treatment, to evaluate the consequences on bone length, and to assess the effects of the different agents on the biomechanical integrity of the skeleton (53). In 2014, using C57BL/6J newborn mice, Lezot et coll. (51) evaluated the different effects on tibia growth of two protocols corresponding to long- and short-term treatments with high doses of zoledronate, similar to the clinical protocol administered in oncopediatric patients (5 or 10 injections of zoledronate 50µg/kg) (51). They found a delay in long bone elongation at the end of treatments due to a huge bone formation observed at the growth plate that was quantified as a significant augmentation in specific bone volume (51). Nevertheless, specific bone volumes were lower in short-term versus long-term treated mice at the time corresponding to the end of the long treatment, probably due to recovery initiated during the period separating the two protocol endpoints (51). For long term-treated mice, they observed enlargement of the growth plate hypertrophic zone, characteristic of osteopetrosis, whereas the size of this zone remained unchanged in short term-treated mice (51). In a second study in 2015, these authors reported that the effects observed on long bones with the long-term treatment were gradually reversed by three months after the end of the treatment (52).

2.2.4.2 N-BPs side effects on growing craniofacial skeleton. The effects of N-BPs on craniofacial skeleton growth have been studied little compared to the effects on axial and appendicular skeletons. The different findings regarding the effects of N-BPs on craniofacial skeleton growth, including teeth, have mainly been obtained in preclinical assays.

Concerning craniofacial bones, Bradaschia-Correa et coll. (50) investigated the effects of alendronate (2.5 mg/kg of sodium alendronate) on the endochondral ossification of the mandibular condyle in newborn Wistar rats, by evaluating the distribution of osteoclasts and the presence of osteoadherin (a small proteoglycan present in bone matrix but absent in cartilage during endochondral ossification) (50). They observed that alendronate treatment in young rats altered endochondral ossification on mandibular condyles but did not alter the recruitment and fusion of osteoclastic cells (50). However, these cells remained latent and unable to remodel the calcified cartilage/primary bone trabeculae into spongy bone at the mandibular ramus. They concluded that N-BP therapy has a potential risk of disturbing maxillofacial growth in young patients (Wilkinson and Little, 2011). Lézot et coll., in the studies previously described (51,52), evaluated the impact of high doses of zoledronate on craniofacial skeleton growth. They found an enlargement of the craniofacial sutures in mice (C57BL/6 and CD1 strains) treated with zoledronate, more severe in longer treatments (51,52). They described a domed skull associated with the suture enlargement. Nevertheless, during the three months following the end of the zoledronate treatment, the suture mineralization defects and dome morphology of the skull gradually normalized (51,52).

Teeth are an important part of the craniofacial skeleton. The formation of the deciduous and permanent dentition begins before birth and is not completed when the mineralized tissues of the crown and root are formed, but continues through post-natal life until teeth erupt and reach the position of functional occlusion, at approximately 15–16 years of age in humans (161)(162). Two events are necessary

for tooth eruption. The first is the resorption of the bone overlying the crown of the tooth and second is the biological process that will result in the tooth moving through this eruption pathway (163). Osteoclast activity is crucial for dental and periodontal development and tooth eruption (163,164), consequently it was not surprising that administration of N-BPs affected both tooth development and function (49,51,165). Some preclinical and clinical studies have reported alterations in dental development related to the use of N-BPs (51,52,162,166,167)

Hiraga et coll., using Wistar rats, examined the effects of zoledronate during tooth formation and eruption (162). Two experimental groups, 7-day-old (early treatment group) and 14-day-old (late treatment group), received single, once-weekly or once-daily, subcutaneous injections of zoledronate for 3 weeks (162). They found that, in the early treatment groups, zoledronate dose-dependently inhibited the eruption of both molars and incisors and the formation of molar roots. In the late treatment groups, the eruption of first molars was not affected as these teeth had already partially erupted at the beginning of the treatment. In contrast, the posterior molars, which were still covered with alveolar bone, failed to erupt normally (162). These results suggested that zoledronate-induced inhibition of tooth eruption depended on the developmental stage of each tooth (162). Hiraga et coll. also identified unresorbed pieces of alveolar bone, ankylozed molars and odontoma-like formations around the basal end of the incisors (162). They concluded that treatment with zoledronate during tooth development has the potential to inhibit tooth eruption, impair tooth formation, and induce several types of dental abnormality (162).

Bradaschia-Correa et coll. (168) evaluated the molar root formation of newborn Wistar rats treated with alendronate (injected 2.5 mg/kg alendronate for 9, 12 and 30 days) (168). Their findings indicated that resorption of the basal portion of the bony crypt was necessary to root formation (168). The authors concluded that in young patients with bone diseases and receiving bisphosphonate therapy, there was a risk of disturbing the development, eruption and root formation of teeth (168). Lézot

et coll. also reported evidence in mouse models of the potential adverse dental effects of zoledronate treatment used in pediatric patients with bone malignancies (51). The treatment applied to new-born mice induced a seizure in incisor and first molar eruption, and a blockage of root elongation with root histogenesis severely impacted for all molars (51). In 2015, the same authors demonstrated that the tooth retention induced by the treatment was irreversible after the end of the treatment (52).

Kamoun-Goldrat et coll. evaluated the dental consequences of bisphosphonate treatments in 33 patients with OI (166). This study concluded that bisphosphonate therapy delays tooth eruption in humans and may increase the number of impacted teeth in patients already suffering from dental disorders (166).

2.2.4.3 N-BPs side effects on adult axial and appendicular skeletons. The main side effect of N-BPs on long bone is the atypical femoral fracture (AFF)(145,169–171).This event was first reported in 2005 and the association with N-BP treatments was immediately questioned (172). Today, there is a consensus concerning the fact that AFF are caused mainly by long-term therapies with N-BPs (145,169–171). The essential features of AFF are classified into major and minor (173). Major features are location (from distal to lesser trochanter to proximal to supracondylar flare), displacement with minimal trauma (or without trauma), negligible comminution, and transverse or short oblique configuration. Minor features include prodromal pain, flaring of the lateral cortex, propensity to be bilateral and delayed healing (42,145,170,173,174).

The pathophysiology of AFF is related to the conjunction of three main factors. First is an impairment of the mechanical properties of bone (42,145,170). Second are morphometric factors such as bone shape, smaller diameter of the shaft, larger femoral offset and increased curvature. These aspects could explain the higher risk in woman than in men (145,170). Third are faults in the correct repair of bone micro-

factures or micro-damage. In bone physiology, micro-fractures are associated with repeat and moderate loading that caused fatigue and the formation of small cracks. This micro-damage, mainly affecting bones in the lower extremity, is normally resorbed by osteoclasts and replaced with new bone (121,170). However, in the presence of N-BPs, due to alterations to remodeling, micro-cracks accumulate, fuse and cause stress fractures (170,175). The main risk factor for the occurrence of AFF is exposure time to N-BPs (42,145,169–171). It is effectively considered that after 4-5 years of treatment with N-BPs, bone fragility has significantly increased and therefore the risk of AFF too (145,170). Other risk factors include ethnic origin (AFFs are 4 to 5 times more frequent in Asians than in Caucasians) (171,176), age and sex (more common in older women) (171,176). Lloyd et coll. found that reduced cortical toughness with bisphosphonate therapy was one of many factors contributing to AFFs (177). They suggested a deficit in intrinsic and extrinsic toughening mechanisms, which contribute to AFFs in patients treated with long-term bisphosphonates (177).

2.2.4.4 N-BPs side effects on adult craniofacial skeleton: BRONJ. Until now, osteonecrosis of the jaw is considered the main side effect of N-BPs on the adult craniofacial (123,178–191) For this reason, initially this side effect was denominated Bisphosphonate Osteonecrosis of the Jaw (BRONJ).

However, others drugs have been involved in the occurrence of the osteonecrosis of the jaw (124,189,192), and for that reason since 2009 the American Association of Oral and Maxillofacial Surgeons (AAOMS) recommended that the term BRONJ was replaced with the new terminology ‘‘Medication Related Osteonecrosis of the Jaw’’ (MRONJ) (185). In this manuscript the main objective is to analyze the bisphosphonates related osteonecrosis of the jaw and the term BRONJ was so used.

BRONJ has received different definitions and its pathophysiology and epidemiology have been subject to wide controversy and investigation, to date without consensus

(123,183). In 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) defined BRONJ as an area of exposed or necrotic bone, or bone that can be probed through an intra-oral or extra-oral fistula, that fails to heal within 8 weeks in patients who have received or are receiving treatment with anti-resorptive drugs (in the absence of maxillary radiotherapy) (178,185). In 2015, the International Task Force on Osteonecrosis of the Jaw (a group of clinicians and researchers with extensive experience with BRONJ patients), defined BRONJ as exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider, with exposure to a BP and no history of radiation therapy in the craniofacial region (193). Notwithstanding, the vast majority of authors in their reports use the definition adopted by the AAOMS (123,178,194).

The prevalence and incidence of BRONJ is not clear and depends on several factors, such as duration of the treatment (more than 3 or 4 years), origin of the treatment (osteoporosis or cancer), route of administration (oral or intravenous) and scientific source (investigator dependence). The prevalence is greater in patients with cancer treated with intravenous N-BPs (0.52-7.4%) than in patients with osteoporosis treated with intravenous N-BPs (0-0.348%) or oral N-BPs (0.001-0.10%) (123,183,185,186,188,190,195,196) The incidence in osteoporotic patients treated by intravenous route N-BPs is also higher (0-90 cases per 100,000 patients/year) than by oral route (1.04-69 cases per 100,000 patients/year). In cancer patients, the incidence ranges from 0.8 to 12% and is considered to be 12.222 per 100,000 patients/year (123,183,189) Interestingly, multiple myeloma patients more frequently develop BRONJ than patients with solid tumors such as breast or prostate cancers(124).

- **Pathogenesis and classification of BRONJ.** The multifactorial pathophysiology of BRONJ is agreed by many authors (184,185,189,193,197–199), and several theories have been proposed for the etiology of BRONJ. The first one is related to bone remodeling inhibition by N-BPs by means of different mechanisms (123,193).

The second is associated with inflammation and infection of the affected bone often occurring after extraction of teeth with advanced dental disease or around teeth with periodontal or periapical infection. As active resorption does not occur in BP-containing bone, the infected tissue is not readily removed and can easily progress to chronic osteomyelitis. There is experimental evidence showing that infection and BP administration are necessary and serve as sufficient conditions for osteonecrosis (123,193). The third is linked to the anti-angiogenic effect of N-BPs. BRONJ is then regarded as the result of a deficiency in blood supply. This effect is considered to be a significant contributor to the pathological process (123,193,199) The fourth theory is known as soft tissue toxicity. Although N-BPs primarily act on osteoclasts, they also have direct toxicity towards soft tissues such as oral epithelial cells. N-BPs suppress the proliferation and displacement of oral keratinocytes that can increase the chances of latent bone exposure and subsequent infection (123,193,199). The fifth theory proposed is related to the potential effect of N-BPs on immunity. N-BPs control the activity of various cells involved in the immune response. In addition, the risk of osteonecrosis after tooth extraction becomes significantly higher if immunosuppressive drugs, influencing the innate/acquired immune system, are given during N-BP administration (123). The last theory is related to hair-line fractures. Throughout life, bone tissue continuously undergoes repetitive micro-fractures and healing processes. Such micro-traumas that slowly accumulate with age, due to the suppressive effect of N-BPs on osteoclasts, result in latent osteonecrosis lesions (123,193).

From another point of view, Lombard et coll. proposed two theories to explain BRONJ, named “inside-outside” and “outside-inside” BRONJ theories (123). The former is based on the inhibition of osteoclastic activity and a decrease in bone turnover, whereas the second is based on local immune-depression, associated with mucosal/dental lesions, which may lead to a local infection and/or inflammation spreading to the bone, and inducing osteonecrosis (123). They suggested three potential etiologies: lack of bone formation caused by the absence of osteogenic

differentiation from mesenchymal stem cells, an imbalance in bone remodeling caused by anti-resorptive drugs, and the disruption in homeostasis between immune and bone systems, with reference to the concept of osteoimmunology (123).

A genetic predisposition has been proposed by some authors (122,193) based on the fact that only certain patients with similar comorbidities and medical management develop BRONJ. Polymorphisms in farnesyl pyrophosphate synthase or several genes such as cytochrome P450 CYP2C8, among others, have been suggested as predisposing certain individuals to BRONJ (122,193).

The staging system currently adopted by most authors was proposed by the AAOMS, and considers four specific stages for BRONJ (184,185,189,193,197–199). At risk: Patients subjected to BP treatments by oral or intravenous routes, without symptoms or apparent bone necrosis. (187,193,200,201). Stage 0 (disease variant without bone exposure): No clinical evidence of necrotic bone, though with clinical findings, radiographic changes and nonspecific symptoms. The main symptoms include toothache, bone and maxillary sinus pain and dysesthesia, among others (187,193,200,201). Stage 1: Exposed bone or intra- or extra-oral fistulation in the maxillofacial region penetrating to the bone, in asymptomatic patients without evidence of infection. Radiographs may show the signs seen in Stage 0. Stage 2: Exposed bone or intra- or extra-oral fistulation in the maxillofacial region, penetrating to the bone, with infection evidenced by pain and erythema or exposed bone with suppuration. Radiographs may show the signs seen in Stage 0 (187,193,200,201). Stage 3: Exposed bone or intra- or extra-oral fistulation in the maxillofacial region penetrating to the bone, with pain, infection and at least one of the following signs: necrotic bone extending beyond the alveolar bone, pathological fracture, extra-oral fistula, oro-antral or oro-nasal communication, osteolysis extending to the inferior margin of the mandible or to the sinus floor (187,193,200,201).

- **Risk factors of BRONJ.** Drug-associated, demographic, systemic and local factors are related to the occurrence of BRONJ (183,185,186,202). Drug-associated factors include the type of N-BPs (nitrogenous or non-nitrogenous), the route of administration (oral or intravenous) and the potency. BRONJ is more common with N-BPs of higher potency administered intravenously (zoledronate and pamidronate) than with orally-dispensed N-BPs (alendronate). In addition, longer therapy appears to be associated with a higher risk of BRONJ (178,183,185,186,189).

Demographic and systemic factors involve age (more common in older patients), gender (more common in women than men), genetic (caucasian ethnic origin) and disease for which the medication was prescribed (more frequently observed in patients treated for cancer than for osteoporosis). Comparing different types of cancer, the risk is greater for patients with multiple myeloma than for patients with breast cancer than for other cancers. Moreover, the risk is also higher when osteopenia/osteoporosis is diagnosed concurrently with cancer (185,186,189). Other systemic factors include disease conditions such as diabetes mellitus, rheumatoid arthritis, hemodialysis, anemia, hypocalcemia, hyperparathyroidism, vitamin D deficit, among others; the concomitant use of corticosteroids, chemotherapeutics or anti-angiogenic agents, and patient habits (smoking and alcohol consumption) (183,185,186,189).

The local risk factors include dento-alveolar surgery (especially extractions and implants, periapical surgery and periodontal surgery involving osseous injury), dental and periodontal infection and local anatomy (specially lower jawbone, torus, mylohyoid ridge) (183,185,186,189). Other local factors associated with BRONJ are bone turnover level, micro-organisms of the oral cavity, and the presence of removable dentures (185,186). Although the theory of a triggering local factor is supported, the spontaneous appearance of BRONJ, with no clear predisposing event, has also been referenced by some authors in clinical reports, without so far having a specific etiology (203,204)

2.2.4.5 Skeleton side effects of N-BPs, prevention and management

- **Recommendations for side effect prevention and management in children.** Studies demonstrated that N-BP treatments in children induced a transient arrest in long bone growth with a grade rescue of bone parameters after the end of the treatment. So there are no specific recommendations. Meanwhile, it will be necessary to evaluate the consequences of these pediatric treatments on the adulthood of the patients (125,143). Concerning the side effects of N-BPs on growing craniofacial skeleton, it seems that the only irreversible effects after the end of the N-BP treatments are tooth inclusion and ankylosis secondary to defective eruption (42,51,52,162,166). Controlling the appearance of these side effects through radiographic and clinical follow-up during the treatment seems to be the best strategy, along with interruption of the treatment.

- **Recommendations for side effect prevention and management in adultsAxial skeleton.** To avoid AFFs, treatment 'holidays' after 5 years of oral N-BPs or 3 years of parenteral N-BPs is an efficient treatment strategy (39,121) independently of the N-BPs administered (42,145,169–171).

  - **Craniofacial skeleton.** Although no protocol has been established, several recommendations have been proposed to manage BRONJ or to prevent the risk of its apparition. Adequate management with specialized dentistry in the care of osteolytic disorders, use of biomarkers as risk predictors, and use of local and systemic pharmacological and non-pharmacological alternatives have been reported and are presented in the following paragraphs.

    - **Management of BRONJ.** In cases of patients with BRONJ the most important consideration is to control the symptoms and associated infection. Treatment strategies range from conservative nonsurgical therapy to early surgical intervention depending on the stage of the disease. There is no treatment indicated in patients

at risk, but correct education on the risk of BRONJ is necessary, and adequate oral hygiene protocols are required (187,193,200,201). For stage 0 patients, systemic management is recommended, including control of pain and administration of antibiotics, with control of localized causes such as dental decay or gingivitis (178,187,193,200,201). For stage 1 patients, the proposed treatment includes the use of an antibacterial oral rinse that may help to control oral micro-organisms. Immediate surgery is not needed and a re-evaluation of the necessity of the bisphosphonate treatment is indispensable (178,187,193,200,201) Pharmacological management in stage 2 includes control of the pain, oral antibiotics, an oral antibacterial mouth rinse protocol and debridement to relieve soft tissue irritation (187,193,200,201). Finally, in stage 3 patients, control of the pain and infection with systemic antibiotics is required, as is the use of antibacterial oral rinses and surgical debridement or resection to control long-term risks (187,193,200,201). There is no data to clarify the most appropriate duration of the antibiotic therapy for BRONJ, but two weeks for patients with persistent stage 1 disease and a course of up to 4-6-weeks for more severe stages are indicated (178,201,205). Table summarizes the main antibiotics used to treat BRONJ.

- **Prevention of the risk of BRONJ.** To prevent the development of BRONJ, dental precautions by the dental professional, the use of biomarkers as risk predictors and the use of local and systemic pharmacological or non-pharmacological alternatives have been proposed. A brief description of these strategies appears below.

- **Dental precautions.** The treating physician must refer the patient to his/her dentist for an evaluation, treatment, and monitoring program focused on attaining and maintaining oral health (179,187,206). A radiographic and clinical assessment of the dentition and periodontal tissues must be performed to identify any existing pathology, and to eliminate acute infections and areas of potential infection before beginning the N-BP therapy to avoid later complications (179,187,206). To maintain oral health, patients at higher risk for BRONJ should have short follow-up intervals

scheduled for prophylaxis every 3 months (179,187,206). If patients on anti-resorptive therapies need an invasive intra-oral procedure, the treating dentist must refer the patient to dental care specialized in the pharmacological and non-pharmacological alternatives for preventing and treating complications related to N-BP management. Before treatment, patients on N-BP therapy must enter a protocol with a drug holiday (3 months before and after the intervention) to reduce the risk of BRONJ (179,187). Non-traumatic surgery should be performed with primary closure and antibiotic coverage until good wound healing is observed to avoid chronic infection (179,187,193). Smoking, as well as intra-oral prosthetics near the surgical site, should be avoided (179,187).

Table 5. Antibiotic management of bisphosphonate related osteonecrosis of the jaw (BRONJ).

TABLE 5: ANTIBIOTIC MANAGEMENT OF BISPHOSPHONATE RELATED OSTEONECROSIS OF THE JAW (BRONJ)			
TYPE OF DRUG	**DRUG**	**USE**	**COMMENTS**
TOPICAL ANTIMICROBIALS	Chlorhexidine gluconate 0.12%	Stage I, and for Stage II or III in addition to other therapies	Decrease total bacterial counts, including potentially pathologic organisms.
	topical minocycline 10% in orabase		Causes a significant stimulation of osteoblasts, increasing bone matrix
ORAL ANTIMICROBIALS	Amoxicillin Amoxicillin + Clavulanate Ampicillin + Sulbactam Clindamycin Fluoroquinolones (levofloxacin-ciprofloxacin) Metronidazole Doxycycline	Stage I: Two weeks Stage II-III: Four - Six weeks.	Broad-spectrum antibiotics are required. Selection of specific antibiotics should be based on patient tolerance, compliance, prior antibiotic exposure and the targeted against common colonizers of BRONJ lesions.
INTRAVENOUS ANTIMICROBIALS	Ampicillin + sulbactam Metronidazole Clindamycin Fluoroquinolones		Intravenous antibiotics should be chosen in patients with pathogenic organisms resistant to oral agents and may provide greater tissue penetration in severe cases.

▪ **Biomarkers as risk predictors.** The use of biomarkers, although an important tool for predicting the risk of BRONJ, is still a topic under construction and leaves areas to explore in future research. Since Marx et coll. proposed the use of the C-terminal telopeptide of type I collagen (CTX) as a biomarker for evaluating the risk of BRONJ (179), different research groups have attempted to detect specific biomarkers in serum, urine, and saliva that identify bone degradation or formation (189,194,207–212).

Biomarkers related to bone degradation are associated with type I collagen. They have two pyridinium derivative cross-link molecules, pyridinoline and deoxypyridinoline, and are detected in blood or excreted in a non-metabolized form in urine. They are specific markers for bone resorption (213,214). CTX, the terminal carboxyl fragment of type I collagen, which cross-links with pyridinoline, is released during bone degradation by osteoclasts as first reported by Marx et coll. in 2007. For some authors CTX is considered a major biomarker for the risk of BRONJ (179). In normal conditions, serum CTX levels must be more than 150pg/ml, but they tend to decrease with the suppression of bone turnover by drugs or other factors. Levels of 100 to 150 pg/ml indicate a moderate risk of BRONJ, while levels of less than 100 pg/ml a high risk (179). Although some studies have validated their applicability (208,215,216), different clinical and preclinical assays and systematic reviews have questioned the use of this biomarker and concluded that its serum level is not a definitive risk predictor for BRONJ (185,189,193,207,211,212,217). NTX, the terminal amino fragment of type I collagen which cross-links with pyridinoline, is a more sensitive marker than CTX and is excreted in the urine following degradation of the bone matrix (210).

• Parathyroid hormone (PTH) indirectly promotes bone resorption by osteoclasts and is considered to be an unspecific marker for bone resorption (208,210). Other biomarkers include proteins involved in bone formation regulation such as under-carboxylated osteocalcin (Glu-OC) (193,207,216), bone-specific alkaline

phosphatase (BAP) (193,210,216), salivary proteins such as metalloproteinase-9 – a proteolytic enzyme involved in wound healing mainly expressed by osteoclasts – and desmoplakin – a component of desmosome structures in different cells (209). Nevertheless, none of them has been related specifically to BRONJ and their role in this pathology requires further research (209).

Serum RANKL/OPG levels have been proposed by some authors as a risk predictor for BRONJ due to its significant role in bone turnover (194). The RANK/RANKL/OPG (receptor activator of nuclear factor-κβ/receptor activator of nuclear factor-κβ ligand/osteoprotegerin) system, is the most important regulator in osteoclast proliferation, differentiation, activation and apoptosis (22,27). Its relationship with osteolytic pathologies has been demonstrated with the presence of an over-expression of RANK or an alteration of the RANKL / OPG ratio in almost all cases of osteolysis of hereditary origin (24,27,97,103,218). However, based on the fact that expression of elements from the RANKL/RANK/OPG system have been implicated in key regulatory functions in other tissues (15,27,57), the RANKL/OPG ratio cannot be considered a specific biomarker for bone and therefore this ratio does not reflect a specific risk for BRONJ (194).

Genotype profiling has been considered a tool for identifying individuals with a risk of developing BRONJ (122,193). A genetic predisposition has been proposed (122,193) based on the identification of patients with similar comorbidities in BRONJ (122,193). Polymorphisms in the farnesyl pyrophosphate synthase and/or several genes such as cytochrome P450 CYP2C8 have been suggested as predisposing for BRONJ (122,193). However, there is little evidence for a pharmacogenetic susceptibility to the development of BRONJ (122,193).

In brief, none of these biomarkers reflect the overall decrease in bone remodeling activity caused by N-BPs and they cannot reflect the suppression of osteoclastic activity induced by these drugs. Regardless, researchers in the field still consider

that this predictive strategy will be significant in the near future for BRONJ treatment (185,193,207,210).

- **BRONJ treatment: Pharmacological and non-pharmacological alternatives**

  - **Local treatment:**

- **Antibacterial mouth rinse:** Antibacterial mouth rinse has been reported as controlling bacterial activity related to BRONJ (179,187,202,205,219). The most common oral rinse is chlorhexidine (CHX) (179,187,202,205,219). The antimicrobial CHX is a synthetic cationic bisguanide (220–222) consisting of two symetric 4-cholorophenyl rings and two bisguanide groups connected by a central hexamethylene chain (220,222,223). Marx et coll. have proposed the use of CHX to control BRONJ, based on its ability to decrease total bacterial counts (179), and consider that the presence of N-BPs both promotes bacterial adhesion and biofilm formation on the surface of the N-BP-treated bone, and increases bacterial colonization and biofilm formation in such areas (200,224). As a result, CHX has been recommended by many authors to control and treat BRONJ in stages 1, 2 and 3 (179,187,202,205,219). All protocols include 0.12% CHX gluconate every 8–12 hours for 15 days, or until the surgical site has healed (179,185,187,193,200,202,205,219). In spite of its wide use, a recent Cochrane meta-analysis concluded that CHX rinse is similar in its application for preventing BRONJ to other mouth-washes, such as saline rinses and water rinses (225). Alternatives such as Benzydamine and other oral antiseptic mouth-washes need to be explored in future research.

- **Local antibiotics:** To avoid the toxic effects of the systemic antibiotics used to treat BRONJ, some protocols have proposed the topical use of tetracyclines (226).Tetracyclines (doxycycline and minocycline among others) are a bacteriostatic group of broad spectrum antibiotics which inhibit protein synthesis by binding to 30s

bacterial ribosomes and preventing the access of aminoacyl tRNA to the receptor site on the mRNA-ribosome complex (94,227). These drugs were described more than fifty years ago and since then have been used in the treatment of skin and soft tissue infections caused by gram-positive cocci, spirochetal infections, infections with Nocardia, Actinomyces and others (94,228). Additionally, numerous studies have reported non-antibiotic properties for tetracyclines, including anti-inflammatory and anti-apoptotic activities, inhibitory effects on proteolysis, and angiogenesis and tumor metastasis, among others (228). Minocycline (7-dimethylamino-6-dimethyl-6-deoxytetracycline) is a second-generation semi-synthetic tetracycline analogue (227,228). It affects the micro-organisms associated with periodontitis and is the most effective tetracycline derivative with other biological properties (227,228). Considering its antibiotic and non-antibiotic properties, Karasneh et coll. have proposed its topical use in the management of BRONJ (226). In a clinical assay, five patients diagnosed with stage 2 or 3 BRONJ lesions were treated with minocycline (10% in orabase), applied to the lesions once a week for sustained local antibiotic delivery, in addition to conventional treatment such as surgical debridement, chlorhexidine irrigation, and systemic antibiotics (226). The authors reported controlling pain, infection and less dependence on home care using topical minocycline (226). They concluded that its use as a treatment for BRONJ requires large and well-controlled prospective clinical studies (226). Although the authors did not explore the non-antibiotic properties of minocycline, evaluating the mechanisms involved in the anti-inflammatory, immunomodulatory and neuroprotective effects of minocycline needs to be considered. The possible effects include six aspects: inhibitory effects on the activities of key enzymes such as iNOS (Inducible nitric oxide synthase), reduction of protein tyrosine nitration due to its peroxynitrite-scavenging properties, inhibition of caspase-1 and caspase-3 activation, enhancement of Bcl-2-derived effects (protecting cells against apoptosis), reduction of p38 MAPK phosphorylation, and inhibition of PARP-1 activity (228).

- **Bone Morphogenetic Proteins (BMPs):** Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor-ß (TGF-ß) superfamily (5,123,229,230) and are considered the most important growth factors implied in bone and cartilage formation during development, growth and healing (229)(5,123,229,230). BMPs act like chemotactic agents, they stimulate angiogenesis and the migration, proliferation and differentiation of stem cells from the surrounding mesenchymal tissues to bone-forming cells in an area of injury (5,123,231). The US Food and Drug Administration (FDA) recognized their effect as bone formation inductors and approved two growth factors: BMP-2 and BMP-7 for the treatment of spinal fusion and long bone fractures with collagen carriers (123,230). Preclinical and clinical studies have shown that BMP-2 can be used in therapeutic interventions such as bone defects, non-union fractures, spinal fusion, osteoporosis and root canal surgery (229,230). In dentistry, BMPs have been used in orthopedics and oral/maxillofacial surgery, including BRONJ (123,185,231,232). Cicciu et coll. analyzed the clinical effect of recombinant human bone morphogenetic protein type 2 (rhBMP-2) in 20 patients with BRONJ (231). In all cases, rhBMP-2 was used alone with the collagen carrier (231). A total dose of 4 to 8 mg of rhBMP-2 was delivered to the surgical site at concentrations of 1.5 mg/mL (231). The patients were followed over a period ranging from 6 to 12 months and for all of them, successful healing of the necrotic area was observed and new bone formation in the surgical area could be clinically evaluated by palpation after 3 to 4 months (231). The authors concluded that using rhBMP-2 without concomitant bone grafting materials was useful in promoting the healing of BRONJ (231). However, adequate randomized clinical trials are needed to ascertain the safety and efficacy of BMPs in the management of BRONJ (123,231).

- **Platelet-derived growth factor-BB (PDGF-BB):** Platelet-derived growth factor (PDGF) is a potent mitogen for diploid fibroblasts, arterial smooth muscle cells and brain glial cells (233,234). It also has major effects on bone cells, especially on osteoblasts (233,234). PDGF exists in three isoforms PDGF-AA, -BB and –AB (234).

PDGF-BB is involved in angiogenesis and osteoformation (123) and is considered to be an important factor in bone remodeling (123,235). PDGF-BB is considered to be a new alternative for the treatment of bone resorptive pathologies (123). In experimental models, treatment with PDGF-BB has been shown to increase vasculogenesis and bone formation (123). Few reports have proposed the use of PDGF in the management of BRONJ (123) To date, few reports have related its use (236).

- **Autologous platelet concentrates (APC):** APC is a source of platelet-derived factors obtained by sequestering and concentrating platelets by gradient density centrifugation (237,238). APC contain many factors that promote healing, such as adhesive proteins, pro-coagulant factors, cytokines and chemokines, anti-microbial proteins and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-b), epidermal growth factors (EGF) and vascular endothelial growth factors (VEGF) (237,238). Its use in dental surgery was proposed by Marx et coll. in 1998 (239) and associated with activation of the proliferation, migration and differentiation of cells in the wound area, especially when the number of cells in the tissue injury site is reduced, increasing the regenerative process (238). Several systematic reviews have identified the use of APC as an adjunct therapy in the prevention and treatment of BRONJ in patients undergoing oral surgery, with a lower incidence on the pathology (205,237,238). However there is still little evidence regarding the benefits in term of BRONJ risk prevention (205,237,238).

- **Low level laser therapy (LLLT):** LLLT is an non-surgical adjunctive therapy in the management of the BRONJ (205,225,232,240–242). It has been reported in combination with surgery and other alternatives (205,225,232,240–242). LLLT can modulate cell metabolism, has a bio-stimulating effect, improves wound healing and relieves pain (240,241). LLLT can also increase the mitotic index of osteoblasts, stimulate their proliferation and differentiation, and enhance their activity and bone

formation (241), have an antimicrobial effect with the proliferation of macrophages, lymphocytes, fibroblasts, endothelial cells and keratinocytes (240–242). When applied to oral tissues, LLLT enhances epithelium formation after periodontal surgery, minimizes edema after third molar surgery, and prevents oral mucositis (240–242). The most common LLLT used in bone is argon, carbon dioxide, helium/neon (He:Ne) and neodymium-doped yttrium-aluminum-garnet (Nd: YAG) (241,242). Several clinical studies have reported the efficacy of LLLT for preventing or treating BRONJ (240,243,244). There is no specific protocol regarding LLLT use as supportive therapy for BRONJ, but applications during and in the 2 days following the operation (240), or cycles once a week for 2 months (243) have been reported.

- **Photo inactivation therapy:** Antimicrobial photodynamic therapy (aPDT) is a method developed to treat bacteria-associated oral diseases, such as periodontitis, peri-implantitis and other local infections of the mouth (245). Its use for BRONJ was reported by Hafner et coll. in a preclinical assay and supported by their clinical experience (245). The aPDT system (HELBO: Bredent medical GmbH & Co KG is CE-certified in Europe for use in maxillofacial surgery (245). To date there is no clinical study related to its application in BRONJ management.

- **Mesenchymal stem cell (MSCs) :** MSCs are multipotent stem cells used in regenerative medicine (123). Concerning BRONJ, MSCs have been used based on their capacity to differentiate into osteoblasts, to stimulate bone formation, to activate bone remodeling, and to induce immunomodulation and consequently decrease inflammation (123)

- **Systemic treatment**

- **Oral and intravenous antibiotics:** Given the polymicrobial characteristics of BRONJ (183,193,199), antimicrobial drugs are essential for its prevention and management (180,185,201). Systemic antimicrobial drugs control normal and

pathogenic oral micro-organisms associated with BRONJ including Actinobacteria, Firmicutes, Fusobacteria, and Bacteroides (201). Several aspects need to be taken into account to select optimums and specific antibiotics, such as systemic patient factors (hepatic and renal function, tolerance, compliance, prior antibiotic exposure and other drugs used) (201,205). It is also important to recognize the characteristics of the most common colonizers of BRONJ lesions (Gram positive or Gram negative and aerobic and anaerobic micro-organisms, as well as the presence of bacteria with resistance to antimicrobial drugs) (201). The vast majority of protocols propose amoxicillin alone or combined with clavulanic acid as the first alternatives (201,205). Other antibiotics, such as clindamycin, metronidazole, fluoroquinolones (moxifloxacin, sitafloxacin), among others, are selected according to the characteristics and background of the patient (201,205,246). Table 5 summarizes the main antimicrobial alternatives indicated to prevent BRONJ.

- **Human parathyroid hormone (PTH):** PTH (*Teriparatide)* is an endocrine regulator for calcium and phosphorus metabolism (15). Low doses of PTH stimulate bone formation through osteoblast activation by Wnt signaling (increase in β-catenin levels and LRP6 signaling in osteoblast, and decrease in sclerostin production) (15,200). Using teriparatide to treat BRONJ is based on its ability to stimulate bone formation and angiogenesis (189). It is indicated in the treatment of BRONJ in patients who do not respond to conservative treatment (189,200,225,232,247). The protocol is a daily dose of 20 µg of subcutaneous teriparatide for 6 months (189,225). Kim et coll. in a retrospective longitudinal study examined twenty-four cases of intractable BRONJ and analyzed whether or teriparatide administration was beneficial for healing BRONJ lesions when compared to conservative management (248). They also studied the factors influencing the response to teriparatide (248). They concluded that teriparatide has beneficial effects on healing BRONJ and identified the importance of achieving optimal serum vitamin D concentrations to maximize its therapeutic outcomes. However, a control trial to evaluate the therapeutic efficacy of teriparatide in the resolution of BRONJ is necessary (248).

Interestingly, several successful case reports have been published using teriparatide to control BRONJ in different stages (242,249,250). However, teriparatide therapies must be limited to a duration of 24 months' cumulative treatment, given that preclinical studies have reported the development of osteosarcoma in treated rats. However, no such cases of osteosarcoma have been associated with teriparatide treatment in humans (112,123,247,249,251). Additionally, this anabolic treatment is not a viable option for most cancer patients due to the cell proliferation induced in the bone marrow by teriparatide (123,247,249,251).

- **Hyperbaric oxygen (HBO):** HBO has been used to manage osteoradionecrosis of the jaw for many years (200,201,242). This therapy is based on the fact that HBO provides greater oxygen to tissues with impaired vascularization, reverses impaired leukocyte function, and also supplies reactive oxygen and nitrogen (123,200,242). Concerning the use of HBO as an adjunct therapy for BRONJ, several reviews and clinical studies have reported its applicability (123,180,190,193,200,201,225,232,242,252,253). Commonly, HBO is recommended as a surgical adjunct therapy and its effects on tissue are associated with improved wound healing and bone turnover, and less pain (123,190,201,242,252). Although HBO is widely reported in the literature, its clinical utility for BRONJ is not clear. The efficacy of this costly and time-intensive treatment still needs to be evaluated (200,201,253).

- **Xanthine derivative (Pentoxifylline):** Pentoxifylline, a xanthine derivative, is used primarily for the treatment of intermittent claudication and other symptoms of peripheral vascular disease (201,254). The pharmacological activity of pentoxifylline includes increased intracellular cAMP, as a competitive nonselective phosphodiesterase inhibitor. It activates PKA, inhibits TNF and leukotriene synthesis, reduces inflammation and innate immunity, improves red blood cell deformability, reduces blood viscosity, and decreases the potential for platelet aggregation and thrombus formation. It is also an antagonist at adenosine 2

receptors and it can increase collagenase activity in vitro (202,254). Combined with tocopherol, pentoxifylline has been shown to have a synergistic effect on the regression of small areas of BRONJ (202). Although there is no clear evidence for BRONJ prevention, pentoxifylline is used during eight weeks, starting one week before the procedure (254) at the dose of 400 mg twice daily with vitamin E 1000 IU (201,254).

- **Tocopherol (Vitamin E):** Tocopherols are a class of organic chemical compounds consisting of various methylated phenols, many of which have vitamin E activity (254). They have been considered a natural, potent and lipid-soluble chain-breaking antioxidant (254,255). The role of vitamin E includes maintaining the structural integrity of human cells by influencing cell signaling, modulating the expression of connective tissue growth factor, regulating gene expression and transcribing and facilitating the protection of wounds against infections (256). Their use in BRONJ has been reported in several protocols as an adjunct therapy with pentoxifylline (200,201,225,254). They are recommended both in the management of the different stages of BRONJ, and preoperatively in those who require surgical treatment for BRONJ (201). The recommended dose is 1000 IU daily (201,254). There is no consensus concerning the duration of the treatment, but it is normally determined by the patient's response, treating physician, and medical team (200,254). To date, there is little evidence regarding the efficacy of vitamin E and pentoxifylline in BRONJ.

- **Medical ozone therapy (MOT):** MOT has been use as antimicrobial, wound healing, vasculogenic, and immunostimulating therapy (123). It acts by preserving the endogenous antioxidant system and blocking the xanthine/xanthine oxidase system (123). MOT has been considered as a complement to conventional therapy and is used as an adjunct therapy in BRONJ treatment (123,225). There is little evidence to support its use in the management of BRONJ (123,186).

Table 6. Complementary treatments and preventions of stages I bisphosphonate related ostenecrosis of the jaw, association with conservative and surgical management.

TABLE 6: COMPLEMENTARY TREATMENTS AND PREVENTIONS OF STAGES I BISPHOSPHONATE RELATED OSTENECROSIS OF THE JAW. ASSOCIATION WITH CONSERVATIVE AND SURGICAL MANAGEMENT	
HUMAN PARATHYROID HORMONE: Teriparatide	Stimulate effectively osteoblast function and proliferation, increase osseous cell signaling (including Wnt), and activate osteoclasts.
XANTHINE DERIVATIVE: Pentoxifylline	Decrease inflammation and reduce blood viscosity by increasing erythrocyte deformability
VITAMIN E	Decreases tissue inflammation and fibrosis, and is a scavenger of free radicals capable of cellular injury.
BONE MORPHOGENETIC PROTEINS (BMPS): Transforming growth factor-ß (TGF-ß) family	Potential treatment to the successful healing of the necrotic area and new bone formation.
PLATELET CONCENTRATES	Autologous platelet concentrate. Regulating the inflammation and stimulation of chemotactic agents, as a topical agent during bone resection.
PLATELET-DERIVED GROWTH FACTOR-BB (PDGF-BB)	Autologous platelet concentrate. Regulating the inflammation and stimulation of chemotactic agents, increase vasculogenesis and bone formation in the experimental animal model.
LOW-LEVEL LASER THERAPY (LLLT) WITH ND	YAG laser or GaAlAs diode laser has Improve vascularization of mucous membrane, bone regeneration, and pain reduction.
HYPERBARIC OXYGEN HBO	Improve healing by increasing oxygen concentration, immunologic regulation, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) production.
MEDICAL OZONE THERAPY (MOT)	Acts as antimicrobial, wound healing, vasculogenic, and immunostimulating therapy
MESENCHYMAL STEM CELL (MSCs)	Preclinical and clinical assays have analyzed their use in MRONJ due their ability to differentiate into osteocytes and due their immunomodulatory properties, must be considered as grafting material in osteonecrosis foci

2.2.5 Future directions for controlling the side effects of N-BPs.

2.2.5.1 Strategies to directly control side effects: Despite their side effects, N-BPs show an elevated benefit/risk ratio concerning the treatment of most ODs. The first set of strategies has thus consisted in specifically targeting the causes of side effects in patients without modifying therapeutic protocols (doses and timing). Such strategies suppose that side effects are linked to at least one effect of N-BPs other than the inhibition of the osteoclast function and the induction of its apoptosis. More precisely, these strategies presume that cell types other than osteoclasts are affected by N-BPs and responsible for the side effects. Several publications have reported that N-BPs can modulate the differentiation/function of immune cells with consequences on the inflammatory response. Macrophages (257–261) and T-lymphocytes (262–265) appear to be the most commonly-affected immune cells but an impact on dendritic cells (266) and neutrophil granulocytes (Hagelauer et al., 2015) has also been reported. The fact that patients treated with a combination of N-BPs and immune modulator drugs such as Adalimumab (268), Sunitinib (269) and aromatase inhibitors (270,271) are evidenced an enhanced frequency of BRONJ compared to patients treated simply enforces the idea that immune modulation by N-BPs may be implicated in the occurrence of side effects. For this reason, using drugs or techniques to stimulate the immune response appears to be a promising strategy for limiting N-BP side effects when compatible with the pathology being treated.

Other publications have established that N-BPs also have an anti-angiogenic effect by inhibiting the proliferation, differentiation, function and induction of apoptosis in endothelial cells (272–278). Vascularization is an important element of bone growth in either endochondral or membranous ossifications, so the anti-angiogenic effect of N-BPs may be implicated in the occurrence of side effects. Moreover, defective vascularization is a key element of osteonecrosis. Balancing the anti-angiogenic effect of N-BPs is therefore a promising strategy for reducing the side effects of N-

BPs. Interestingly, activated protein C (APC) and sphingosine-1-phosphate (S1P) have been shown to inhibit N-BP-induced endothelial cell death (275,279) and are thus promising candidates for limiting the side effects of N-BPs.

Another cell type affected by N-BPs is the osteoblast (132,280–287). N-BPs induce a decrease in the osteoblastic expression of pro-angiogenic factors (132), the pro-osteoclastic factor RANKL (281,284,285), pre-osteoblast maintaining factors BMP-2 and MSX1 (281,284), the adhesion/migration factors integrin aVb3 and tenascin C (282) and the matrix factor collagen type I (286). The decrease in expression of all these factors can favor the occurrence of the side effects of N-BPs. Interestingly, the cytoprotectant dexrazoxane was shown to protect osteoblasts from N-BPs in vitro and in vivo (280) and could have a place in the arsenal for limiting N-BP side effects.

Bone marrow mesenchymal stem cells (BMMSCs) that can differentiate into osteoblasts and chondroblasts are also affected by N-BPs (288–290). N-BPs seem to induce a blockage in BMMSC differentiation (288,289), which is suggested as inducing BRONJ, as injections of naive (regarding N-BPs) autologous BMMSCs in patients with BRONJ makes it possible to cure them (289,290). In this context, protecting BMMSCs from N-BPs may be a fruitful approach for suppressing N-BP side effects.

Fibroblasts (287) and keratinocytes (291) have also been reported as being affected (proliferation inhibition) by N-BPs. The conclusion that can be drawn from all these publications on various cell types is that N-BPs affect the cell proliferation process. The mechanism of this inhibition is supposed to be based on alteration of the mevalonate pathway (MVP) by N-BPs. Interestingly, treating different cell types (endothelial cells, fibroblasts and osteogenic cells) with geranylgeraniol, the metabolite of MVP and whose expression is significantly decreased by N-BPs, rescues the BRONJ induced by N-BPs and is a highly promising strategy for eradicating these N-BP side effects.

2.2.5.2 Strategy based on the reduction of remnant BPs in the organism after the end of the treatment. N-BPs are characterized by very significant stability on the bone matrix (hydroxyapatite), which can be a disadvantage in certain situations. For instance, bone and dental surgeries are not recommended in patients who have been treated with N-BPs and rescuing growth in children after the end of their treatment may take a long time, with the risk of side effects. A strategy for reducing remnant N-BPs after the end of the treatment was evaluated in rats using systemic and local chelation (292). A reduction of 20% was observed, which might be of considerable benefit for patients when it comes to avoiding N-BP side effects.

2.2.5.3 Strategies based on reducing N-BP doses, frequency and treatment duration. Another set of strategies developed to minimize the risk of N-BP side effects is to reduce the N-BP treatment dose, frequency and/or duration, as observed for zoledronate in dogs (293). However, such strategies suppose compensation with another drug in order to either maintain the same level of bone resorption inhibition or to normalize the balance between bone apposition and resorption.

The first approach was to use another type of bone resorption inhibitor in combination with N-BPs, taking into account that this inhibitor might induce similar side effects, as reported for the RANKL blocking antibody (Denosumab) (294). However, synergic effects have been reported, for instance between zoledronate and a RANKL blocking antibody (52), with the opportunity to significantly reduce the dose used and increase the interval between two injections. Interestingly, several studies have reported that non-nitrogenous N-BPs can be used in combination with N-BPs with a significant reduction in side effects in adults (260,295,296). The mechanism of action has not yet been elucidated but may implicate the fact that non N-BPs do not induce similar side effects than N-BPs (297) and/or do not inhibit the same phosphate transporters as N-BPs (129). The BP/N-BP co-treatment strategy merits further investigation.

The second approach was to combine N-BP treatments with drugs that stimulate bone anabolism. This approach was underestimated up to now, despite the fact that studies with teriparatide have reported promising effects on BRONJ (242,251).

2.2.5.4 Strategies based on local delivery of BPs. The last set of strategies developed to avoid the side effects is based on the observation that in most cases these effects concern areas initially not affected by ODs, for instance the mandible of patients with prostate tumors, but receiving active N-BPs according to the systemic route of administration chosen. Limiting the use of systemic diffusion of active N-BPs with local administration is the leitmotiv of these strategies. Implants covered with BPs (298) and sponges (299,300) or matrices (301,302) full of N-BPs have been shown to be an efficient strategy for minimizing side effects. An alternative strategy was using carriers for local delivery of active N-BPs (298,303,304). This last strategy is more promising than when linked to the carrier as the N-BPs can be inactivated and are consequently incapable of inducing side effects even if injected by the systemic route. Unfortunately, such strategies for local administration or targeting of N-BP action cannot be used to treat systemic ODs, which represent a large proportion of these diseases. Finally, this suggests that different strategies might be needed to avoid N-BP side effects, depending on the OD for which the BPs were prescribe*d.*

2.2.5.5 Strategies based on combining N-BPs with non N-BPs. Some reports have discussed the antagonistic effect of non N-BPs such as crodronate and etidronate on N-BPs (129,260,305). Based on the fact that N-BPs may enter soft tissue cells by means of specific phosphates transporters such as SLC20 and/or SLC34 (129), and those transporters could be blocked by non N-BPs such as etidronate or clodronate, some authors have suggested combining these non N-BPs with N-BPs in patients who are thought to be at risk for BRONJ, in order to limit or

prevent inflammatory and necrotic effects (129). However, as the authors concluded, more evidence is necessary in future experimental and clinical studies.

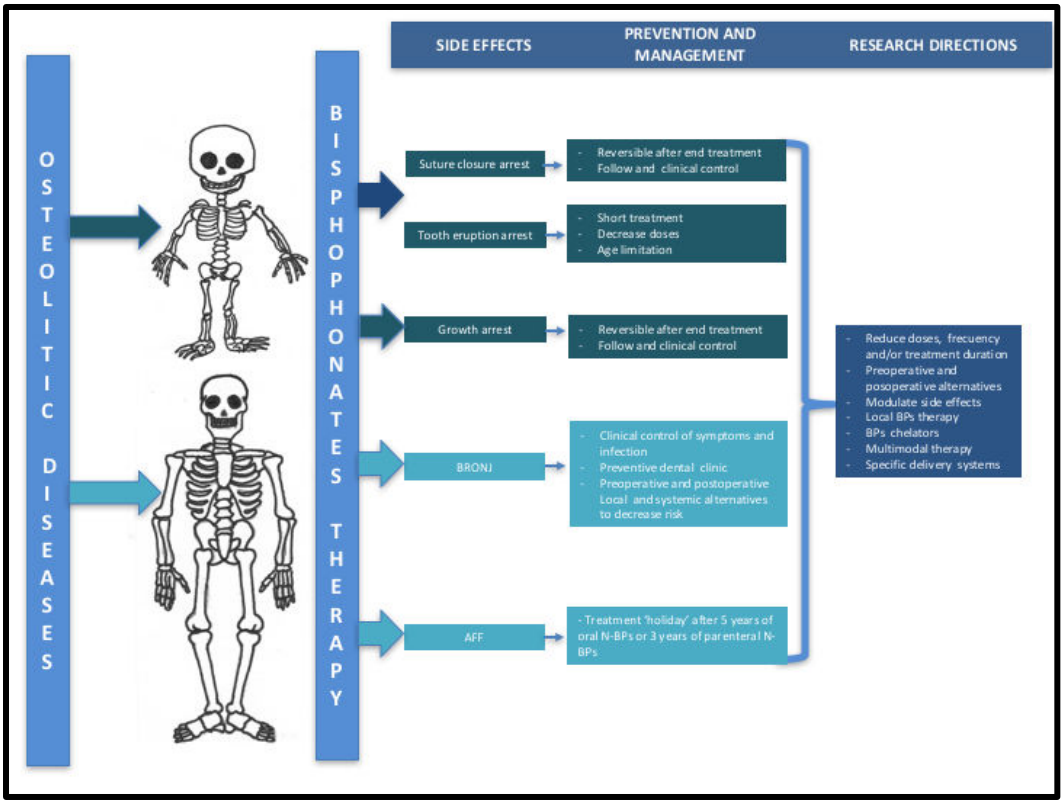

Figure 9. Summary NBPs side effects, control, prevention and future directions.

2.2.5.6 Focus on BRONJ research limits. Up to now, studies on BRONJ have mainly focused on determining how agents linked to BRONJ affect cell characteristics. Ex vivo studies using oral cavity cells (oral epithelial cells and fibroblasts) and bone cells (osteoclast and osteoblasts) have revealed that N-BPs reduce cell proliferation, induce apoptosis, and slow down cell migration (306–308). In vivo studies have been carried out with animals to identify the pathophysiology of BRONJ and the role of N-BPs in its development. Models of BRONJ were first developed in rodents (mice, Rice rats, Wistar rats and Sprague-Dawley rats) with short- and long-term studies involving either oral or intravenous N-BPs (306,309–311). However, several limitations were encountered with these animal studies, the main one being the difficulty of simulating all the conditions implicated in human

BRONJ, including genetic and environmental factors, co-existing systemic diseases, concomitant medications along with the precise dosage for each animal model (306,309). In fact, animal models developed to study the pathogenesis of BRONJ had variable success in simulating the conditions of the clinical lesions reported in humans (309). Rodent models have numerous advantages, such as the ability for genetic manipulation and the possibility for controlling all the variables. However, bone remodeling suppression is an important component of BRONJ and the human mandible has high rates of intra-cortical remodeling, contrasting with the lack of remodeling within cortical bone (osteonal or intra-cortical remodeling) observed in rodents in the absence of interventions (306,312). This is the reason why several studies have been carried out using large animal models (dogs and pigs) with a cortical bone physiology more similar to that of humans (306,313,314). The main challenge facing research in the field of BRONJ is establishing a preclinical model as close as possible to the clinical situation. Despite the accumulation of data with time on the pathogenesis of BRONJ, the “perfect” model is still missing and is a block for progress in both BRONJ management and prevention.

3. SCIENTIFIC RATIONALE

3.1 PROBLEM STATEMENT

Zoledronic acid (ZOL), one of the most potent amino-bisphosphonate (N-BPs), is currently used in clinical practice to control ODs in children and adults, regardless of their genetic, endocrine, inflammatory or oncogenic origins (for review (315)). Despite its proven efficacy in the treatment of these systemic or local diseases, several side-effects affecting axial, appendicular and craniofacial skeletons have been reported in either preclinical or clinical studies (49,51,157,160,162,165,316). During growth, the most critical side effect recognized in axial and appendicular skeletons has been the long bone arrest (28–33,317) Concerning the craniofacial system during growth, studies have mainly focused on side effects on the dental and periodontal tissues and the alveolar bone (28–33,317). Dental eruption failure, periodontal disease, alveolar bone loss, hyperminiralization in enamel and dentin, root resorption and root structure alterations were any of the craniofacial consequences reported (28–33,317). Some of these side-effects were presented as reversible after the end of treatment, for instance the long bone growth arrest, other as definitive, for instance, the dental eruption failure (For review (315)). Some authors have linked these side effects to an increase of the osteoclast activity secondary to an imbalance in the RANKL/RANK/OPG triad (28–33).

The main clinical side effect of N-BPs use in adults concerning the craniofacial skeleton, is the Bisphosphonate Related Osteo-Necrosis of the Jaw (BRONJ) (39,42–49).The origin of such side effects remains controversial (39,44,45). Preclinical models of BRONJ have been developed in *WT* and transgenic mice but until now no study is conclusive and related the origin of the BRONJ questions remain unanswered (50–54). Moreover, clinical reports have described the spontaneous appearance of the osteonecrosis of the jaw, even in absence of N-BPs therapy (203,204).

Significant variations in the penetrance of the N-BPs side-effects have been reported in the human population as in animal models (51,52,189,315). Regarding the origins associated to such effects and side-effects variations several factors have been proposed like drug-related causes as pharmacological properties of the drug (pharmacokinetics and pharmacodynamics), doses, duration of the therapy, drug interactions and pharmacogenetics. On the other hand host-related causes as expression levels in bone cells of genetic and epigenetic factors, imbalance in the RANKL\RANK\OPG triad and the composition in cytokines/chemokines of the bone microenvironment has been evocated (42,51,52,175,189,315,318). Surprisingly, none of these causes have been analyzed in-depth so far.

Concerning the RANKL\RANK\OPG triad in bone microenvironment, few studies have analyzed the impacts of N-BPs on the relative expressions of the elements of this triad (319,320). At the contrary, the consequences of perturbations of RANKL receptors expression levels onto N-BPs effects and side-effects on skeleton have never been evaluated. This is all the more surprising giving that the osteolytic diseases associated with a gain in RANK function (namely the Expansile Skeletal Hyperphosphatasia (ESH), the Familial Expansile Osteolysis (FEO) and the Paget Disease of Bone 2, early-onset (PDB2)) (321), or OPG loss function (namely the Paget Disease of Bone 5, juvenile-onset (PDB5)) (14,322) and osteoporosis age related (37,110) are currently treated with N-BPs (14,37,110,323).

To achieve this goal, combinations of two transgenic mouse models, plus already established and validated protocols of pediatric and adult ZOL treatment that induce craniofacial and appendicular skeletons side-effects, will be used (34,51,52,110) The analysis will involve both the appendicular and craniofacial skeletons, at the end of growth (one-month post-treatment) and in the longer-term (at ten months of age), as well as the effects and side effects of the treatment with ZOL in adult craniofacial skeleton at ten months age. In this way, it will be possible to clarify if the variation in response to N-BPs could be in part associated with the activity level of the TNFSF11

(RANKL) in the host.

3.2 HYPOTHESES

3.2.1 Null hypothesis (H0). Mice with different expression levels of RANK and OPG will have the same response to ZOL treatment during growth as in adult.

3.2.2 Alternative hypothesis (H1). Mice with different expression levels of RANK and OPG will respond differently to ZOL treatment during growth as in adult.

3.3 OBJECTIVES

3.3.1 General. To establish the consequences of disturbances to the expression levels of RANKL receptors on the effects and side-effects of ZOL during growth and in adulthood.

3.3.2 Specifics.

- To characterize the different skeletal phenotypes of mice with different expression levels of RANK and OPG at the end of growth (six weeks) and at adult age (10 months).

- To analyze how the different expression levels of RANK and OPG module the response to ZOL treatment during growth and in adult mice.

3.4 EXPERIMENTAL STRATEGY

3.4.1 To generate and characterize mouse with different RANKL signaling activity levels. Two transgenic mouse models on a C57BL/6J background were combined to generate six different RANKL signaling activity levels:

- The Tnfrsf-11b (osteoprotegerin: Opg) knock–out model (Strain B6.129S4-Tnfrsf11b^{tm1Eac}/J, Stock 010672, The Jackson Laboratory, Bar Harbor, ME, USA)

- The *Tnfrsf-11a* (receptor activator of nuclear factor kappa B: *Rank*) over-expression model (RANK^{tg}) which overexpress RANK receptor in cells of the monocyte/macrophage lineage by the Mrp8 (myeloid related protein 8 promotor). This model was previously created by our collaborator Dr Chistopher Mueller (324).

This signaling was chosen in relation to its importance during growth in the osteoclastogenesis in either the axial, the appendicular or the craniofacial skeletons (15,27,325), in the immune/inflammatory response (15,27) and in the cell-to-cell communications necessary to the development of many organs including all skeleton components (21,57,326). Both transgenic mouse models correspond to over-activation of the RANKL signalization achieved by two complementary approaches that make possible combinations to obtain a series of mice with graded levels of RANKL signaling activation. The genotypes of th emice of this series are *Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/+}$*Rank*$^{Tg+}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg+}$, *Opg*$^{-/-}$*Rank*$^{Tg-}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$. The *wild-type* (*Opg*$^{+/+}$*Rank*$^{Tg-}$) mouse was considered as the control mouse for all the analyses.

3.4.2 To applicate ZOL treatments mimicking those used in onco-pediatric and adult patients.

3.4.2.1 Growing mice: To analyze the effects of N-BPs on the skeleton of growing mice, the protocol of ZOL administration was designed according to the pharmacokinetics data of ZOL and mimicking the clinical protocol administered in pediatric patients with primary bone tumors as previously described (51,52). Briefly, mouse pups were randomly divided into treated and none treated groups. In the treated group each pup received 4 subcutaneous injections of 50 µg/kg of ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basel, Switzerland) at day 1, 3, 5 and 7 after birth (Fig. 10). In none treated group each pup received four injections of sterile normal saline solution. In order to analyze the consequences of ZOL treatment at the end of growth mice were sacrificed at one month and a half. In order to analyze the long-term consequences of ZOL treatment during growth, a group of twelve mice (two for each genotype) was maintained and followed until ten months of age.

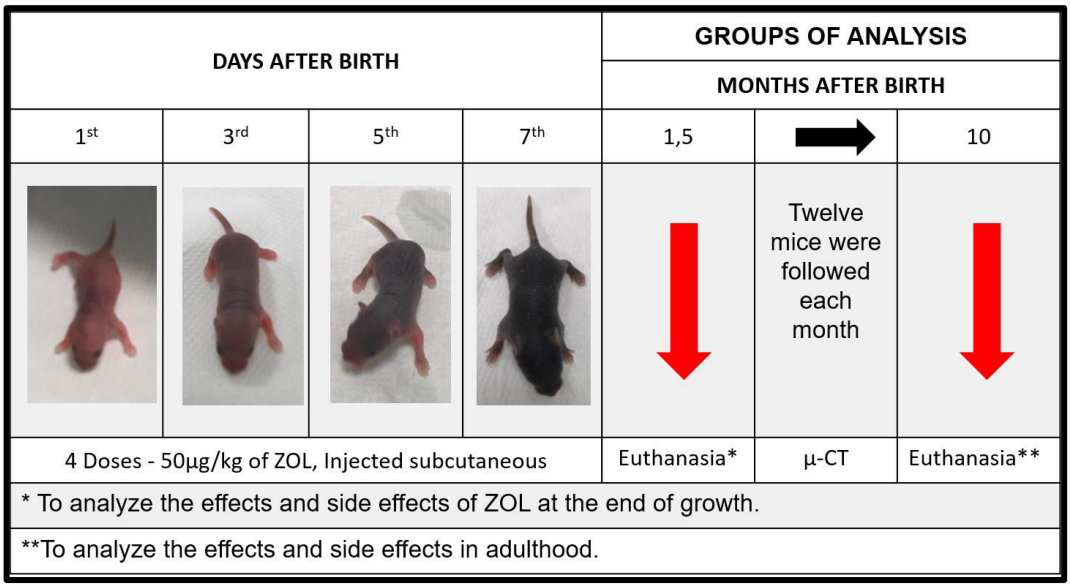

Figure 10. Zoledronic acid (ZOL) administration protocol in growing mice. From day one other birth mouse pups received four subcutaneous injections of ZOL at the dose of 50µg/kg at intervals of two days. Genotypes were determined at 1 month on tail biopsies by PCR on extracted genomic DNA. At one and half months, some of the treated mice were sacrificed to analyze the skeletal phenotypes at the end of growth. Twelve mice were maintained until ten months, with control micro-tomography every two months, to analyze the skeletal phenotypes at a distance from the treatment.

3.4.2.2 Adult mice: To analyze the effects of N-BPs on the skeleton of adult mice, the protocol of ZOL administration was designed according to the pharmacokinetics data of ZOL and miming the clinical protocol administered in adult patients with osteolytic disease. Briefly, 22 mice with different RANKL signaling activity levels were chosen aleatory and treated with ZOL (Table 7). Each mouse received two subcutaneous injections of 2 µg/kg/wk of ZOL (Zometa®, Novartis Pharmaceuticals Corporation, Basel, Switzerland) per week, during eight weeks (Fig. 11). In none treated group each mouse received the same number of injections with a sterile saline solution. In order to analyze the consequences of ZOL treatment in adulthood, mice were followed by micro-CT analysis each month and sacrificed at ten-month of age.

Table 7. Distribution on mice treated with ZOL at the beginning of the experimental period.

	GENOTYPE					
	WT= $Opg^{+/+}Rank^{tg-}$	*$Opg^{+/-}Rank^{tg-}$*	*$Opg^{-/-}Rank^{tg-}$*	*$Opg^{+/+}Rank^{tg+}$*	*$Opg^{+/-}Rank^{tg+}$*	*$Opg^{-/-}Rank^{tg+}$*
♀	2	4	0	2	4	2
♂	2	4	1	1	4	0

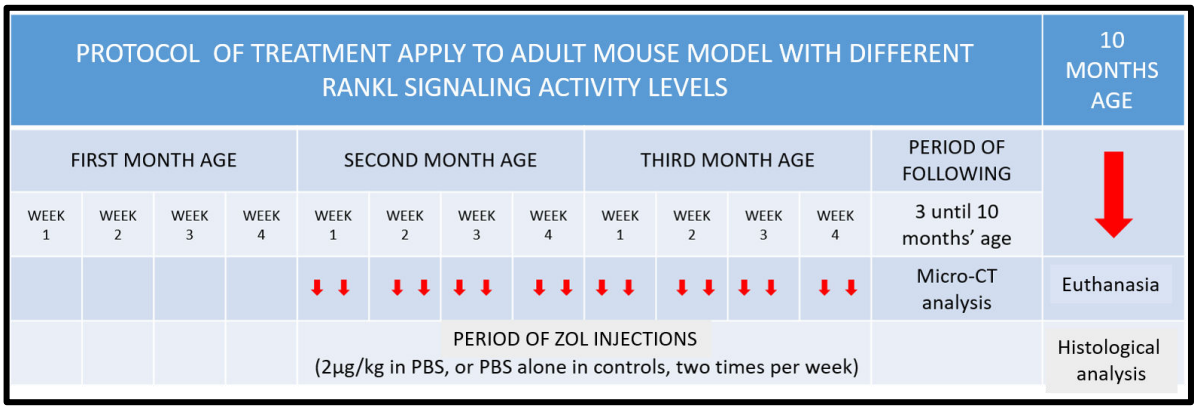

Figure 11. Protocol of Zoledronic acid (ZOL) administration in adult mice.

4. MATERIAL AND METHODS

4.1 ANIMALS

4.1.1 Mice handling. All mice, male and female, were housed from birth until ten months, with the same diet and light conditions, under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France) in accordance with the institutional European guidelines (EU directive 2010/63/EU). All protocols applied in the present study were first validated by the French ethical committee of the “Pays de la Loire” (CEEA-PdL-06) and authorized by the French ministry of agriculture and fisheries (authorization # 11208-2017083115577055). The mice were handled and sacrificed by authorized investigators in strict respect of these protocols.

4.1.2 Genotype identification. The genotypes were determined by PCR, using genomic DNA extracted from the tail of each mouse (317,325). The primers information and the PCR protocol are presented in table 8. Once the PCR has been done, the genotype was obtained by identification of the amplified fragments, using agarose gel electrophoresis (2%) (Fig. 12).

Table 8. Information concerning the genotype identification by PCR.

GENE	PRIMER	SECUENCE	PCR PROTOCOL		
			(µl/Rxn)	REACTIVES	PCR steps
OPG +/+ (375 pb) -/- (572pb)	OPG $^{+/+}$ _ FW	TGC-CCT-GAC-CAC-TCT-TAT-ACG-GACC	10	-2x phire tissue direct PCR MM	98ºC 5min 98ºc 5 sec
	OPG $^{-/-}$ _ FW	TGA-CCG-CTT-CCT-CGT-GCT-TTA-C	1 1	-Primer F (+/+) -Primer F (-/-)	58ºC 5sec 72ºC 20 sec
	OPG REV	GGT-CCT-TGA-TTT-TTC-TAT-GCC	1 1 6	-Primer Rev -DNA -H_20	Rep (2-4 x 40) 72ºC 1 min 4ºC forever
RANK (300pb)	Tg _ FW	TGT-CTC-TGT-GTG-AAT-GGA-CC	2 0,6	-Tp mix -$MgCl_2$	93ºC 5min 93ºc 5 sec
	Tg _ Rev	GTC-CGA-GAT-GCT-CAT-AAT-GC	0,4 0,4 0,4 1 14,8	-dNTPs -Primer Tg F -Primer Tg R -DNA -H_20	58ºC 5sec 72ºC 20 sec Rep (2-4 x 40) 72ºC 1 min 4ºC forever

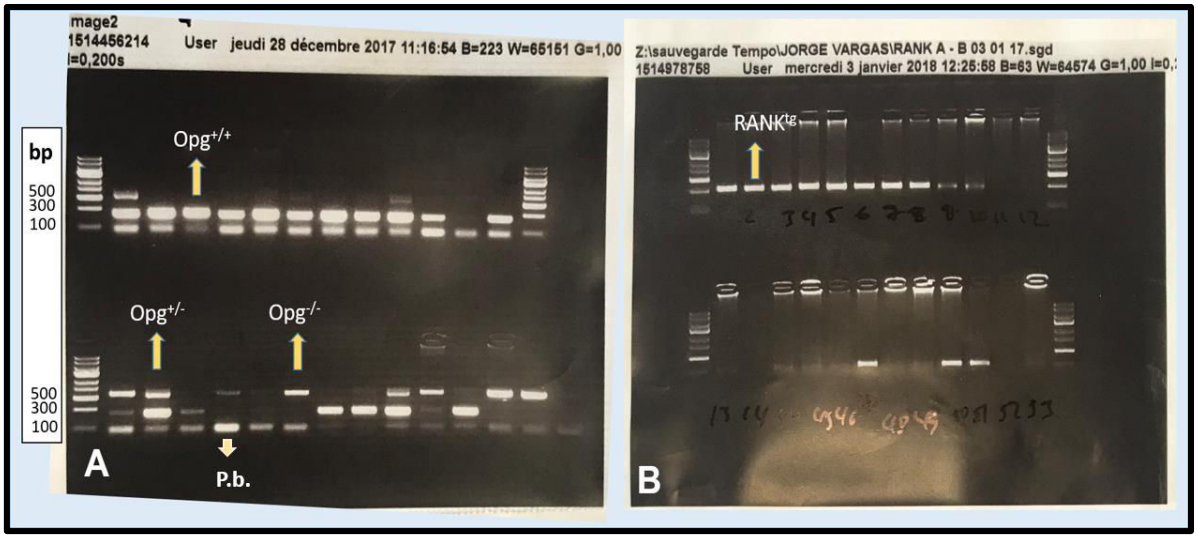

Figure 12. Electrophoresis analysis of PCR results. A: Amplicons observed enable to identified each mouse genotype. Opg$^{-/-}$ have only a 620 bp fragment. Opg$^{+/+}$ have only a 580bp fragment and OPG$^{+/-}$ have both 620-bp and 580-bp fragments. While the overexpression RANK (RANK^{tg}) shows positive band 300 bp. The primer band (P.b) indicated has 100bp. The electrophoresis was done in agarose gel (2%).

4.2 MICRO-CT ANALYSIS

A Skyscan 1076 micro-CT scanner (Skyscan, Kontich, Belgium) was used to analyze the mouse skeleton structures. Tibia, heads, mandibles and dental structures were analyzed according to the objective of experiments. This aspect will be detailed below for each experiment.

All mice were scanned using the same parameters (pixel size 9µm, 50kV, 0.5mm Aluminum filter, 20 minutes of scanning). The reconstruction was carried out using NRecon and the analyses were performed using CTAnalyzer (CTAn), CTVox and DataViewer software (Skyscan). The different measurements were made using IMAGE-J software (National Institutes of Health, Bethesda, MD, USA). In this way, the acquisition of the image in CTVox (camera viewing angle 20º) was systematically calibrated with a phantom of 5mm (known size) and all measurements were finally sized using the analysis scale in the IMAGE-J software.

4.2.1 Morphometric and mineral analysis in growing mice skeleton. The Skyscan 1076 micro-CT scanner acquisitions were used to analyze on the one hand the morphometric and mineral parameters of representative bones of appendicular and craniofacial skeletons (tibia and maxillary basal and alveolar bones), and on the other hand the eruption status of molars and incisors.

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In order to analyze the ZOL effects onto the growing appendicular skeleton, the right tibia was measured for all mice. Concerning vertical growing, the longitudinal distance was seized. Concerning axial growing, the external and the internal diameters were measured and the difference between those measures enable to determine the cortical thickness. The marks of reference used for tibia measurements are indicated in Figure 13A.

In order to analyze the effects onto the growing craniofacial skeleton, measures were realized accordingly to Vora and collaborators (327) and Simon and collaborators (328). Indeed, eight measures relative to the sagittal, the vertical and the transversal planes of craniofacial growth were realized (Figure 13A). The marks of reference used to perform these craniofacial measures are presented in Figure 13A.

In order to obtain the bone mineral parameters, more specifically the tissue mineral density (TMD) for the cortical bone and the percentage of bone volume (BV/TV), the trabecula number (Tb.N) and the trabecula thickness (Tb.Th) for trabecular, basal and alveolar bones, a sample volume of 2.0mm of length and 1.1mm x 1.1mm of width surface was sectioned using the Data Viewer software, and analyzed using the CTAn software. The different points chosen for the analysis are presented in Figure 13B.

In order to facilitate the identification of changes in the different structures, a “color density range” was used in CTAn software that enables to adjust the correspondence of color and brightness values with image gray scales. For tibia and head images, the level 39 of brightness and level 45 of contrast from the color density range of CTAn software were used systematically.

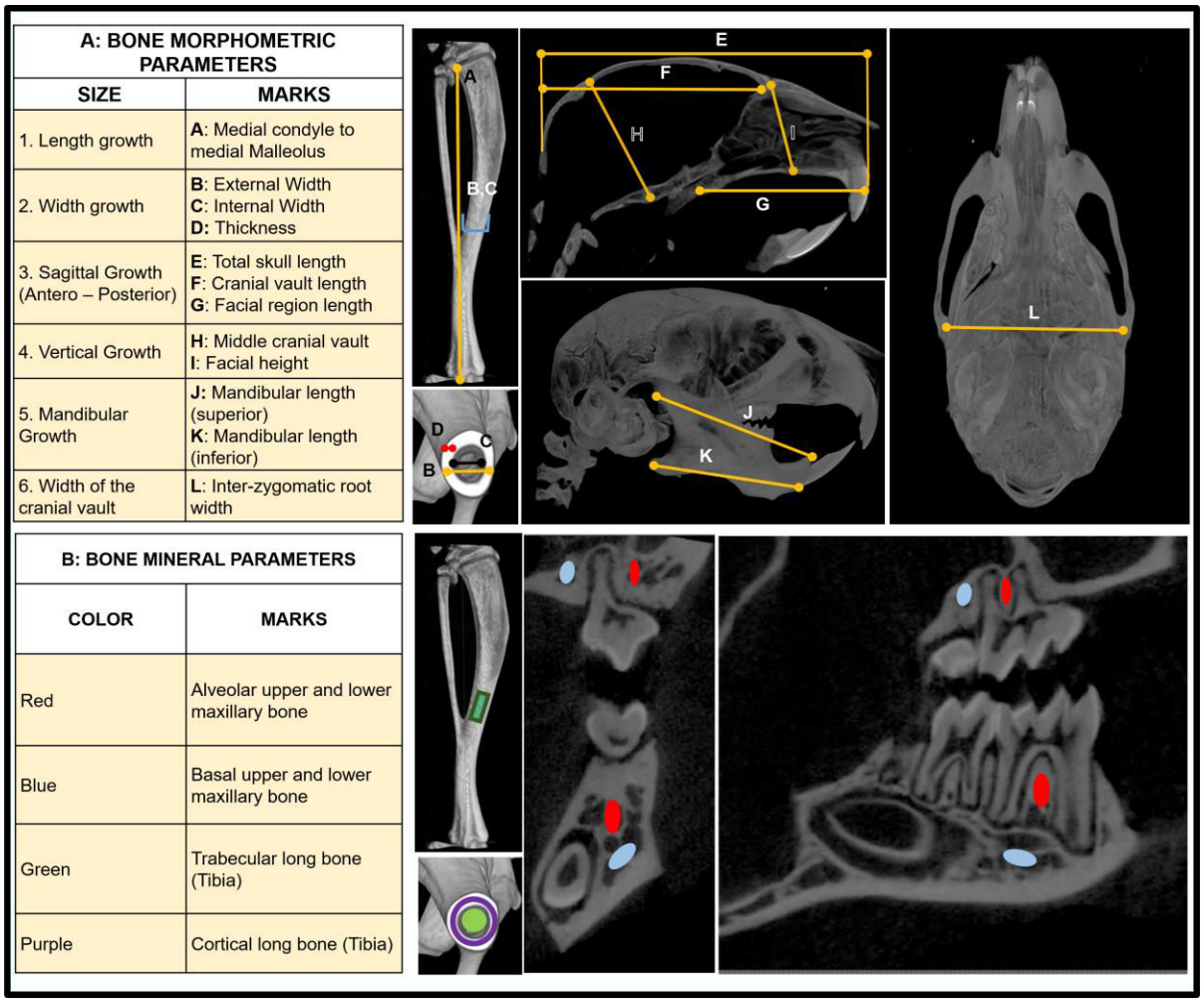

Figure 13. Bone morphometric and bone mineral parameters in growing mice. To analyze the effects on long bone morphometric parameters (A), the tibia was measured in its length, width and thickness using specific reference dots making it possible to determine different values, named A to D. To analyze the effects on craniofacial morphometric parameters (A), the skull was measured in all its planes using landmarks that made it possible to determine 8 representative measurements, named E to L. A phantom of known size (5mm) was used to calibrate and standardize all the measurements in CTVox and IMAGE-J software.

To analyze the effects on long bone mineral parameters (B), a region of interest was chosen in the region of the tibia diaphysis, localized precisely 200 µm over the furcation between tibia and fibula. The TMD (tissue mineral density) was measured for cortical bone (purple color), and the BV/TV (bone volume/tissue volume), Tb.N (trabecular number) and Tb.Th (trabecular thickness) were measured for trabecular-like bone (green color). To analyze the effect on craniofacial bone mineral parameters (B), eight regions of interest were chosen, four in the upper and four in the lower maxillary, corresponding to the inter-radicular areas of the first molars for alveolar bone (red color) and the underlying dental crypt areas for basal bone (blue color). The BV/TV (bone volume/tissue volume), Tb.N (trabecular number) and Tb.Th (trabecular thickness) were measured for both alveolar and basal bones.

4.2.2 Morphometric and mineral analysis in adult mice craniofacial skeleton.

To analyze the impact of RANKL signaling activity level on the morphometric and mineral homeostasis of craniofacial bone, the following measurements were analyzed: First, in order to examine the impact of the different genotypes onto the morphometric bone structure, five mandibular dimensions were obtained, considering the coronal (CrP), frontal (FrP) and sagittal (SgP) planes of right mandible. The marks of reference used to perform these craniofacial measurements are presented in Figure 14 and were made according to Voral et al. (327) and Simon et al. (328). Secondary, in order to identify the effects on the bone mineral parameters, the bone volume (BV mm^3) for cortical bone or the percentage of bone volume/total volume (%BV/TV), the trabecula thickness (Tb.Th), the trabecula number (Tb.N) and the trabecula separation (Tb.Sp) for cancellous and alveolar bones were measured. A sample volume of 2.0 mm in length, and 1.1 mm x 1.1 mm in surface width was sectioned using the Data Viewer software and analyzed using the CTAn software. The different points chosen for this analysis are presented in Figure 15A.

In order to examine the impact of the RANKL signaling activity level on the periodontal status, the total periodontal bone loss was sized, taking as a reference the distance between the cementum enamel junction (CEJ) and the alveolar bone crest in three different positions of the first lower right molar mesial (M), central (C) and distal (D), both in the buccal and the lingual side (Figures 15B and C).

In order to identify how the RANKL signaling activity level affects the root morphometric and mineral parameters, the roots, mesial and distal, of the first lower right molar were analyzed. The total length was analyzed in a sagittal section, taking as a reference the CEJ until the latest apical point of both roots (Fig 16A). To analyze the root width, three different points (cervical, middle and apical) both in sagittal and frontal views of the mesial root were considered (Fig. 16B and C). Finally, the impact of RANKL signaling activity level onto the root resorption was analyzed by

identification of the presence of resorption lacunas using the CTvox and CTAn software (Fig. 33C). In that way, the roots of all six lower molars (3 right and 3 left) were considered for each mouse.

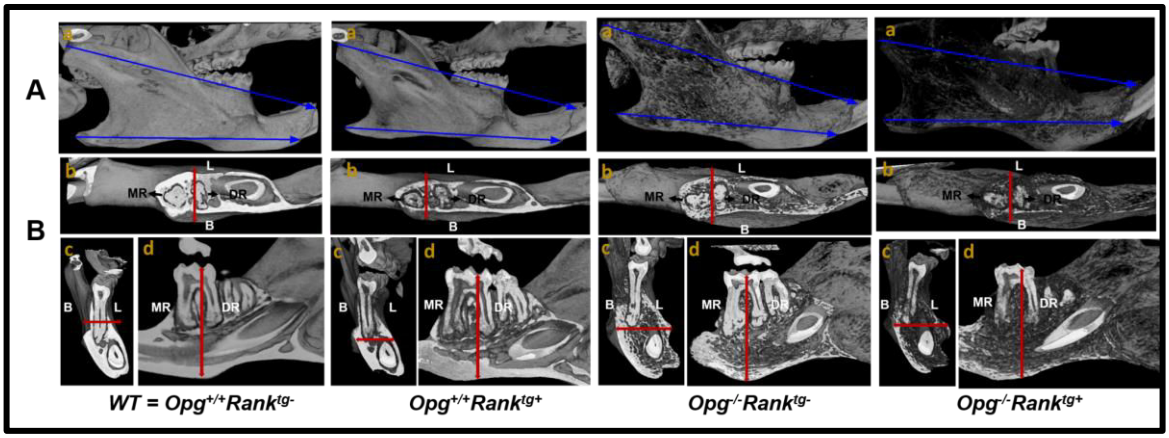

Figure 14. Mandible morphometric analysis, parameters for craniofacial dimensions in adults mice. µCT images obtained by CTvox software of right mandibles of mice with different RANKL signaling activity levels. A: The total mandibular length (a) was seized for all mice considering the sagittal view plane, according the marks presented. Measurements were made using the method proposed by Vora et al. (327) and Simon et al (328). B: Measurements guide of mandibles dimensions for morphometric analyses. (b) Coronal plane, (c) Frontal plane and (d) sagittal plane. The analyses were done taking as a reference the mesial root (MR) and distal root (DR) of the first lower right molar. Buccal (B) and Lingual (L).

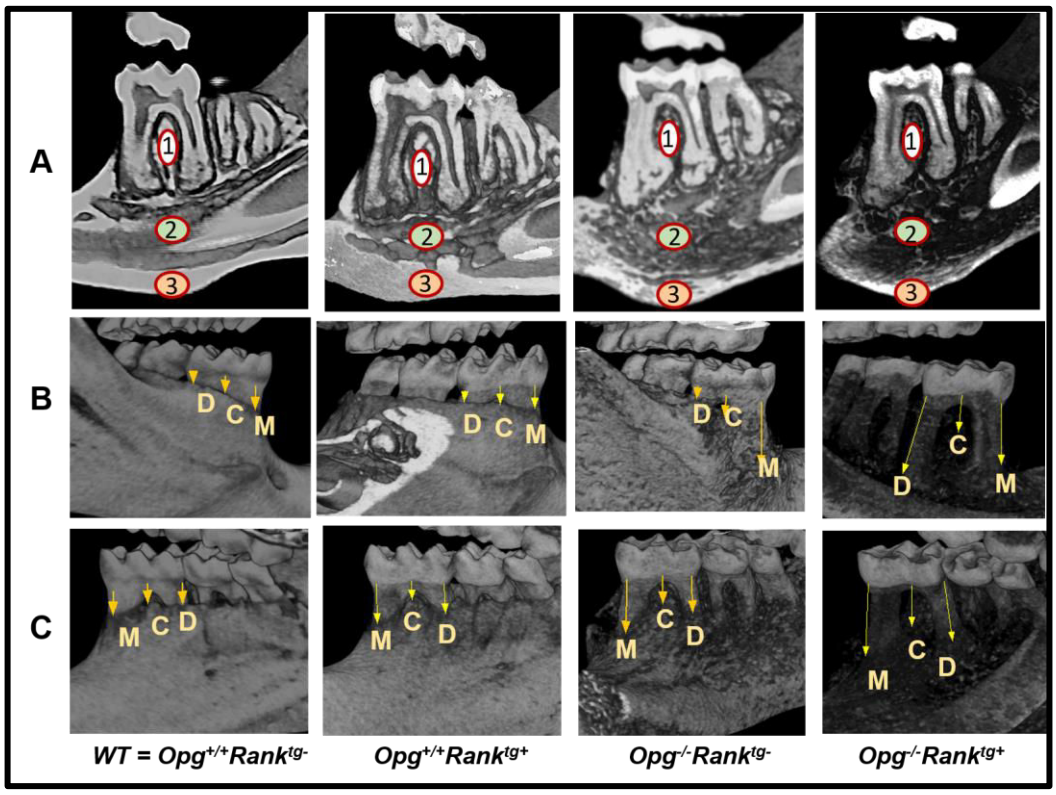

Figure 15. Bone morphometric and mineral parameters for periodontal analysis in adult mice. µCT images obtained by CTvox software of right mandibles of mice with different genotypes, at the level of the first lower molar. A: The analysis of mineral parameters: percentage of bone volume/total volume (%BV/TV) trabecula thickness (Tb.Th), trabecula number (Tb.N) and trabecula separation (Tb.Sp) for alveolar (1) and cancellous (2) bone, and bone volume (BV) in cortical bone (3) were quantified for each sample using the CTAn software. B and C: Three different points of reference in the right first lower molar (mesial - M, central – C and distal - D) were chosen to analyze the alveolar bone loss, both in buccal (B) and lingual (C) sides. The analysis was done considering the cementum enamel junction (CEJ) (start of the yellow arrow) and the alveolar bone crest (head of the yellow arrow) as points of reference for the measures.

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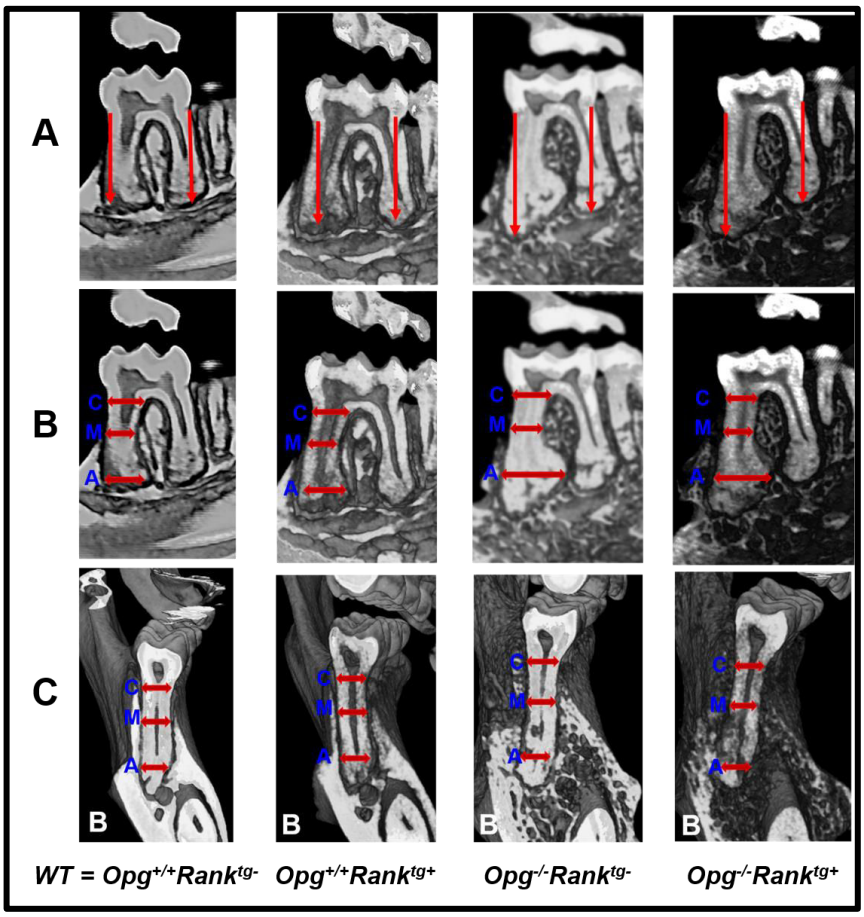


Figure 16. Parameters for root analysis in adult mice. µCT images obtained by CTvox software of right mandibles of mice with different genotypes, showing the measurements guide of the first lower molar root dimensions analyses: A: Total root length size in the sagittal plane, considering the length between the cement enamel junction (CEJ) (arrow start) and the latest apical point of the root (arrow end). B: Width size in the sagittal plane. C: Width size in the frontal plane. The width was measured in three different points of the root: cervical (C), middle (M) and apical (A).

4.3 HISTOLOGY

Tibias and heads, collected from euthanized mice, were fixed in 4% buffered paraformaldehyde (Sigma-Aldrich, Saint-Quentin Fallavier, France). The samples were then decalcified in a buffered pH 7.4 solution containing 4.13% EDTA (Sigma-Aldrich) and 0.2% paraformaldehyde over four days in KOS sw10 (Milestone, Sorisole, Italy). All the samples were then dehydrated and embedded in paraffin according to the conventional methods. For the histological analysis the tissue of

interest was sectioned (3 µm-thick sections) and either stained with Masson's trichrome to analyze the morphology or with tartrate-resistant acid phosphatase (TRAP) to identify multinucleated osteoclasts as previously described (51). Both stainings were carried out using an HMS 740 automated staining machine (Microm) in which the samples are dewaxed and rehydrated by successive baths of xylene and ethanol of decreasing concentration (100, 95, 80 and 70%).

Specifically, Masson's trichrome (three-color staining protocol which produces red muscle fibers, green collagen and bone, light red cytoplasm, and dark brown nuclei) was done according the following protocol: Groat hematoxyline (15 min), distilled water (5min), acid fuchsine (5 min), acetylated water 1% (10 sec), molybdenum-reducing and Orange G (5 min), acetylated water 1% (10 sec), light green (5 min), acetylated water 1% (10 sec), alcohol 100% (30 sec) x 2, toluene (1 min) x 2. For TRAP staining the protocol correspond to a 90 minutes of incubation in 1 mg/mL of Naphthol AS-TR phosphate, 60 mmol/L N,N-dimethylformamide, 100 mmol/L sodium tartrate, and 1 mg/mL Fast Red TR Salt solution (all from Sigma Chemical Co., St. Louis, MO, USA), follows by a counterstaining with hematoxylin (32).

Histological images were then acquired using a Nano-Zoomer 2.0-RS slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan) before being visualized and analyzed using Nano-Zoomer software.

4.4 STATISTICAL ANALYSIS

All the measurements were analyzed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, California, USA). To determine the normality of the data, a Brown-Forsythe test was carried out, considering significantly standard deviations ($p<0.05$). Two-way ANOVA followed by Tukey multiple comparisons test was performed to evidence statistically significant differences $p<0.05$ (*), $p<0.01$ (**); $p<0.001$ (***) and $p<0.0001$ (****).

5. RESULTS

5.1 SKELETON PHENOTYPES OF MICE WITH DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS AT THE END OF PEDIATRIC GROWTH

Bone morphometric (Fig. 17 and 20) and mineral parameters (Fig. 18, 19 and 21) were measured and compared at the end of pediatric growth (one and half month) on micro-CT scans of tibias and heads obtained for mice from the different genotypes.

5.1.1 Appendicular skeleton phenotypes associated with different RANKL signaling activity levels at the end of growth. Regarding the morphometric parameters, the analysis of mice of the different genotypes associated to different RANKL signaling activity level (Fig. 17), evidenced no significant difference in either the vertical or the axial growth (mean global length: 12.31±1.04; global mean external diameter: 0.86±0.09; mean global internal diameter: 0.58±0.06 and mean global thickness: 0.14±0.03). Concerning the bone mineral parameters, in absence of treatment, no significant difference was observed between the different genotypes (Fig. 18) with however the TMD that appeared slightly inferior in *Opg*$^{-/-}$ (*Rank*$^{Tg-}$ and *Rank*$^{Tg+}$) mice than in mice from all other genotypes (mean 3.6±0.4 vs 4.4±0.3).

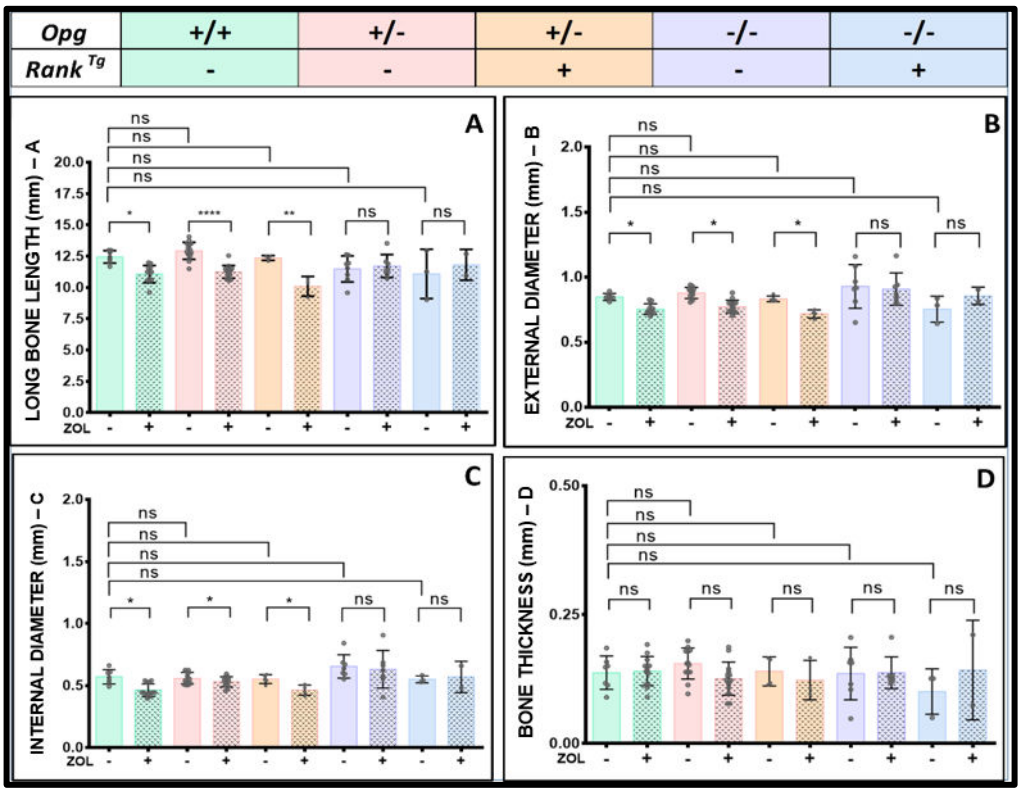


Figure 17. Comparative analysis of long bone morphometric parameters between mice with different genotypes, treated or not with ZOL. Tibia length (A), tibia external width (B), tibia internal width (C), and tibia cortical thickness (D) presented no significant difference between non-treated mice regardless of the genotype. In treated mice, tibia length (A), tibia external width (B), and tibia internal width (C) were significantly reduced for $Opg^{+/+}$\$Rank^{Tg-}$, $Opg^{+/-}$\$Rank^{Tg-}$ and $Opg^{+/-}$\$Rank^{Tg+}$ mice compared to untreated animals with respect to the genotype. The cortical thickness (D) was unaffected by the treatment with ZOL, regardless of the genotype considered. None of the parameters were affected by the treatment with ZOL for $Opg^{-/-}$\$Rank^{Tg-}$ and $Opg^{-/-}$\$Rank^{Tg+}$ mice (A-D). ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

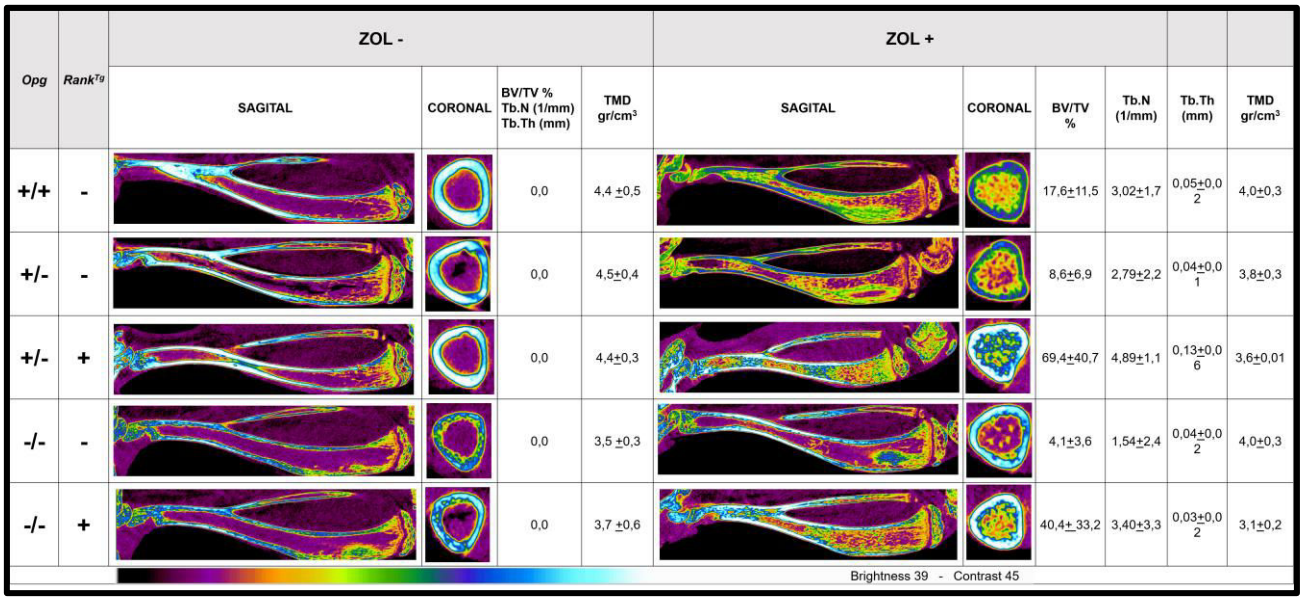


Figure 18. Comparative analysis of long bone mineral parameters between mice with different genotypes, treated or not with ZOL. Micro-CT scan sections in the sagittal and axial planes of the tibias with different genotypes with and without treatment were presented. Axial sections were realized in the diaphysis region of the tibias (the most affected by the ZOL treatment protocol chosen) in which measurements of mineral parameters were realized. The color density range revealed visually identifiable changes in the different structures. In the absence of treatment, the bone mineral parameters (BV/TV, Tb.N and Tb.Th) were null and the TMD around 4.4 gr/cm^3 except in Opg$^{-/-}$ (regardless of Rank^{Tg} status), for which the TMD was around 3.6 gr/cm^3. In the treated group, the TMD was not significantly affected compared to the untreated group in respect to each genotype, while two situations emerged for the bone mineral parameters. In the absence of RANK over-expression (Rank^{Tg}), the BV/TV and Tb.N values decreased following the Opg allelic decrease, while in the presence of RANK over-expression (Rank^{Tg+}), these parameters were high.

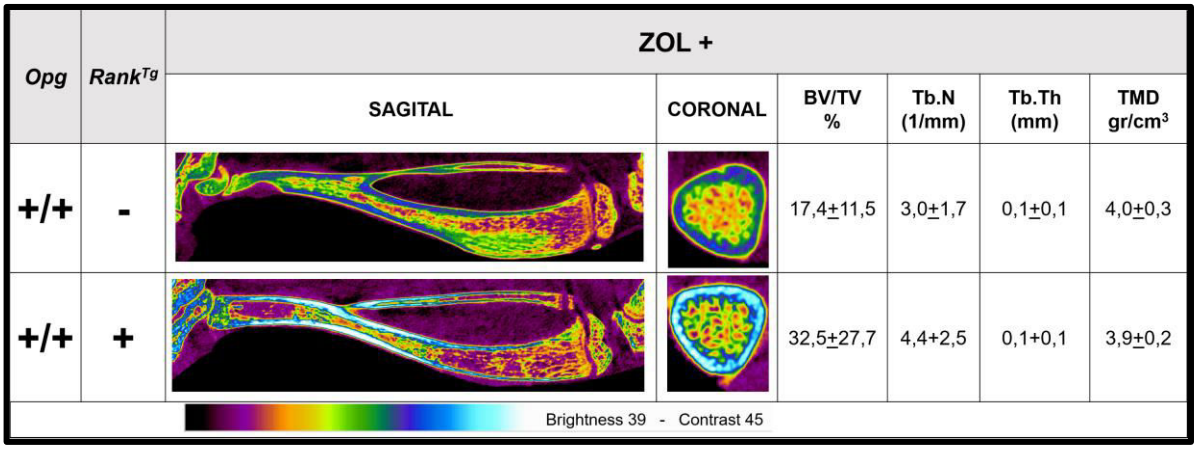


Figure 19. Long bone minerall parameters comparative analysis between Opg$^{+/+}$\Rank^{Tg-} and Opg$^{+/+}$\Rank^{Tg+} mice treated with ZOL. Micro-CT scans sections in the sagittal and axial planes of the tibias of the two genotypes with treatment were presented. Axial sections were realized in the diaphysis region of the tibias in which measures of mineral parameters were realized. The color density range lets visually identify changes in the different structures. The Tb.Th and the TMD were not significantly different in the two genotypes whereas the Tb.N and the BV/TV appeared softly higher in the Rank overexpressing mouse.

5.1.2 Craniofacial skeleton phenotypes associated with different RANKL signaling activity levels at the end of growth. The craniofacial morphometric parameters and the mineral parameters of maxillary basal and alveolar bones were measured at the end of growth (one and half month) on the micro-CT scans of heads obtained for mice of the different genotypes treated or not with ZOL. Regarding the morphometric parameters, the analysis of the mice of the different genotypes of the not treated group (Fig. 20) did not reveal any significant difference, with the exception of the facial height which appeared higher in the *Opg*$^{-/-}$ mice (Fig. 20E). For the bone mineral parameters, the analysis of the different genotypes of the not treated group (Fig. 21), revealed that basal and alveolar bones were not similarly modulated regarding the BV/TV and the Tb.Th while close concerning the Tb.N. Effectively, the Tb.N in both bones was significantly lower only in the *Opg*$^{-/-}$*Rank*$^{Tg-}$ mice (Fig. 21C and D). For the two other parameters, the alveolar bone appeared more affected than the basal bone. Regardless, BV/TV appeared to decrease gradually and significantly compared to the *Opg*$^{+/+}$*Rank*$^{Tg-}$ from the *Opg*$^{+/-}$*Rank*$^{Tg-}$ toward the *Opg*$^{-/-}$*Rank*$^{Tg+}$ genotypes (Fig. 21A and B). For the Tb.Th, a similar graded decrease was observed for the alveolar bone (Fig. 21E) while for the basal bone this parameter being solely significantly decreased for the *Opg*$^{-/-}$*Rank*$^{Tg-}$ mice (Fig. 21F).

The analysis of the dental structures in the absence of treatment showed some interesting aspects. Regardless the genotypes considered, normal eruption was observed for either incisors or molars, and the dental structures evidenced a normal development (Fig. 22A and 23). The main specific feature observed in these untreated mice concerned the *Opg*$^{-/-}$ mice (*Rank*$^{Tg-}$ or *Rank*$^{Tg+}$) where small root resorption lacunas associated to the presence of numerous TRAP positive cells (Fig. 23). A reduced diameter of the root was also present in the *RANK*$^{Tg+}$ mice (for instance visible in Fig. 22A for *Opg*$^{-/-}$*Rank*$^{Tg+}$ comparatively to *Opg*$^{-/-}$*Rank*$^{Tg-}$).

5.2 CONSEQUENCES OF ZOL TREATMENT ON SKELETON PHENOTYPES OF MICE WITH DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS AT THE END OF PEDIATRIC GROWTH

Bone morphometric (Fig. 17 and 20) and mineral parameters (Fig. 18 and 21) were measured at the end of pediatric growth (one and half month) on micro-CT scans of tibias and heads obtained for mice from the different genotypes treated with ZOL and compared with not treated groups.

5.2.1 Appendicular skeletons of transgenic mice, with genetically-achieved grade RANKL activity levels, were differentially affected at the end of growth following treatment with ZOL. Comparison of treated and not treated mice from the same genotype evidenced a significant reduction of all morphometric parameters except the bone thickness in treated mice of the *Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$ *and* *Opg*$^{+/-}$*Rank*$^{Tg+}$ genotypes (Fig.17). On the contrary, no such reduction was observed (Fig. 17) regardless the parameter considered for the *Opg*$^{-/-}$*Rank*$^{Tg-}$ mice (mean length 11.71±0.90 for treated vs 11.48±1.03) and *Opg*$^{-/-}$*Rank*$^{Tg+}$ mice (mean length 11.82±1.20 for treated vs 11.08±1.90).

Regarding the mineral parameters, in the treated group, the TMD appeared not significantly affected compared to the not treated group, with respect to the genotypes, except in the *Opg*$^{+/-}$*Rank*$^{Tg+}$, while all the other parameters, which value are zero in the not treated group, appeared increased (Fig. 18 and Fig. 19). Interestingly, the observed values of these parameters were different with respect to the genotypes. Considering only the *Opg* status, a relationship is observed between the values of the parameters and the allelic reductions, the *Opg*$^{-/-}$ evidencing the lower parameters and the *Opg*$^{+/+}$ the higher (Fig.18). Considering the *Rank* status, the genetically-achieved over-expression induced very significant elevations of all the parameters independently of the *Opg* status (Fig. 18 and Fig.19) except the Tb.Th in the *Opg*$^{-/-}$*Rank*$^{Tg+}$

5.2.2 Craniofacial skeletons of transgenic mice, with genetically-achieved grade RANKL activity levels, were differentially affected at the end of growth following treatment with ZOL. In the craniofacial skeleton, the comparison of treated and not treated mice from the same genotype evidenced two different situations. First, for the *Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$ and *Opg*$^{+/-}$*Rank*$^{Tg+}$ mice the total skull length, the cranial vault length, the facial region length, the upper maxillary and the lower maxillary were significantly reduced in the treated group (Fig. 20 A, B, C, F and G) whereas the middle cranial vault, the facial height and the cranial vault width were not affected (Fig. 20 D, E and H). Secondly, in the *Opg*$^{-/-}$*Rank*$^{Tg-}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$ mice none of the parameters was significantly affected by the ZOL treatment (Fig. 20).

Due to a defective inter-radicular alveolar bone formation associated to blocked eruption and root elongation by ZOL treatment, measurements of bone mineral parameters were only possible for the basal bone in the treated group. Interestingly the BV/TV and the Tb.N were significantly increased in the treated group compared to the not treated group whatever the genotype (Fig. 21B and D) with very close values in all treated mice. Surprisigly, the Tb.Th was also significantly increased by the treatment, excepted in mice over-expressing *Rank* with however a noticeable tendency for increase (Fig. 21F).

Teeth are important elements of the craniofacial skeleton whose histogenesis/organogenesis was known to be sensitive to treatment with ZOL. More specifically, the eruption and the root elongation processes have been shown perturbed by treatment with ZOL in C57BL/6J wild-type mice. In order to determine the consequences of the OPG and RANK expression levels on the severity of these perturbations, these two processes have been comparatively analyzed in mice harboring the different genotypes. For the eruption process, micro-CT scans of heads have been used (Fig. 22) to determine the eruption status in a three-steps classification corresponding to fully erupted (yellow), partially erupted (clear blue)

and not erupted (dark blue) teeth. For the root elongation, histology was used focusing on the first mandibular molars (Fig. 23).

In the treated group, the dental phenotype was affected in all mice with however different proportions according to genotypes (Fig. 22 and 23). Alteration to the eruption of the incisors (upper and lower) and first molars (upper and lower) were observed in all genotypes, but with a globally-graded decrease in penetrance from *Opg*$^{+/+}$*Rank*$^{Tg-}$ toward the *Opg*$^{-/-}$*Rank*$^{Tg+}$ mice (Fig. 22B). Interestingly, all teeth were not similarly affected in each mouse regardless of the genotype. For the eruption of the first molars, the most affected was the upper molar, 100% in *Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$ and *Opg*$^{+/-}$*Rank*$^{Tg+}$ mice, while the lower molar was touched in around 80% in these mice (Fig. 22B). For the eruption of the incisors, a similar response was reported for these mice for the upper incisor (more than 80%) whereas the lower incisor eruption was partially rescued (less than 40% affected). For the *Opg*$^{-/-}$mice, eruption of first molars and incisors was clearly less affected with a maximum of 60% for the upper first molar of *Opg*$^{-/-}$*Rank*$^{Tg-}$ (impacted 40%, partial erupted 20%) and a minimum of 0% for the lower incisor of either *Opg*$^{-/-}$*Rank*$^{Tg-}$ or *Opg*$^{-/-}$*Rank*$^{Tg+}$ mice* (Fig. 22B). It is important to emphasize that the eruption of the second and the third molars were unaffected whatever the genotype considered (Fig. 22A).

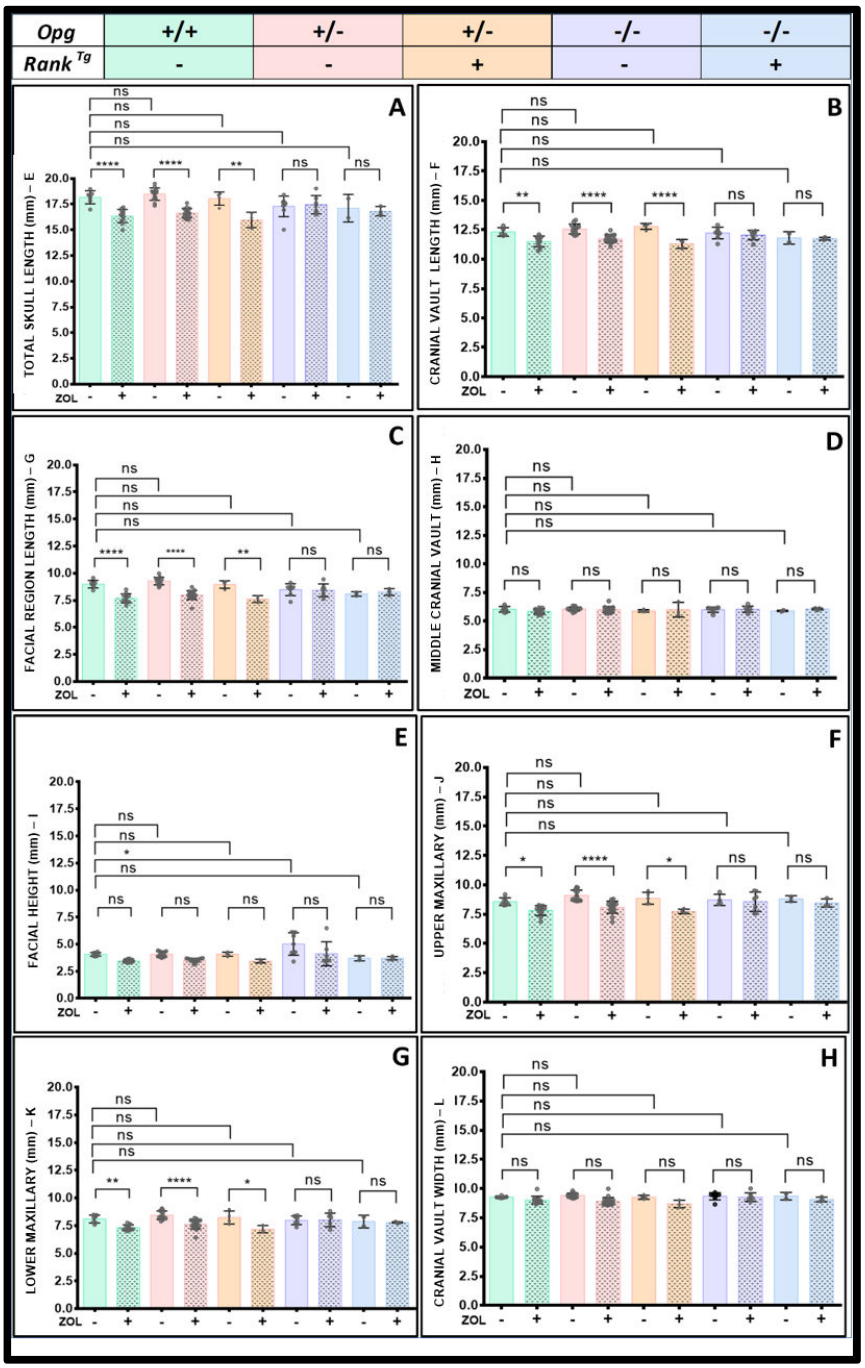


Figure 20. Comparative analysis of craniofacial morphometric parameters between mice with different genotypes, treated or not with ZOL. Measurements were carried out in the sagittal (A, B, C, F, G), vertical (D, E) and transversal (H) planes. In the absence of treatment, no difference was observed regardless of the measurement considered between the different genotypes, with the exception of facial height, which was in the *Opg*$^{-/-}$*Rank*^{Tg} mice (E). In the treated group, none of the parameters measured were significantly affected in the *Opg*$^{-/-}$ mice regardless of *Rank*^{Tg} status, while for all the other genotypes the sagittal parameters were significantly reduced and the vertical **and** transversal ones unaffected. ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

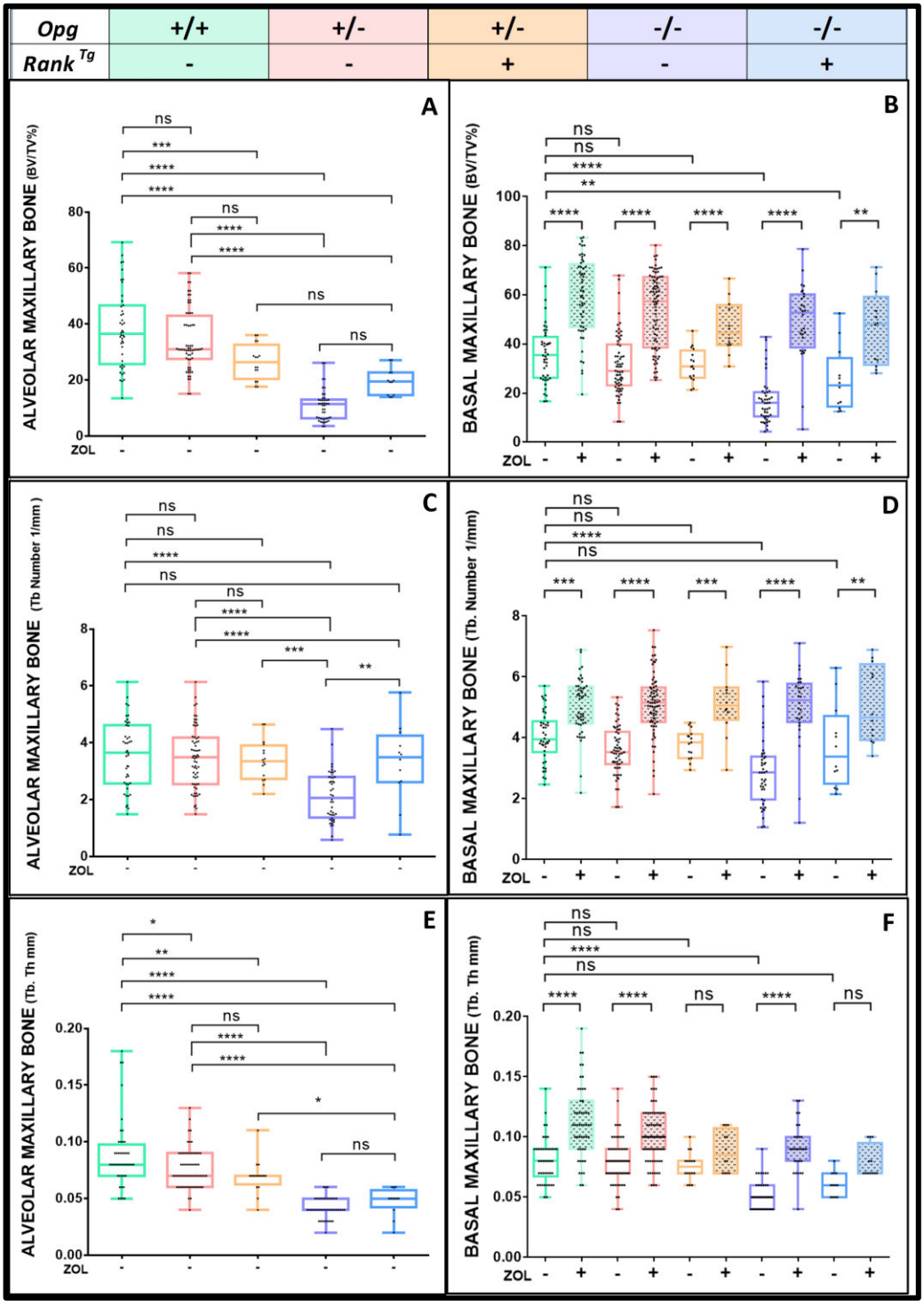


Figure 21. Comparative analysis of craniofacial bone mineral parameters between mice with different genotypes, treated or not with ZOL. Alveolar (A, C, E) and basal (B, D, F) maxillary bone mineral parameters were measured. In the absence of treatment, a graded decrease in the BV/TV and Tb.Th was observed in the alveolar bone (A, E) following the genotype severity in terms of osteolytic potential, while the Tb.N was stable except in $Opg^{-/-}$\$Rank^{Tg-}$ mice (C). In the basal bone, all parameters were significantly lower in the $Opg^{-/-}$\$Rank^{Tg-}$ mice and only the BV/TV in the $Opg^{-/-}$\$Rank^{Tg+}$ mice, while no difference was observed for the other genotypes (B, D, F). After treatment,

the BV/TV and Tb.N were significantly increased in the basal bone regardless of the genotype considered (B, D),), while the Tb.Th was significantly increased only in the basal bone of mice that did not over-express RANK ($Rank^{Tg-}$). ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

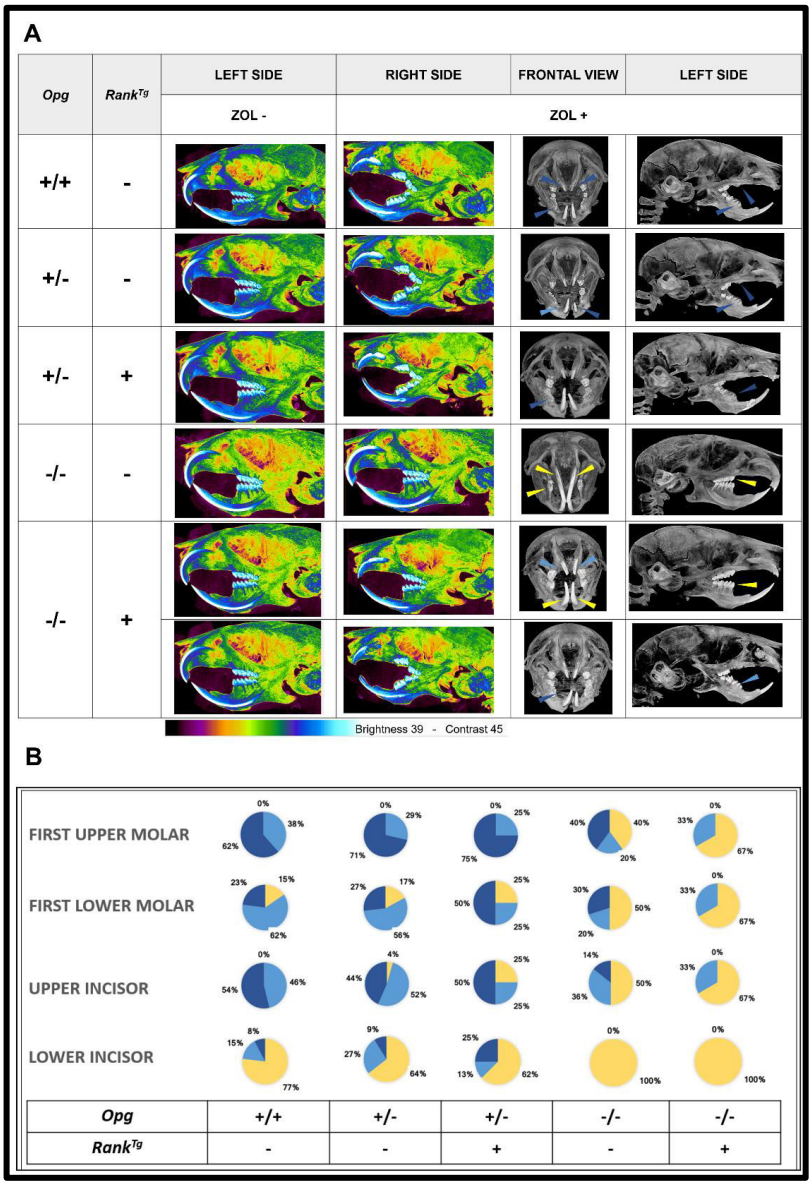


Figure 22. Comparative analysis of dental eruption between mice with different genotypes, treated or not with ZOL. Lateral right and left side views and a frontal view of the micro-CT scans of heads (A) made it possible to classify the eruption of the first molars and incisors at three different stages: fully-erupted (yellow arrow-heads), partially-erupted (clear blue arrow- erupted regardless of the genotype considered (A), while after treatment the percentage of teeth in the different categories of the classification varied depending on the genotypes (B). A graded increase in the percentage of fully-erupted teeth was observed in relation to the severity of the genotype, with the $Opg^{-/-}$\$Rank^{Tg+}$ mice evidencing more fully-erupted teeth (B).

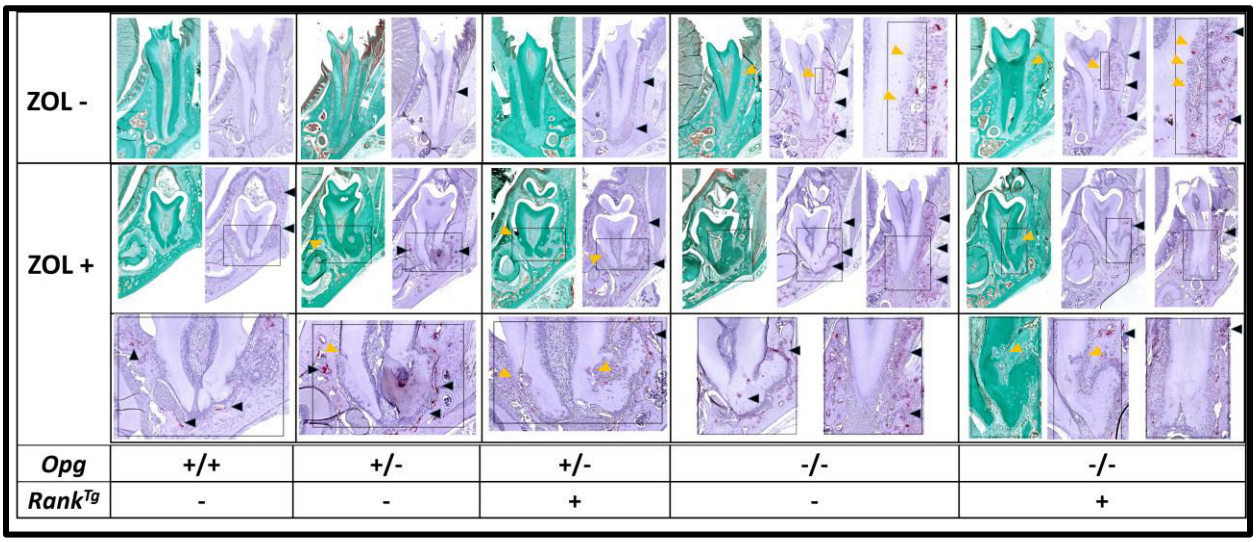


Figure 23. Comparative analysis of dento-alveolar histology between mice with different genotypes, treated or not with ZOL. Masson trichrome staining and TRAP histo-enzymology were used on frontal sections of the head in the plane of the first mandibular molars for the different genotypes, treated or not with ZOL. In the absence of treatment, the first molars were fully-erupted and a graded increase in the number of TRAP positive cells at the alveolar bone surface was visible, depending on the severity of the genotype (black arrow-heads). Small root resorption lacunas were visible (yellow arrow-heads) in the $Opg^{-/-}$ mice over-expressing or not RANK. In the treated groups, the first molars were included, except in certain $Opg^{-/-}$ mice over-expressing or not RANK. Root resorption lacunas were visible in all the included molars (yellow arrow-heads) while surprisingly not in all the fully-erupted molars, despite the fact that those teeth belonged to $Opg^{-/-}$ mice. Enlargements of the views of the apical regions made it possible to observe a graded increase in the TRAP staining at the alveolar bone surface (black arrow-heads) following the Opg allelic reduction, only in the absence of RANK over-expression ($Rank^{Tg-}$). In the RANK-over-expressing ($Rank^{Tg+}$) mice, few TRAP positive cells were present at the alveolar bone surface regardless of eruption status.

For the histology of the mandible first molar root, treatment with ZOL induced a blockage of the root elongation correlated to defective eruption regardless of the genotype considered (Fig. 23). On the contrary, when tooth eruption was effective (mainly in $Opg^{-/-}$ mice whatever the $Rank^{Tg}$ status) a close to normal root elongation was observed with surprisingly no visible root resorption lacunas comparatively to what can be seen in untreated $Opg^{-/-}$ mice (Fig. 23). Interestingly, a graded increase of the TRAP positive cell number (black arrow-heads) was observed at the alveolar bone surface from the $Opg^{+/+}\backslash Rank^{Tg-}$ to the $Opg^{-/-}\backslash Rank^{Tg+}$ in the absence of treatment whereas in the treated group two situations were observed. First in mice not transgenic for *Rank* ($Rank^{Tg-}$), a grade increase of the number of TRAP positive cells at the alveolar bone surface was observed from the $Opg^{+/+}$ to the $Opg^{-/-}$ mice

independently of the eruption/root elongation status (Fig. 23). On the contrary, in the *Rank*$^{Tg+}$ mice, regardless the *Opg* genotype, a close to wild-type mice number of TRAP positive cells were observed at the alveolar bone surface (Fig. 23). Surprisingly, in contrast to the two situations observed for the number of TRAP positive cells at the alveolar bone surface, the number of TRAP positive cells at the root surface and in the root resorption lacunas of not erupted tooth was similar for all genotypes despite the fact that lacuna sizes were gradually greater with the lose of *Opg* alleles (Fig. 23). Other classical features of none erupted tooth were observed for all genotypes as anatomical malformations and hypercementosis (Fig. 23).

In order to go further in the analyses of the craniofacial consequences of ZOL treatment in the different genotypes, the potentiality of a relationship between the dental phenotype severity and morphometric parameters alteration has been researched (Fig. 24). Skull length and the facial region length have been analyzed dependently from the first upper molar eruption status (respectively Fig. 24B and C) and mandibular length dependent on the eruption status of the first lower molar (Fig.24D). The table (Fig. 24A) summarized the mice distribution in the different genotypes according to the dental eruption status. A clear relationship was observed between the full molar eruption and the absence of craniofacial parameters alteration while in all cases of defective eruption a significant reduction of the craniofacial parameters was observed (Fig.24). Interestingly, taking into account that full eruption after ZOL treatment was observed only for *Opg*$^{-/-}$*Rank*$^{Tg-}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$ genotypes, the few mice from those genotypes with defective eruption (red asterisk) were analyzed apart and evidenced as for all the other genotypes a reduction of the craniofacial parameters (Fig. 24).

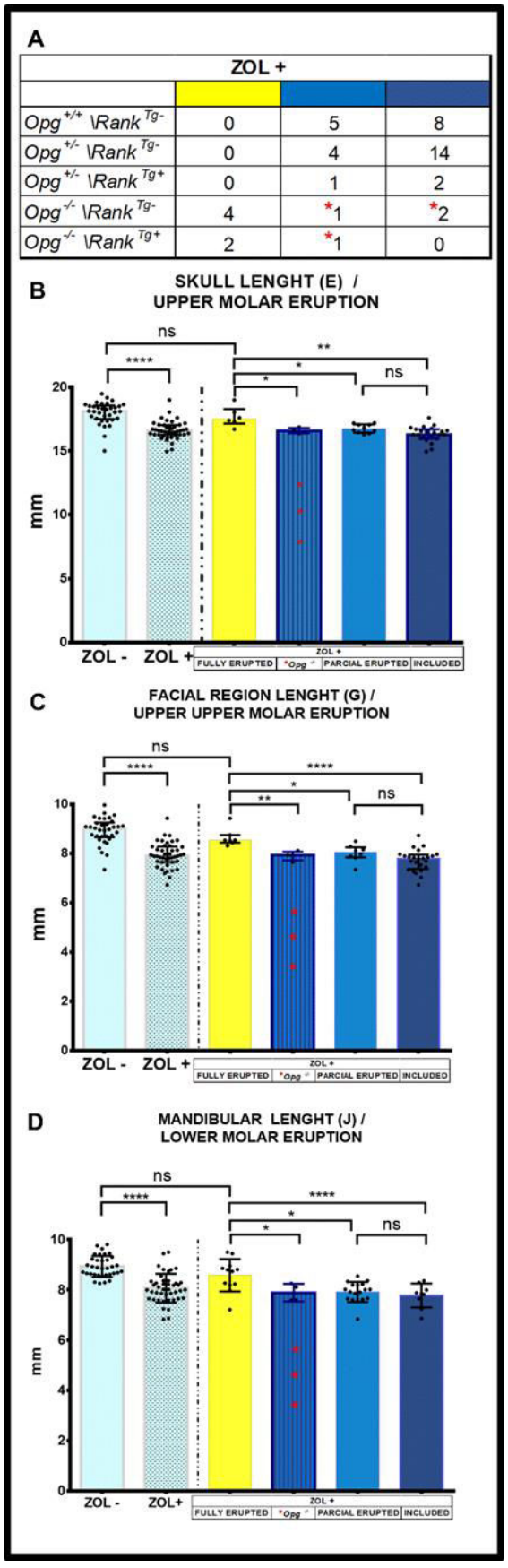


Figure 24. Correlation between defective eruption and a reduction in craniofacial sagittal morphometric parameters. In order to establish the existence of a correlation between effective tooth eruption and normal craniofacial sagittal parameter measurements, skull length (B), facial region length (C) and mandibular length (D) measurements were reanalyzed based on the eruption status of the first molars independently of genotype (A). To deal with the fact that all fully-erupted molars (yellow) were observed only in $Opg^{-/-}$ mice, the four cases of non-fully-erupted molars in $Opg^{-/-}$ mice (red stars) were analyzed separately. The results revealed a strict correlation between defective eruption and reductions in craniofacial morphometric sagittal parameters. Moreover, these parameters associated with the fully-erupted teeth of treated mice were not significantly different from those of untreated mice. ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

5.2.3 Appendicular and craniofacial skeletons of transgenic mice with different genetically-achieved RANKL activity levels, were still differentially affected a long time (10 months) after the end of the ZOL treatment. In order to analyze the long-term consequences of ZOL administered in newborn mice, twelve mice (two for each genotype) were followed from birth until ten months of age using micro-CT scans of the tibia (Fig. 25) and the head (Fig. 26). Unfortunately, at three and six months of age respectively five and three mice were sacrificed for ethical reasons due to the deterioration of their health consecutively to the development of abnormal massive tumor-like structures in the proximal region of either upper or lower incisors (Figure 26). Consequently, at ten months only four animals have been analyzed using the same parameters than for analyses realized at one and half month. Two of these mice corresponded to *Opg*$^{+/-}$*Rank*$^{Tg-}$, one to *Opg*$^{+/-}$*Rank*$^{Tg+}$ and one to *Opg*$^{-/-}$*Rank*$^{Tg+}$. In order to analyze the consequences of the ZOL treatment during growth on the bone morphometric and mineral parameters at ten months, six untreated mice with the same genotypes were used as references.

Regarding the appendicular skeleton, the BV/TV analyses evidenced that while this percentage was null for all untreated mice, it remained very high in *Opg*$^{+/-}$*Rank*$^{Tg+}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$ mice (Fig. 25A). In contrast in the *Opg*$^{+/-}$*Rank*$^{Tg-}$ mice this percentage was low comparatively to values reported at one and half month (Fig. 25A). The TMD analyses evidenced more important values in treated mice except for the *Opg*$^{+/-}$*Rank*$^{Tg+}$ mice (Fig. 25A) contrasting with the situation at one and half month. Concerning the bone morphometric parameters of the tibia at the exception of bone thickness (total length, external diameter and internal diameter), lower values were observed for the four treated mice compared to untreated mice (Fig. 25B and C).

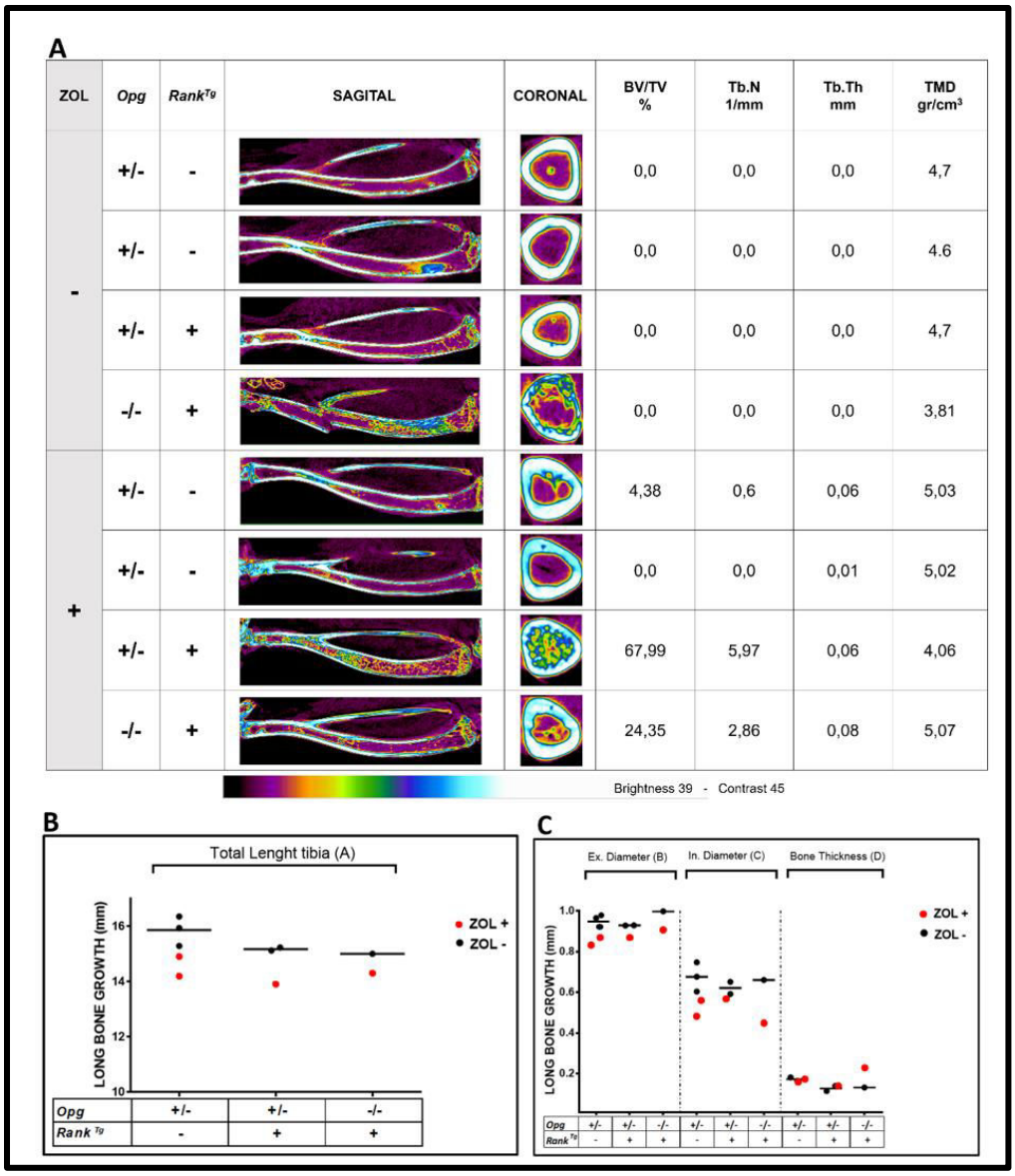


Figure 25. Remnant consequences of treatment with ZOL on bone morphometric and mineral parameters in tibias at ten months of age. Sagittal and axial micro-CT images of ten month-old mouse tibias, corresponding to the three genotypes for which mice (N=4) survived to tumor-like structures induced by ZOL treatment, were analyzed for bone mineral parameters compared to untreated mice (N=6) from the same genotypes (A). The TMD appeared slightly higher in the treated mice, except in $Opg^{+/-}$\$Rank^{Tg+}$. Interestingly, the most significant difference was observed in the $Opg^{-/-}$\$Rank^{Tg+}$ mice. The BV/TV and Tb.N were back to low values for the $Opg^{+/-}$\$Rank^{Tg-}$, close to the zero observed for untreated mice. In contrast, for RANK over-expressing mice, $Opg^{+/-}$\$Rank^{Tg+}$ and $Opg^{-/-}$\$Rank^{Tg+}$, these parameters remained high. The Tb.Th was not null, but low in the treated mice. The bone morphometric parameters in the same mice were measured (B, C). Regardless of the genotype considered, tibia length (B), and the external and internal diameters (C) appeared to decrease in the treated mice. In contrast, bone thickness was not affected (C).

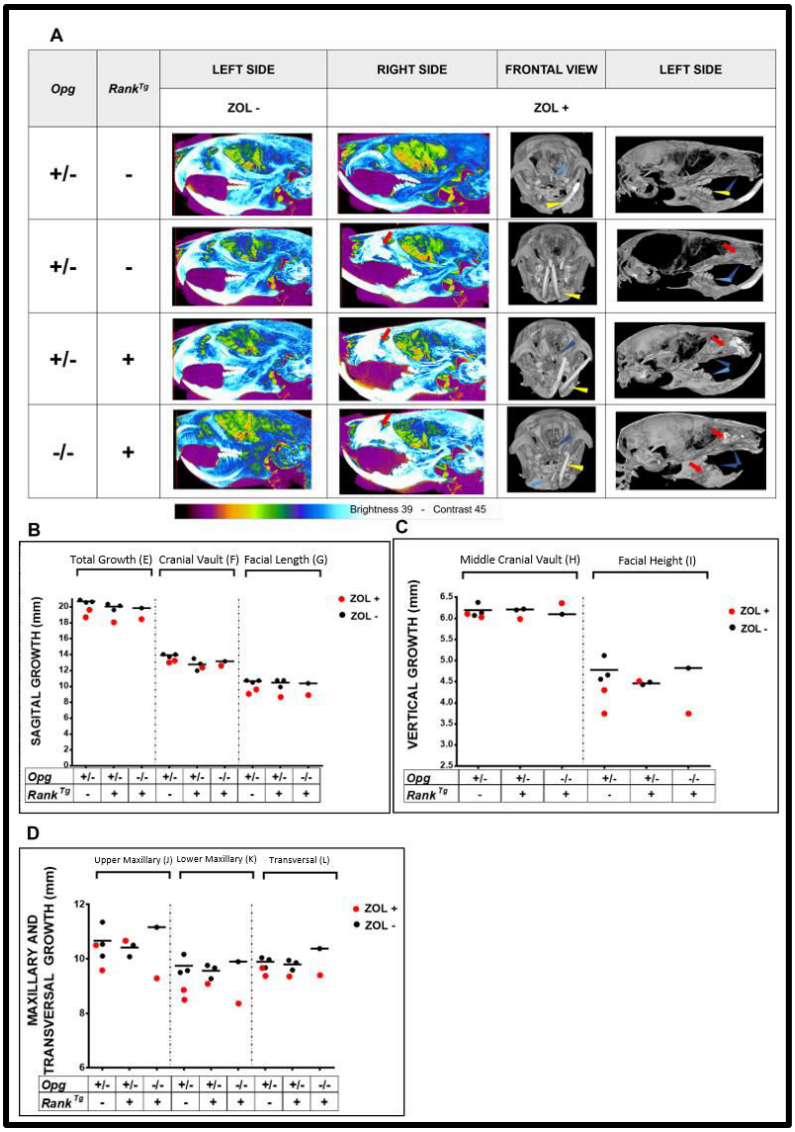


Figure 26. Remnant consequences of treatment with ZOL on dental eruption and craniofacial morphometric parameters at ten months of age. Lateral right and left side views and a frontal view of micro-CT scans of ten-month-old mouse heads (A) made it possible to reveal that parts of the molars and incisors had still not erupted (blue arrow-heads) with repercussions on the morphology of the erupted teeth (yellow arrow-heads). Tumor-like structures associated with included, continuously growing incisors were also visible (red arrow-heads). The craniofacial morphometric parameters of the four mice treated with ZOL that survived up to ten months were measured and compared to those obtained for non-treated mice from the same treatment with ZOL on dental eruption and craniofacial morphometric parameters at ten months of age. Lateral right and left side views and a frontal view of micro-CT scans of ten-month-old mouse heads (A) made it possible to reveal that parts of the molars and incisors had still not erupted (blue arrow-heads) with repercussions on the morphology of the erupted teeth (yellow arrow-heads). Tumor-like structures associated with included, continuously growing incisors were also visible (red arrow-heads). The craniofacial morphometric parameters of the four mice treated with ZOL that survived up to ten months were measured and compared to those obtained for non-treated mice from the same genotypes (B, C, D). For the measurements of sagittal growth (B), total growth and facial length were clearly lower in treated mice while the differences were less obvious for cranial vault length. For the measurements of vertical growth (C), facial height

seemed to be the only parameter reduced, except in the $Opg^{+/-}$\$Rank^{Tg+}$ mouse. For transversal growth (D), all parameters appeared to have decreased, although less significantly in the $Opg^{+/-}$\$Rank^{Tg+}$ mouse.

For the craniofacial skeleton, the micro-CT scans of the heads evidenced that the eruption defects of both incisors and first molars were still present (Fig. 26A) with in addition the presence in three of the four treated mice of tumor-like structures associated to not erupted upper incisors (red arrows in Fig. 26A). Morphometric analyses (Fig. 26B, C and D) evidenced that all mice treated (red dotes) still have smaller measures in all planes of growth (sagittal, vertical, maxillary and transversal) comparatively to untreated mice of the same genotypes (black dotes), except for the cranial vault length and the Middle cranial vault (measures F and H).

5.3 CRANIOFACIAL SKELETON PHENOTYPES OF MICE WITH DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS AT THE ADULT AGE OF TEN MONTHS

5.3.1 OPG deficiency alters the craniofacial bone homeostasis and induces morphometric alterations while RANK overexpression has a limited effect. The mandible morphometry was analyzed in order to identify the impacts of the different RANKL signaling activity level on the craniofacial bones of ten months-old mice. No significant difference was observed comparatively to *WT* regarding the total mandible length (P > 0, 005; data do not show) among the different genotypes, neither for male nor for female (mean upper mandible 9.8mm, lower 8.7mm) (Fig. 14A). On the contrary, the analysis of mandible sizes in the three different space planes (CrP, FrP and SgP) using *WT* as a reference, showed an impact of the genetic background on the mandibular proportions, without difference between sexes (Fig. 14B and Table 9). This effect was mainly associated to the absence of *Opg* (*$Opg^{-/-}$*), being more pronounced in the presence of Rank overexpression (*$Opg^{-/-}Rank^{tg+}$*). In this sense, the increases of all dimensions for female and male *$Opg^{-/-}Rank^{tg-}$* and *$Opg^{-/-}Rank^{tg+}$* (Table 9 and Fig. 27) were statistically significant (P<0.05)

compared to *WT.* Regarding the other genotypes, although no significant difference was observed, a gradual increase in the mandible dimensions was perceived in the absence of one Opg allele (*Opg*$^{+/-}$*Rank*$^{tg-}$ and *Opg*$^{+/-}$*Rank*$^{tg+}$) for both female and male (Table 9 and Graphic 27). In female a sificant increase was observed for all dimentions in the *Opg*$^{+/-}$*Rank*$^{tg+}$ mice. Interestingly, the sole presence of *Rank* (*Opg*$^{+/+}$*Rank*$^{tg+}$*)* did not affect the mandible dimensions whatever the plane considered (Table 9 and Graphic 27).

Table 9. Morphometric sizes of right mandibles of mice with different genotypes according three different spacial planes: Coronal, Frontal and Sagittal.

	WT = Opg^{++}*Rank*$^{-}$				*Opg*$^{+-}$*Rank*$^{-}$				*Opg*$^{--}$*Rank*$^{-}$				*Opg*$^{++}$*Rank*$^{+}$				*Opg*$^{+-}$*Rank*$^{+}$				*Opg*$^{--}$*Rank*$^{+}$			
	FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE	
PLANES	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD
CORONAL (CrP)	1,3	0,1	1,3	0,1	1,5	0,1	1,4	0,1	1,7	0,1	1,7	0,2	1,3	0,1	1,4	0,1	1,6	0,2	1,5	0,2	1,9	0,3	1,9	0,1
FRONTAL (FrP)	1,3	0,1	1,3	0,1	1,4	0,1	1,4	0,1	1,9	0,1	1,8	0,1	1,3	0,1	1,4	0,1	1,6	0,2	1,4	0,1	1,9	0,3	1,9	0,0
SAGITTAL (SgP)	3,0	0,2	3,0	0,2	3,3	0,2	3,1	0,4	3,5	0,2	3,4	0,4	2,9	0,2	2,9	0,3	3,4	0,2	3,1	0,2	3,9	0,3	3,5	0,2

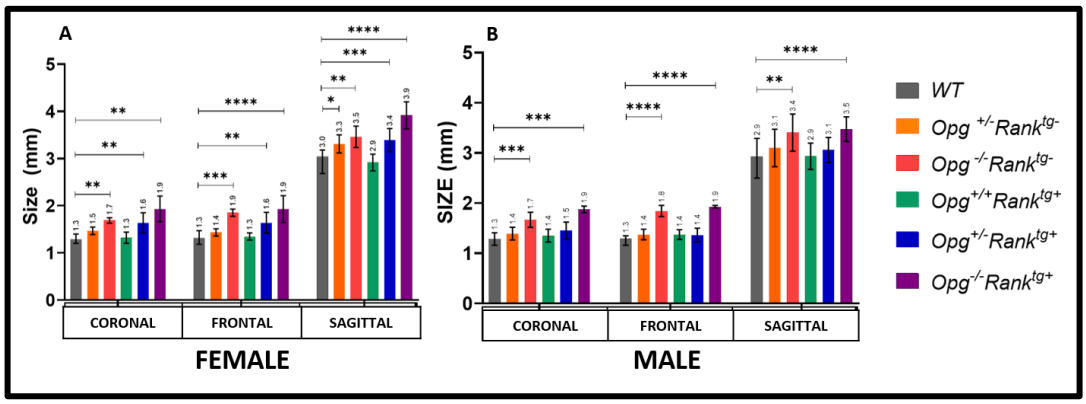


Figure 27. Comparative analysis of morphometric dimensions of right mandibles of male and female mice with different genotypes. No difference between measures analysed in females and males from the same genotype was observed. Comparisons among WT (control) with the other genetic groups evidenced statically significant difference in all planes for $Opg^{-/-}$ $Rank^{tg-}$ and $Opg^{-/-}$ $Rank^{tg+}$; and also in females $Opg^{+/-}$ $Rank^{tg+}$. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

5.3.2 Analysis of the consequences of the different genetically-achiveved RANKL activity levels on periodontal bones.

5.3.2.1 OPG expression level, not RANK, impacts the periodontal bones mineral parameters

Using the CTAn software (Fig 15), periodontal bones mineral and morphometric parameters were analyzed at ten months age. Bone mineral parameters were measured in the cortical, the cancellous, and the alveolar bone, at the level of the right lower first molar level (Fig. 15A). The %BV/TV, Tb.Th, Tb.N and Tb.Sp for cancellous and alveolar bone (Fig. 15A) and the BV for the cortical bone (Fig. 15A), were quantified for each sample.

All mineral parameters evidenced a difference between male and female from the same genotype whatever the genotype considered, being systematically lower in male (Fig 28 A, B and 29A, B, D and E). This aspect was evident for *WT* (alveolar %BV/TV mean 82.0 female and 58.6 male, cancellous %BV/TV mean 57.0 female and 48.9 male) and for $Opg^{+/-}Rank^{tg-}$ (alveolar %BV/TV mean 83.6 female and 55.6 male, cancellous %BV/TV mean 58.4 female and 37.0 male). The sex difference

was consistent for all genotypes, with presence of the same characteristic for the different bone mineral parameters (Fig. 28A, B and 29A, B, D and E), except the Tb.Sp which was higher in male than in female (Fig. 29 C and F).

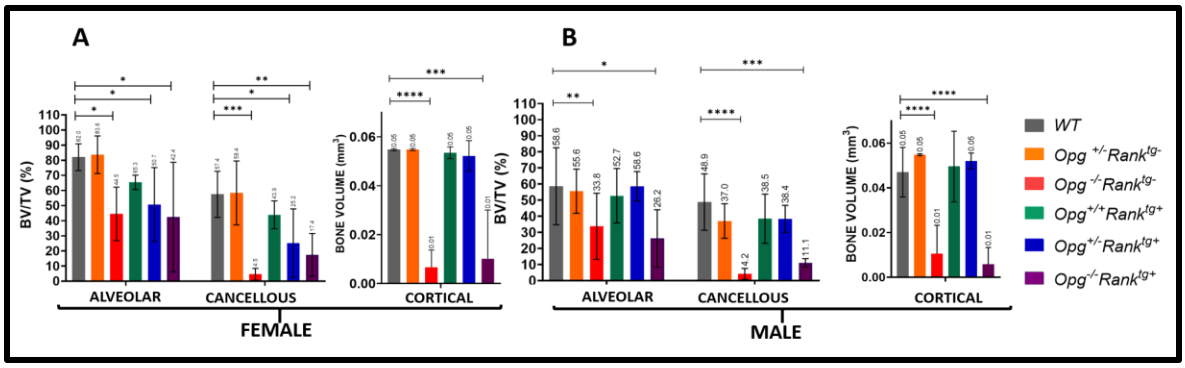


Figure 28. Analysis of porphometric dimensions of right mandibles of male and female mice with diferent genotypes. Female results; B-Male results. The %BV/TV in the three types of bone was lower for males, compared with females. A statistitcally significant reduction of the % was observed in $Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$ mice of both sex comparatively to WT. In female a significant decrease of the %BV/TV was also observed comparatively to WT in $Opg^{+/-}Rank^{tg+}$ mice. Concerning the cortical bone, BV showed a statistically significant reduction for mice of the genotypes $Opg^{-/-}Rank^{tg-}$ and $Opg^{-}Rank^{tg+}$in both sex. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

On the other hand, the analysis of the results comparing the different genotypes to the *WT*, revealed that bone mineral parameters were differently affected. In this sense, the %BV/TV was significantly lower in *$Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$* groups, both in male and female in alveolar and cancellous bone, a similar situation was observed for the BV in the cortical bone. Interestingly in *$Opg^{+/-}Rank^{tg+}$* females the %BV/TV of alveolar and cancellous bones were also statistically different from WT while the BV of cortical bone not (P<0.05). Concerning the other mineral parameters different situations were observed. First regarding Tb.Sp no significant difference was observed comparatively to *WT* (Fig. 29 C and F), whatever the genotype; te sex or the bone (alveolar or cancellous) considered. Concerning the Tb.N parameter; a significant decrease was reported only in the cancellous bone of *$Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$* mice of both sex (Fig. 29 B and E). Regarding the Tb.Th parameter, a significant decrease was reported in both alveolar and cancellous bone of *$Opg^{-/-}Rank^{tg+}$* mice for both sex (Fig. 29 A and D). A significant decrease was also observed for this parameter only in the cancellous bone of *$Opg^{-/-}Rank^{tg-}$* mice,

whatever the sex considered (Fig. 29A). Surprisingly, in females only, a significant decrease of the Tb.Th was also observed in the *Op*$^{+/-}$*Rank*$^{tg+}$ mice for the two types of bone (Fig.29 A–D).

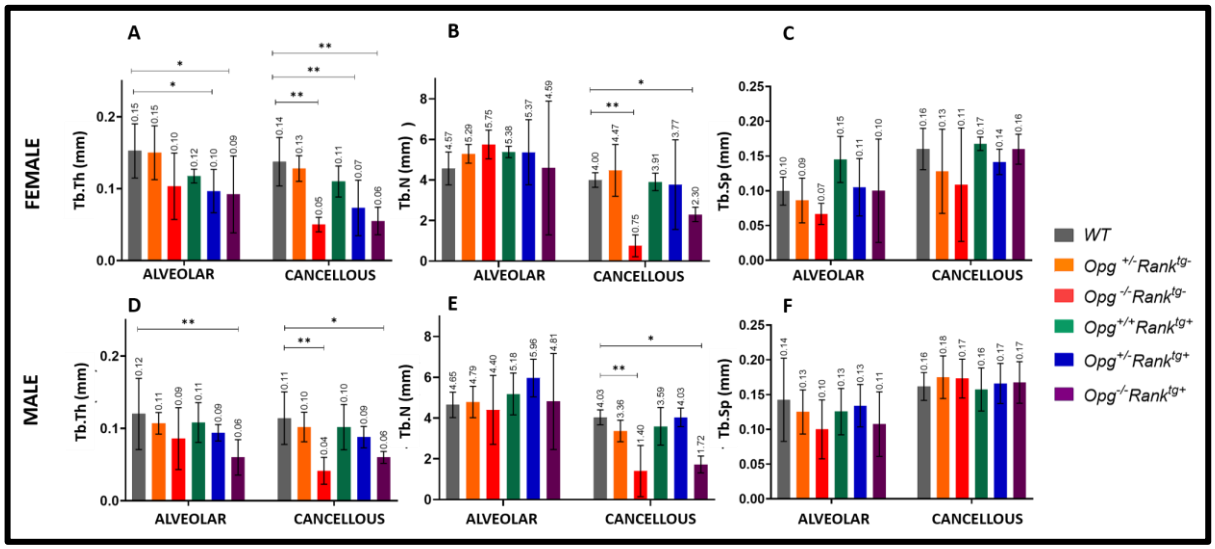


Figure 29. Comparative analysis of the trabecular thickness (Tb.Th), the trabecular number (Tb.N) and the trabecular separation (Tb.TSp) between mice female and male with different genotypes. A,B,C Female results; D,E,F Male results. The different situations were observed for the three parameters. The Tb.Th parameter was reduced in Opg$^{-/-}$Rank^{tg+} males and females in both bones, while only in the cancellous bone of male and females Opg$^{-/-}$Rank^{tg-} mice. Moreover, in Opg$^{+/-}$ Rank^{tg+} female only a significant decrease was also observed in both bones. The Tb.N parameter was reduced only in the cancellous bone of Opg$^{-/-}$Rank^{tg-} and Opg$^{-/-}$Rank^{tg+} and Opg$^{-/-}$Rank^{tg+} males and females. Whereas the Tb.Sp parameter was unaffected significantly in all mice. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

5.3.2.2 Alveolar bone height is highly dependent of the RANKL signaling activity level

The impact of the RANKL signaling activity level on the alveolar bone height was analyzed using µCT images of the right mandible mice with different genotypes with the CTvox software (Fig. 15 B and C). No differences considering the sexes were observed for each genotype whatever the site of measurements (Table 10 and Fig. 30 A and B). Moreover, whatever the sexes and the site of the measurement (buccal or lingual; mesial, central or distal) no significant reduction of the alveolar bone height was observed in the *Opg*$^{+/-}$*Rank*$^{tg-}$ and *Opg*$^{++}$*Rank*$^{tg+}$, comparatively to the

WTmice (Fig. 30A-B; Table 10). In the *Opg*$^{+/-}$*Rank*$^{tg+}$ female mice only a significant bone loss was observed only in the mesial part of the lingual region (Fig. 30). Concerning the *Opg*$^{-/-}$*Rank*$^{tg-}$ and the *Opg*$^{-/-}$*Rank*$^{tg+}$mice, two situations were observed. First, in the mesial part of both buccal and lingual region as in the distal part of the lingual region a significant bone loss was observed for the two genotypes, whatever the sex considered. Secondly, in the other parts of the alveolar bone (central part of the buccal and lingual region and distal part of the buccal region) a sex-genotype alveolar association was observed only for the *Opg*$^{-/-}$*Rank*$^{tg+}$ genotype, whereas in the male such a significant alveolar bone loss was observed only in the *Opg*$^{-/-}$*Rank*$^{tg-}$ genotype (Fig. 30A-B).

Table 10. Ten months-old mice right mandible alveolar bone loss measured in the mesial, central and distal parts of the buccal and lingual regions of right mandibles of ten months-old mice taking as a reference the first molar and sized in the mesial, the central and the distal point of the first molar.

	WT = Opg$^{+/+}$Rank^{tg-}				*Opg$^{+/-}$Rank^{tg-}*				*Opg$^{-/-}$Rank^{tg-}*				*Opg$^{+/+}$Rank^{tg+}*				*Opg$^{+/-}$Rank^{tg+}*				*Opg$^{-/-}$Rank^{tg+}*			
	FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE	
	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEA(mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEA(mm)	SD
MESIAL BUCCAL	0,28	0,07	0,27	0,03	0,27	0,04	0,31	0,10	0,87	0,07	0,92	0,26	0,38	0,04	0,33	0,08	0,38	0,11	0,35	0,03	0,78	0,41	0,54	0,37
MESIAL LINGUAL	0,55	0,10	0,54	0,12	0,59	0,13	0,57	0,13	0,77	0,23	0,84	0,12	0,62	0,17	0,70	0,19	0,83	0,23	0,73	0,09	0,94	0,14	0,81	0,01
CENTRAL BUCCAL	0,23	0,02	0,23	0,03	0,23	0,03	0,23	0,04	0,31	0,04	0,54	0,36	0,27	0,05	0,25	0,04	0,28	0,04	0,27	0,05	0,55	0,45	0,41	0,23
CENTRAL LINGUAL	0,24	0,03	0,22	0,03	0,26	0,03	0,25	0,03	0,51	0,18	0,49	0,21	0,37	0,12	0,31	0,10	0,42	0,16	0,40	0,09	0,60	0,29	0,45	0,16
DISTAL BUCCAL	0,17	0,04	0,17	0,04	0,17	0,03	0,19	0,04	0,25	0,10	0,36	0,28	0,27	0,02	0,24	0,06	0,27	0,06	0,26	0,06	0,56	0,55	0,27	0,03
DISTAL LINGUAL	0,32	0,06	0,29	0,05	0,35	0,04	0,38	0,11	0,57	0,11	0,54	0,15	0,40	0,08	0,45	0,15	0,56	0,13	0,46	0,08	0,60	0,22	0,60	0,04

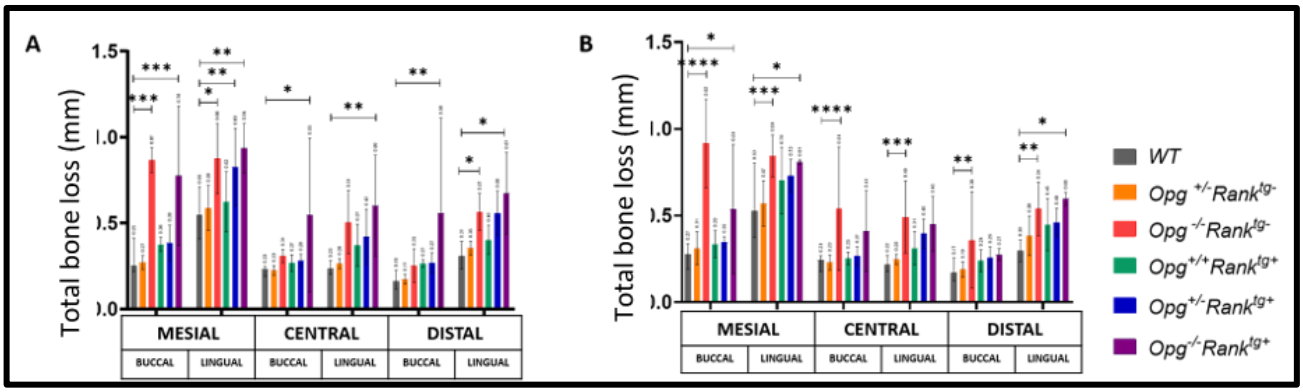


Figure 30. Comparative analysis or right mandible alveolar bone loss between mice with different genotypes, at the level of the right lower first molar. A-Female results; B-Male results. A si. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001. A significant alveolar bone loss was observed for Opg$^{-/-}$Rank^{tg-} and for Opg$^{-/-}$Rank^{tg+} mice, whatever the sex considered in the mesial part of the lingual and buccal region as in the distal part of the lingual region. In other part a sex-genotype association was observed, the Opg$^{-/-}$Rank^{tg+} female ant the Opg$^{-/-}$Rank^{tg-} male being only affected.

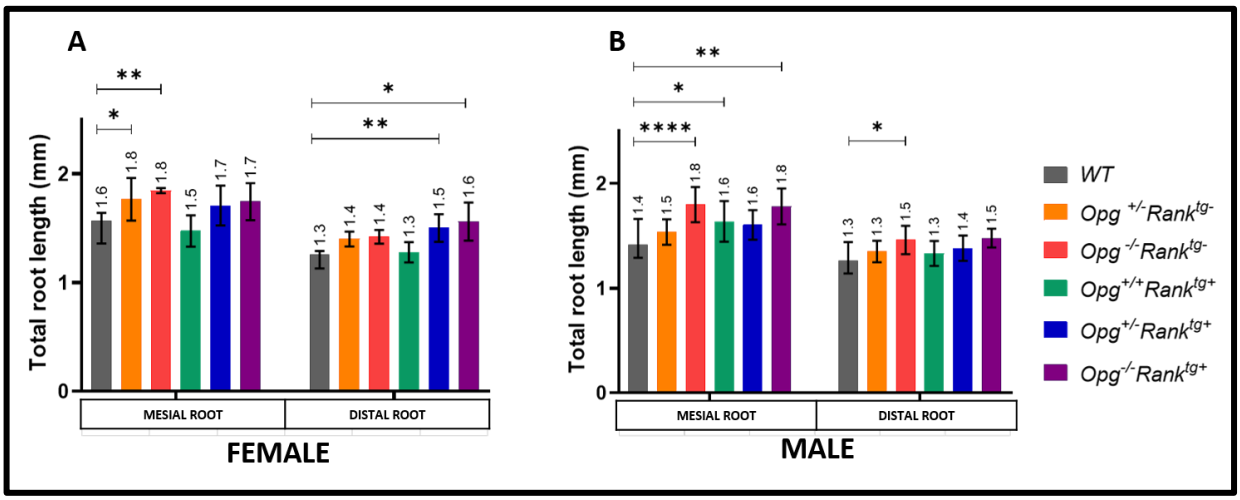


Figure 31. Comparative analysis of total root lenght results. A-Female. B-Male. Comparing with Wt, both sex showed a tendency to increase the root length in all genotypes, except for $Opg^{+/+}Rank^{tg+}$. However, there was not statistically significant in all of them. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

5.3.3 Analysis of the consequences of the different genetically-achieved ANKL ctivity levels on the morphology of the mandible first molar roots.

5.3.3.1 OPG expression level, not RANK, impacts the root structure. In order to analyze the impact of RANKL signaling activity levels onto the structure of the roots; the right mandible first molar roots were measured (Fig 16). The total length of the mesial and distal roots (figure 16 A) and the width of the mesial root in sagittal and frontal planes were sized (figure 16 B and C). No difference was observed between males and females of the same genotype, whatever the genotype considered. Concerning the root length, a tendency to increase was observed with the allelic reduction of OPG ($Opg^{+/-}$ and $Opg^{-/-}$) whatever the RANK expression did not seem to have an impact (Fig. 31). The mesial root seemed to be more sensitive than the distal root. The significance of the difference to WT was mainly observed for the $Opg^{-/-}$mice overexpressing or not RANK.

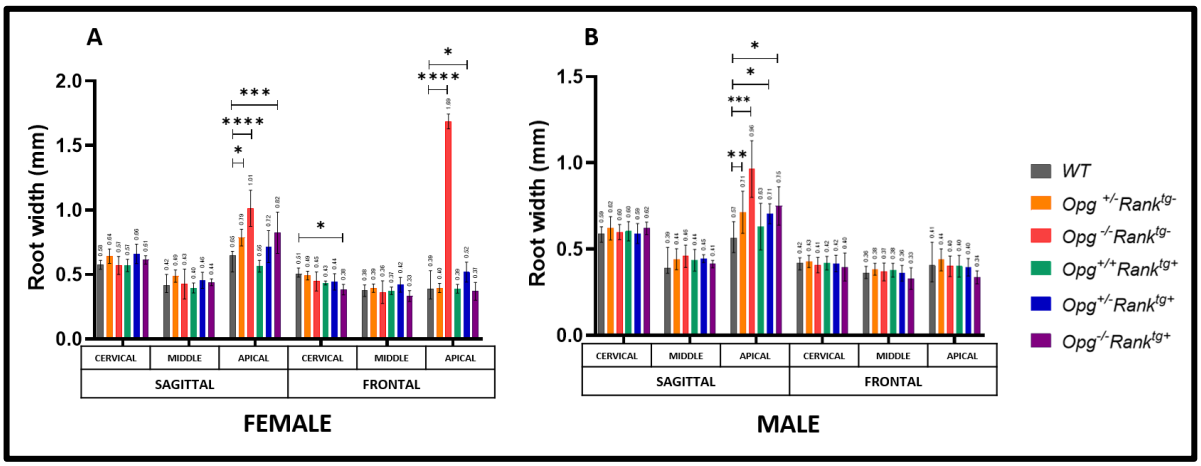


Figure 32. Comparative analysis of root width results. A-Female. B-Male**.** Comparing with WT an increase in the apical root width was identify for $Opg^{-/-}Rank^{tg-}$, both male and female. Other groups showed a tendency to increase in the absence of OPG in the cervical and middle region *: P<0.05; **: 0P<0. 1; ***: P<0.001; ****: P<0.0001.

Concerning the mesial root width (Fig. 32 A and B), no significant differences were observed in the cervical and middle areas of the root in male or female whatever the plane of analysis considered. Only a slight difference was detected in the cervical areas on the frontal plane for females (Fig. 32 A). Regarding the apical area pf the mesial root a tendency to increase was observed with the allelic reduction of the OPG (*$Opg^{+/-}$ and $Opg^{-/-}$*) mainly in the sagittal plane, whereas the RANK overexpression did not see to have an impact (Fig. 32A-B). Significantly differences from *WT* measures were mainly observed in *$Opg^{-/-}$* mice, independently of RANK status.

5.3.3.2 OPG deficiency is associated with the presence of root resorption: In order to analyze the impact of RANKL signaling level onto the presence of root resorption, the number of roots which evidence at least one resorption lacuna was quantified using μCT images and histological sections (Fig.33). The roots of the six lower molars (3 right and 3 left) were taken in account for this analysis. Results evidenced no differences between sexes (Fig. 34), males and females showing the same patron of root resorption; except for the $Opg^{+/-}Rank^{tg+}$ genotype in which a smaller number of root lacunes were observed in females (10% versus 33% in males). The comparative analysis among the different genotypes evidenced no difference between WT and $Opg^{+/-}Rank^{tg-}$ mice. Interestingly, in absence of OPG ($Opg^{-/-}$) an important increase of the number of roots with resorption lacunas was observed (Fig. 34), being more severe when combined to RANK overexpression ($Opg^{-/-}Rank^{tg-}$ 44% female and 42% male and $Opg^{-/-}Rank^{tg+}$ 83% female and 78% male) comparatively to WT (6% female and 7% male) (Fig. 34). On the other hand, the sole Rank overexpression induced a slight increase of the number of root resorption lacunas ($Opg^{+/+}Rank^{tg+}$ 27% female and 20% male). Clearly, the absence

of OPG ($Opg^{-/-}$), and to a lesser extent, the RANK overexpression increased the number of root resorptions (Fig. 34).

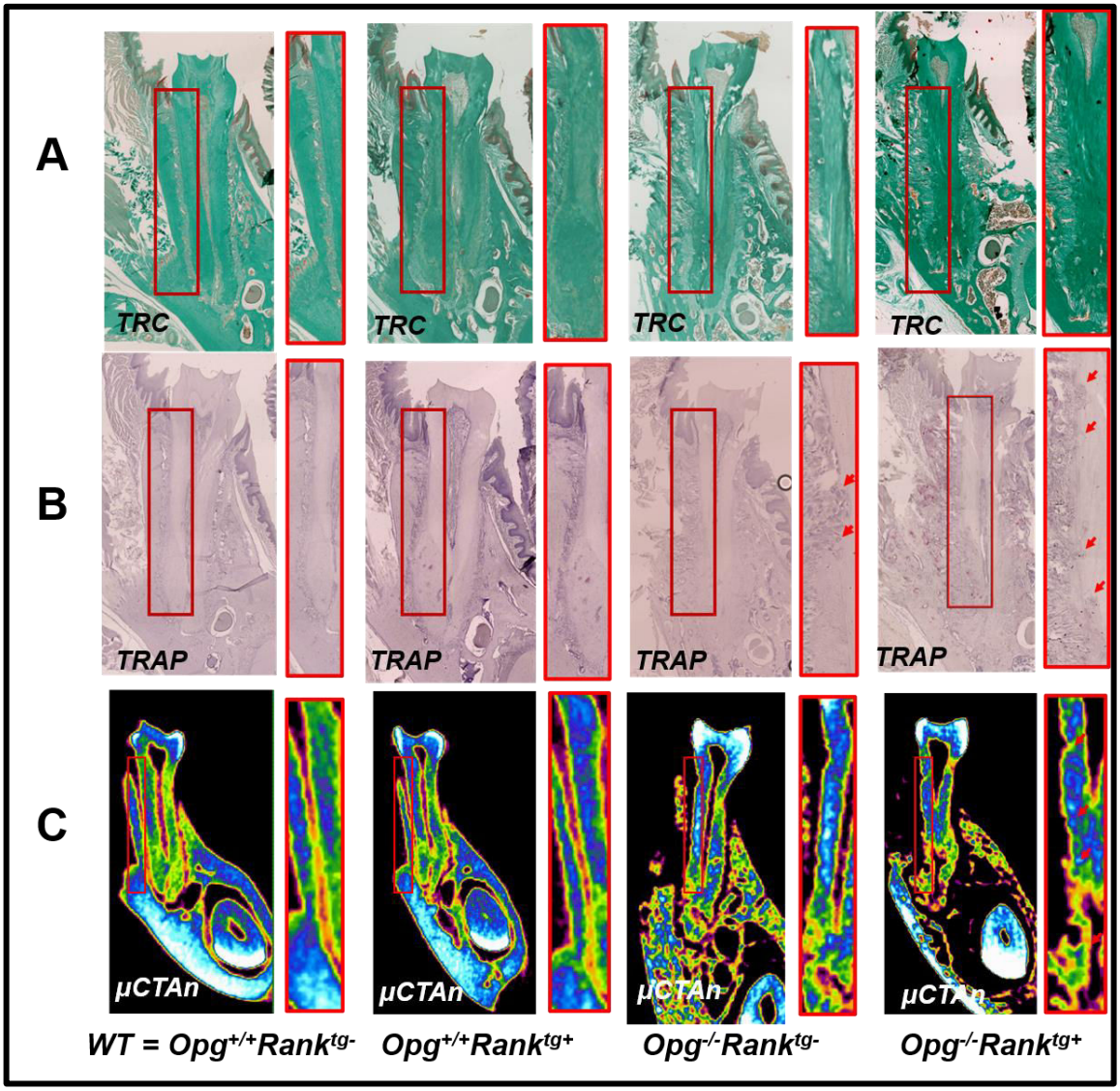


Figure 33. Analysis of the root resorption number in the frontal plane of right mandible of mice with different genotypes. A: histological analysis by Masson's trichrome staining to identify morphological alterations. B: Histological analysis by TRAP staining to identify the OCs cells. C: µCT image acquired by CTAn software. Red arrows indicate different points of root resorption.

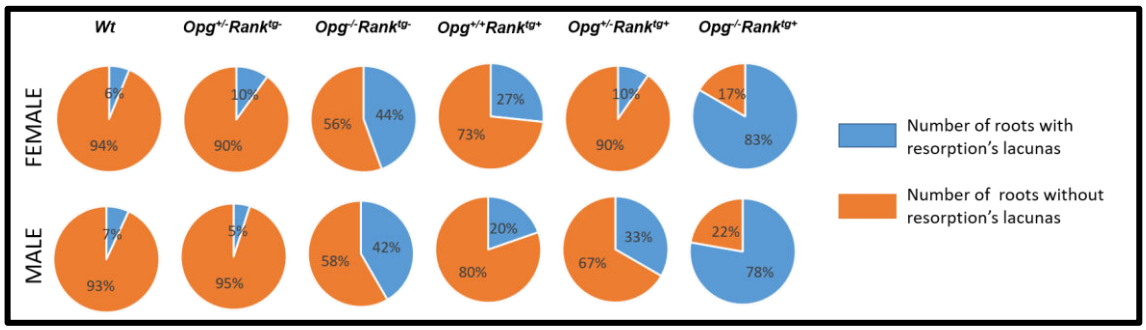


Figure 34. Pie chart of root resorption results according to the different genotypes. The number of roots with almost one lacuna of resorption was quantified for each mice (3 right and 3 left molars per mice) in lower maxillary. The number of roots with resorption increase significantly in the absence of OPG ($Opg^{-/-}Rank^{Tg-}$) and was higher in the presence of $Rank^{tg}$ ($Opg^{-/-}Rank^{Tg+}$).

5.3.4 Impact on the osteoclast number of the different RANKL signaling activity levels.

An histological analysis was performed, in order to identify morphological alterations associated to the different genotype by Masson's trichrome staining and osteoclast cell activity staining (tartrate-resistant acid phosphatase (TRAP)) on the right mandibles of mice (Fig. 35). No difference between sexes was founded. Regarding the analysis of the mice from the different genotypes comparatively to *WT* mice (Fig. 35 A), the only mouse genotypes that showed morphological alterations were $Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{t+}$, with the presence of changes not only in the cortical, the cancellous and the alveolar bone but also in the root structure (Fig 35 A). This result is consistent with those of macroscopical analysis (Fig. 14) that showed changes in bone dimensions related to cortical and cancellous width. Regarding the OCs number, the TRAP staining evidenced a dramatic increase of the OCs number, principally in $Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$ mice (Fig. 35 B). Mice of the other genotypes did not show a difference in osteoclast cell number (TRAP staining) which was quite similar to *WT* (figures not shown). Finally, the morphological alterations in

the cortical and the cancellous bones and the root resorptions appeared to be associated with the increment in the osteoclast cells (Fig. 35 B).

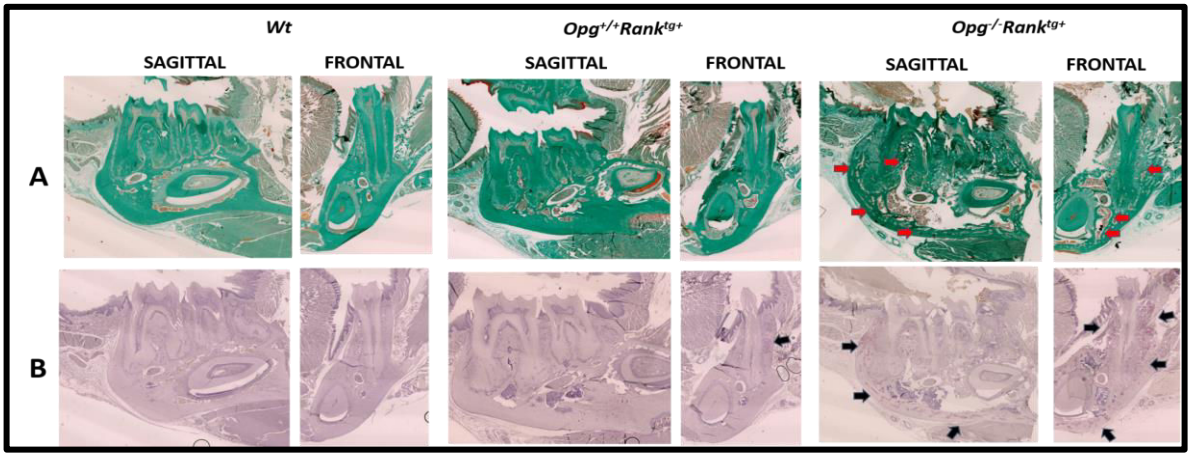


Figure 35. Histological analysis of the right mandible of mice from the different genotypes. A: Masson's trichrome staining to analyze the morphology. B: Tartrate-resistant acid phosphatase staining (TRAP) to identify multinucleated osteoclast cell numbers in sagittal and frontal sections. A: $Opg^{-/-}Rank^{tg+}$and $Opg^{-/-}Rank^{tg-}$ mice models presented more alterations of bones (cortical, cancellous, and alveolar) comparatively to WT mice. B: An important number of OCs were observed in the $Opg^{-/-}Rank^{tg+}$ mice, affecting the cortical, the cancellous and the alveolar bones. A less number of osteoclasts was found in $Opg^{-/-}Rank^{tg-}$ mice comparatively to WT.

5.4 ZOL TREATMENT CONSEQUENCES ON THE ADULT CRANIOFACIAL SKELETON OF MICE WITH DIFFERENT GENETICALLY-ACHIEVED RANKL ACTIVITY LEVELS

Table 11. Distribution of adult mice that have been treated with ZOL during the experimental period and that survive up to 10 months.

	GENOTYPE					
	WT= $Opg^{+/+}Rank^{tg-}$	*$Opg^{+/-}Rank^{tg-}$*	*$Opg^{-/-}Rank^{tg-}$*	*$Opg^{+/+}Rank^{tg+}$*	*$Opg^{+/-}Rank^{tg+}$*	*$Opg^{-/-}Rank^{tg+}$*
♀	1	4	0	0	4	2
♂	0	4	1	1	3	0

The effects of ZOL treatment on the craniofacial skeleton of adult mice with different RANKL signaling activity levels have been analyzed continuously with rigorous periodic checking using an in-vivo scanner to follow the phenotypical appearance, from two months of age to ten months. During this period 3 *WT=Opg*$^{+/+}$*Rank*$^{tg-}$ (two males and one female), two *Opg*$^{+/+}$*Rank*$^{tg+}$ females and one *Opg*$^{+/-}$*Rank*$^{tg+}$ male mice, which have been treated with ZOL, died before ten months and were so excluded from the analysis (Table 12). Regarding the mandible structure, no density modifications related to atraumatic necrotic areas in the craniofacial skeleton were found at ten months, whatever the genotype considered (Fig. 36 and 37). This means that the hypothesis related to the spontaneous occurrence of the BRONJ, at least in the macroscopic findings, was not confirmed. The data related to the periodontal bones response to ZOL are summarized in figures 38 and 40. The analysis of basal bone (cortical and cancellous) and alveolar bone showed a similar phenotype in each genetic background, in treated and untreated groups (Fig. 29, 30 and 40). Indeed, concerning the bone morphometric parameters, no major (significative) variations were found comparatively to untreated animals of the same genotype. Considering deeply those results reported (Fig. 40) slight increases of the BV/TV and the Tb.N parameters were observed mainly in the cancellous bone of the treated groups (Fig. 28, 29 and 38) while the Tb.Th parameter seems to be similar to untreated groups (Fig. 29 and 38) and the Tb.Sp parameter decrease in all treated groups (Fig. 29 and 38). Although these observations concerning the mineral parameters were consistent in all groups, the most important response was found associated to the absence of OPG (*Opg*$^{-/-}$*Rank*$^{tg-}$*, Opg*$^{-/-}$*Rank*$^{tg+}$) and independently of the RANK overexpression (Fig. 39). Concerning the alveolar bone loss, the treatment with ZOL induces a general decrease for all measurements, except in the *Opg*$^{+/+}$*Rank*$^{tg-}$, *Opg*$^{-/-}$*Rank*$^{tg-}$ and *Opg*$^{-/-}$*Rank*$^{tg+}$ which all showed a slight similar increase of bone loss for the MB and DL measures (Fig. 40 and 41). Regarding the root analyses, the ZOL treatment induces a slight decrease of all length measurements while for the width measurement a decrease was observed for all genotypes in the transversal section and an increase in the frontal section, except

for the wild-type group (Fig. 42 and 43). Concerning the number of root resorption lacunas a decrease was observed following the ZOL treatment, except of th *Opg*$^{-/-}$ *Rank*$^{tg-}$ group for which an increase was surprisingly observed (Fig. 43 and 44).

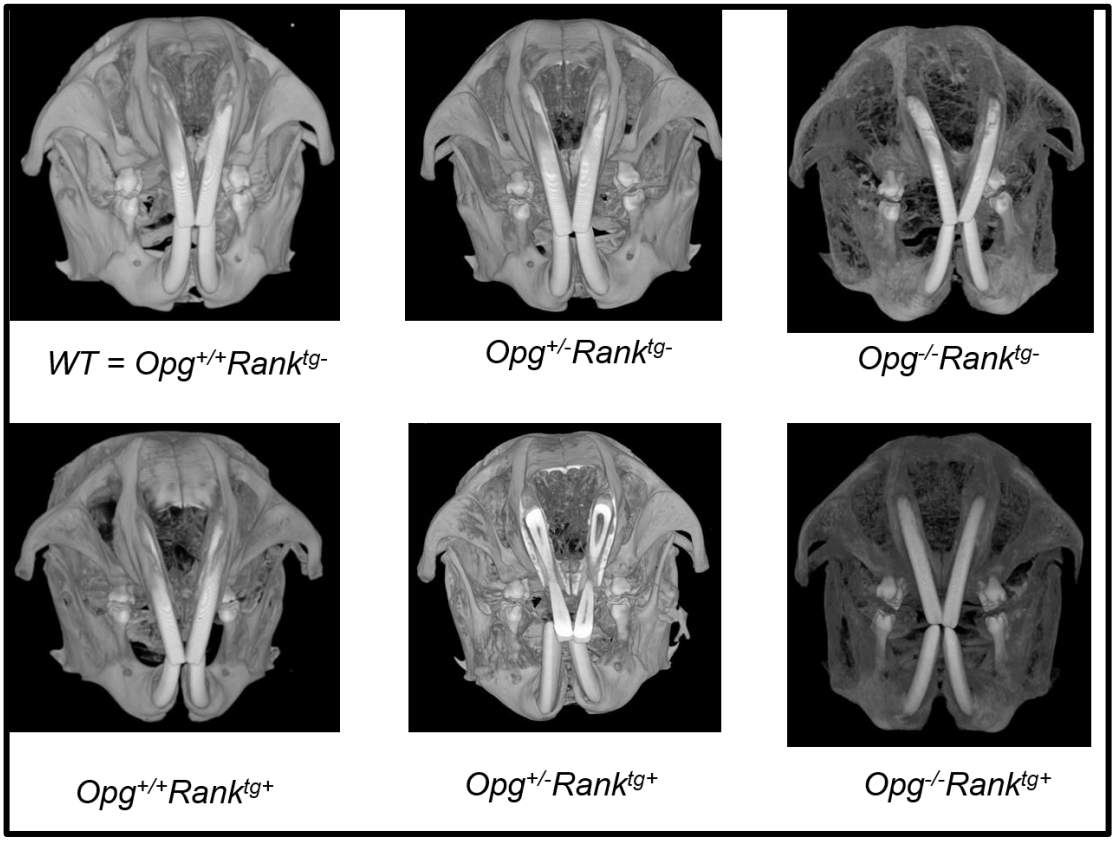


Figure 36. Frontal view of 3D reconstructions of heads of mice the different genotypes. Craniofacial phenotypes of adult mice with different RANKL signaling activity levels treated with ZOL.

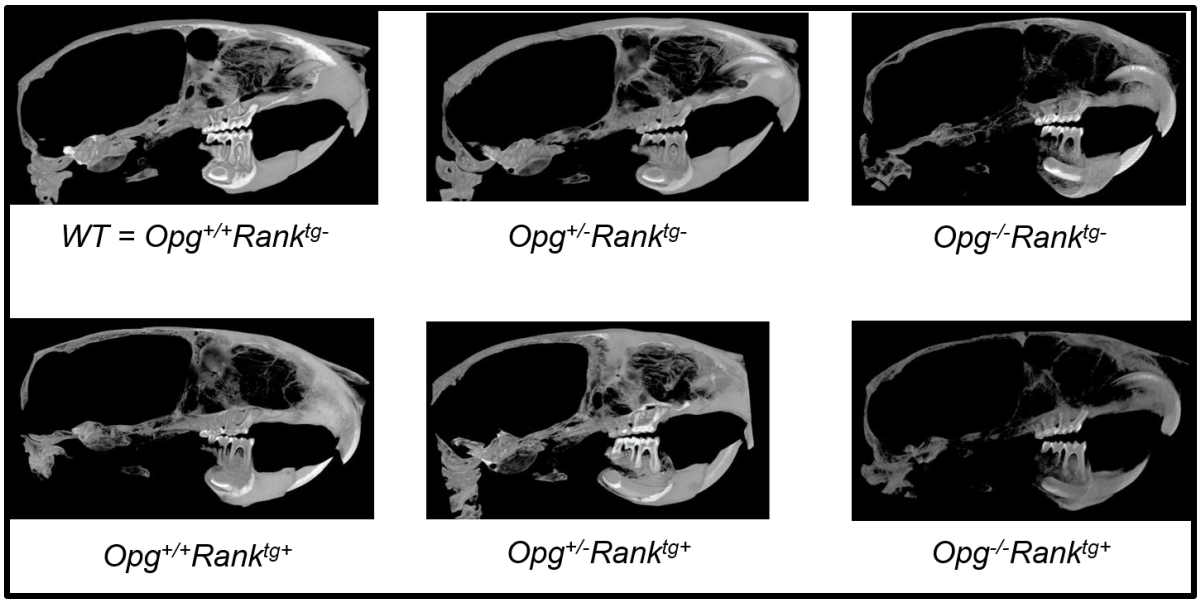
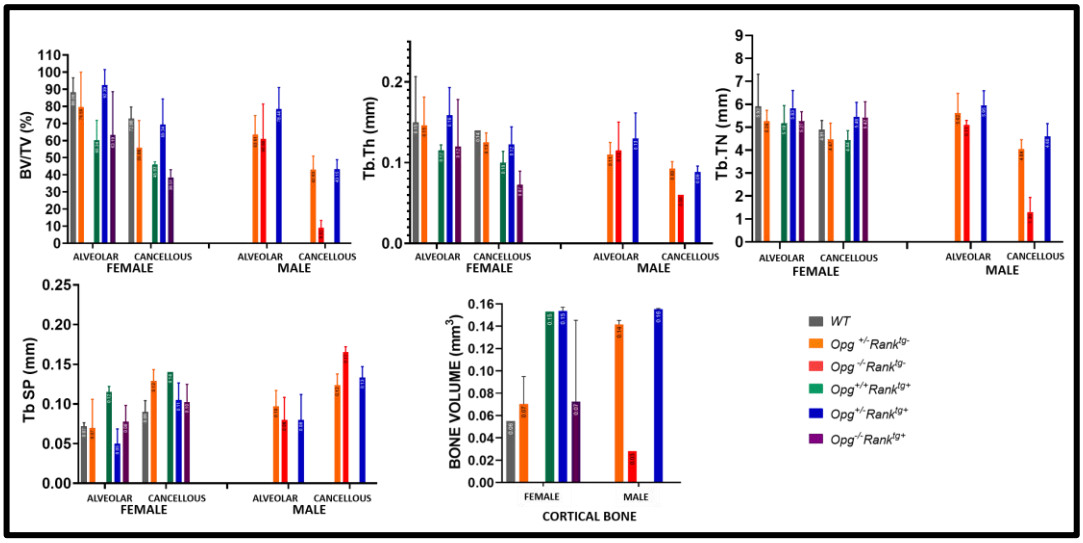


Figure 37. Sagittal view of 3D reconstructions of heads of mice the different genotypes. Craniofacial phenotypes of adults mice with different RANKL signaling activity levels treated with ZOL.



BV/TV	WT = Opg+/+Rank+/+			Opg-/-Rank+/+			Opg-/-Rank+/-			Opg+/+Rank+/+			Opg+/+Rank+/-			Opg-/-Rank+/-		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
FEMALE ALVEOLAR	88,09	8,57	2	79,56	20,42	8				60,34	11,51	2	92,39	9,11	8	63,19	25,37	4
MALE ALVEOLAR				63,61	10,99	8	60,96	20,35	2				78,46	12,57	6			
FEMALE CANCELLOUS	72,88	6,68	2	55,88	15,88	8				45,93	1,63	2	69,34	14,95	8	38,51	4,46	4
MALE CANCELLOUS				42,93	8,01	8	8,92	4,44	2				43,15	5,70	6			

Tb.Th	WT = Opg+/+Rank+/+			Opg-/-Rank+/+			Opg-/-Rank+/-			Opg+/+Rank+/+			Opg+/+Rank+/-			Opg-/-Rank+/-		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
FEMALE ALVEOLAR	0,15	0,06	2	0,15	0,04	8				0,12	0,01	2	0,16	0,03	8	0,12	0,06	4
MALE ALVEOLAR				0,11	0,02	8	0,12	0,04	2				0,13	0,03	6			
FEMALE CANCELLOUS	0,14	0	2	0,13	0,01	8				0,10	0,01	2	0,12	0,02	8	0,07	0,02	4
MALE CANCELLOUS				0,09	0,01	8	0,06	0,00	2				0,09	0,01	6			

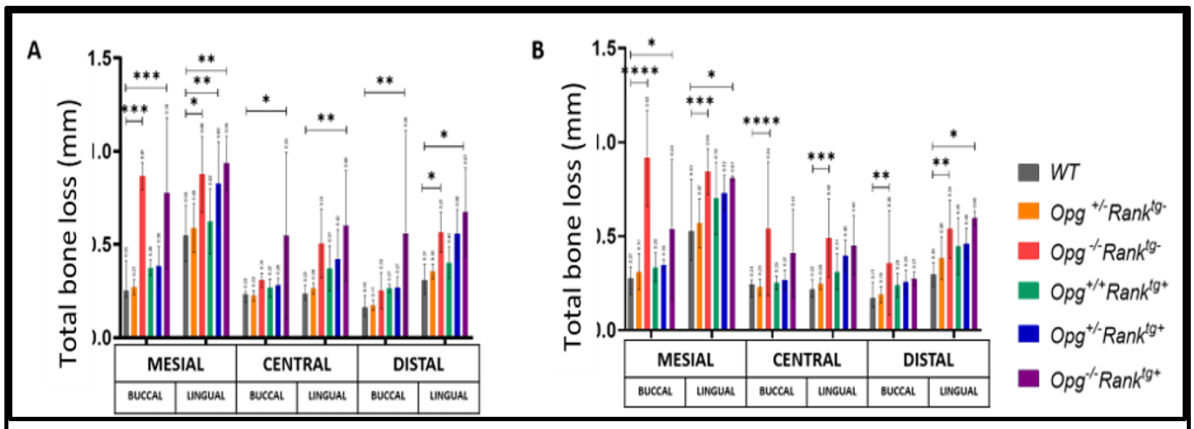
Tb.N	WT = Opg+/+Rank+/+			Opg-/-Rank+/+			Opg-/-Rank+/-			Opg+/+Rank+/+			Opg+/+Rank+/-			Opg-/-Rank+/-		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
FEMALE ALVEOLAR	5,92	1,39	2	5,26	0,47	8				5,18	0,77	2	5,83	0,77	8	5,27	0,40	4
MALE ALVEOLAR				5,62	0,85	8	5,11	0,19	2				5,95	0,64	6			
FEMALE CANCELLOUS	4,91	0,39	2	4,47	0,70	8				4,44	0,42	2	5,46	0,62	8	5,42	0,69	4
MALE CANCELLOUS				4,04	0,42	8	1,30	0,64	2				4,60	0,56	6			

Tb.Sp	WT = Opg+/+Rank+/+			Opg-/-Rank+/+			Opg-/-Rank+/-			Opg+/+Rank+/+			Opg+/+Rank+/-			Opg-/-Rank+/-		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
FEMALE ALVEOLAR	0,07	0,00	2	0,07	0,04	8				0,12	0,01	2	0,05	0,02	8	0,08	0,02	4
MALE ALVEOLAR				0,10	0,02	8	0,08	0,03	2				0,08	0,03	6			
FEMALE CANCELLOUS	0,09	0,01	2	0,13	0,01	8				0,14	0,00	2	0,11	0,02	8	0,10	0,02	4
MALE CANCELLOUS				0,12	0,01	8	0,17	0,01	2				0,13	0,01	6			

Figure 38. Bone mineral parameters of ten months-old mice-treated with ZOL at two months age and during eight weeks. Craniofacial bones mineral parameters measured in ten months-old mice of the different genotypes treated with ZOL during eight weeks starting at two months of age.

PARAMETER \ GENOTYPE		WT=Opg+/+Rank^{Tg-}		Opg+/-Rank^{Tg-}		Opg-/-Rank^{Tg-}		Opg+/+Rank^{Tg+}		Opg+/-Rank^{Tg+}		Opg-/-Rank^{Tg+}	
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
BV/TV	Alveolar	↑	N/A	↓	↑	N/A	↑↑	↓	N/A	↑↑	↑	↑	N/A
	Cancellous	↑↑	N/A	↓	↑	N/A	↑↑	↑	N/A	↑↑	↑	↑↑↑	N/A
Tb.Th	Alveolar	=	N/A	=	=	N/A	↑	=	N/A	↑	↑	↑	N/A
	Cancellous	=	N/A	=	↓	N/A	↑	=	N/A	↑	=	↑	N/A
Tb.N	Alveolar	↑	N/A	↓	↑	N/A	↑	↓	N/A	↑	=	↑	N/A
	Cancellous	↑	N/A	↑	↑	N/A	↓	↑	N/A	↑	↑	↑↑	N/A
Tb.Sep	Alveolar	↓	N/A	↓	↓	N/A	↓	↓	N/A	↓	↓	↓	N/A
	Cancellous	↓	N/A	=	↓	N/A	=	↓	N/A	↓	↓	↓	N/A

Figure 39. Analysis of the variations of the bone mineral parameters induce by the ZOL treatment for the different genotypes. ↑ (Increase of the parameter. ↓(Decrease of the parameter). = (No modification of the parameter). N/A (not applicable).

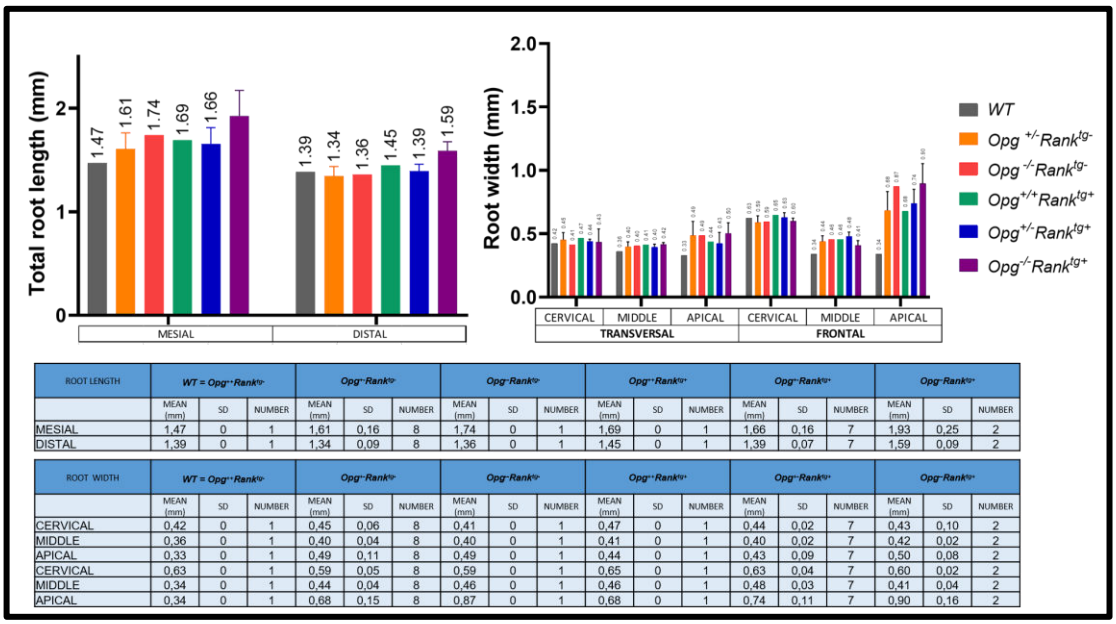


	WT = Opg+/+Rankтg-				Opg+/-Rankтg-				Opg-/-Rankтg-				Opg+/+Rankтg+				Opg+/-Rankтg+				Opg-/-Rankтg+			
	FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE		FEMALE		MALE	
	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEA(mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEAN (mm)	SD	MEA(mm)	SD
MESIAL BUCCAL	0,28	0,07	0,27	0,03	0,27	0,04	0,31	0,10	0,87	0,07	0,92	0,26	0,38	0,04	0,33	0,08	0,38	0,11	0,35	0,03	0,78	0,41	0,54	0,37
MESIAL LINGUAL	0,55	0,10	0,54	0,12	0,59	0,13	0,57	0,13	0,77	0,23	0,84	0,12	0,62	0,17	0,70	0,19	0,83	0,23	0,73	0,09	0,94	0,14	0,81	0,01
CENTRAL BUCCAL	0,23	0,02	0,23	0,03	0,23	0,03	0,23	0,04	0,31	0,04	0,54	0,36	0,27	0,05	0,25	0,04	0,28	0,04	0,27	0,05	0,55	0,45	0,41	0,23
CENTRAL LINGUAL	0,24	0,03	0,22	0,03	0,26	0,03	0,25	0,03	0,51	0,18	0,49	0,21	0,37	0,12	0,31	0,10	0,42	0,16	0,40	0,09	0,60	0,29	0,45	0,16
DISTAL BUCCAL	0,17	0,04	0,17	0,04	0,17	0,03	0,19	0,04	0,25	0,10	0,36	0,28	0,27	0,02	0,24	0,06	0,27	0,06	0,26	0,06	0,56	0,55	0,27	0,03
DISTAL LINGUAL	0,32	0,06	0,29	0,05	0,35	0,04	0,38	0,11	0,57	0,11	0,54	0,15	0,40	0,08	0,45	0,15	0,56	0,13	0,46	0,08	0,60	0,22	0,60	0,04

Figure 40. Total alveolar bone loss meassured in ten months-old mice treated with ZOL at two months age and during eight weeks.

PARAMETER / GENOTYPE		WT=Opg+/+Rankтg-	Opg+/-Rankтg-	Opg-/-Rankтg-	Opg+/+Rankтg+	Opg+/-Rankтg+	Opg-/-Rankтg+
MESIAL	Bucal	↑	=	↑	↓	↓	↑
	Lingual	↓	=	=	↓	↓	=
CENTRAL	Bucal	↓	↓	↓	↑	↓	↓
	Lingual	↓	↓	↓	↓	↓	=
DISTAL	Bucal	↓	↓	↓	↓	↓	↓
	Lingual	↑	=	↑	=	=	↑

Figure 41. Analysis of the variations of the alveolar bone loss in presence of ZOL induce by the ZOL treatment for the different genotypes. ↑ (Increase of the parameter. ↓(Decrease of the parameter). = (No modification of the parameter). N/A (not applicable).



ROOT LENGTH	WT = Opg+/+Rank tg-			Opg+/-Rank tg-			Opg-/-Rank tg-			Opg+/+Rank tg+			Opg+/-Rank tg+			Opg-/-Rank tg+		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
MESIAL	1,47	0	1	1,61	0,16	8	1,74	0	1	1,69	0	1	1,66	0,16	7	1,93	0,25	2
DISTAL	1,39	0	1	1,34	0,09	8	1,36	0	1	1,45	0	1	1,39	0,07	7	1,59	0,09	2

ROOT WIDTH	WT = Opg+/+Rank tg-			Opg+/-Rank tg-			Opg-/-Rank tg-			Opg+/+Rank tg+			Opg+/-Rank tg+			Opg-/-Rank tg+		
	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER	MEAN (mm)	SD	NUMBER
CERVICAL	0,42	0	1	0,45	0,06	8	0,41	0	1	0,47	0	1	0,44	0,02	7	0,43	0,10	2
MIDDLE	0,36	0	1	0,40	0,04	8	0,40	0	1	0,41	0	1	0,40	0,02	7	0,42	0,02	2
APICAL	0,33	0	1	0,49	0,11	8	0,49	0	1	0,44	0	1	0,43	0,09	7	0,50	0,08	2
CERVICAL	0,63	0	1	0,59	0,05	8	0,59	0	1	0,65	0	1	0,63	0,04	7	0,60	0,02	2
MIDDLE	0,34	0	1	0,44	0,04	8	0,46	0	1	0,46	0	1	0,48	0,03	7	0,41	0,04	2
APICAL	0,34	0	1	0,68	0,15	8	0,87	0	1	0,68	0	1	0,74	0,11	7	0,90	0,16	2

Figure 42. Analysis of root length and width of the mandible first molar of ten months-old mice of the different genotypes treated with ZOL.

PARAMETER	GENOTYPE		WT=Opg+/+Rank Tg-	Opg+/-Rank Tg-	Opg-/-Rank Tg-	Opg+/+Rank Tg+	Opg+/-Rank Tg+	Opg-/-Rank Tg+
ROOT LENGTH	MESIAL ROOT		↓	↓	↓	↓	↓	↓
	DISTAL ROOT		↓	↓	↓	=	↓	↓
MESIAL ROOT WIDTH	TRANSVERSAL SECTION	Cervical	↓	↓	↓	↓	↓	↓
		Middle	↓	↓	↓	=	↓	↓
		Apical	↓	↓	↓	↓	↓	↓
	FRONTAL SECTION	Cervical	↓	↑	↑	↑	↑	↑
		Middle	↓	↑	↑	↑	↑	↑
		Apical	↓	↓	↑	↑	↑	↑
ROOT RESORPTION	Number of roots with presence of resorption lacunas		↓	↓	↑	↓	↓	↓

Figure 43. Analysis of the variations of root measurements and the number of resorption lacunas induce by the ZOL treatment in the different genotypes. ↑ (increase of the parameter. ↓(Decrease of the parameter). = (No modification of the parameters). N/A (not applicable). A tendency to decrease of all the root parameters was observed in *WT.* However when OPG and RANK were modulated some variations of the parameters were reported, mainly in total absence of OPG.

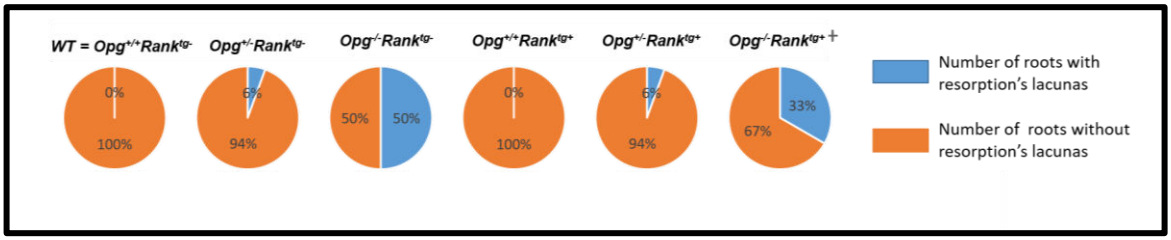


Figure 44. Pie chart of the number of roots resorption results according to the different genotypes treated with ZOL. The number of roots with almost one lacuna of resorption was quantified for each mice mandible molars(3 right and 3 left per mice) in lower maxillary.

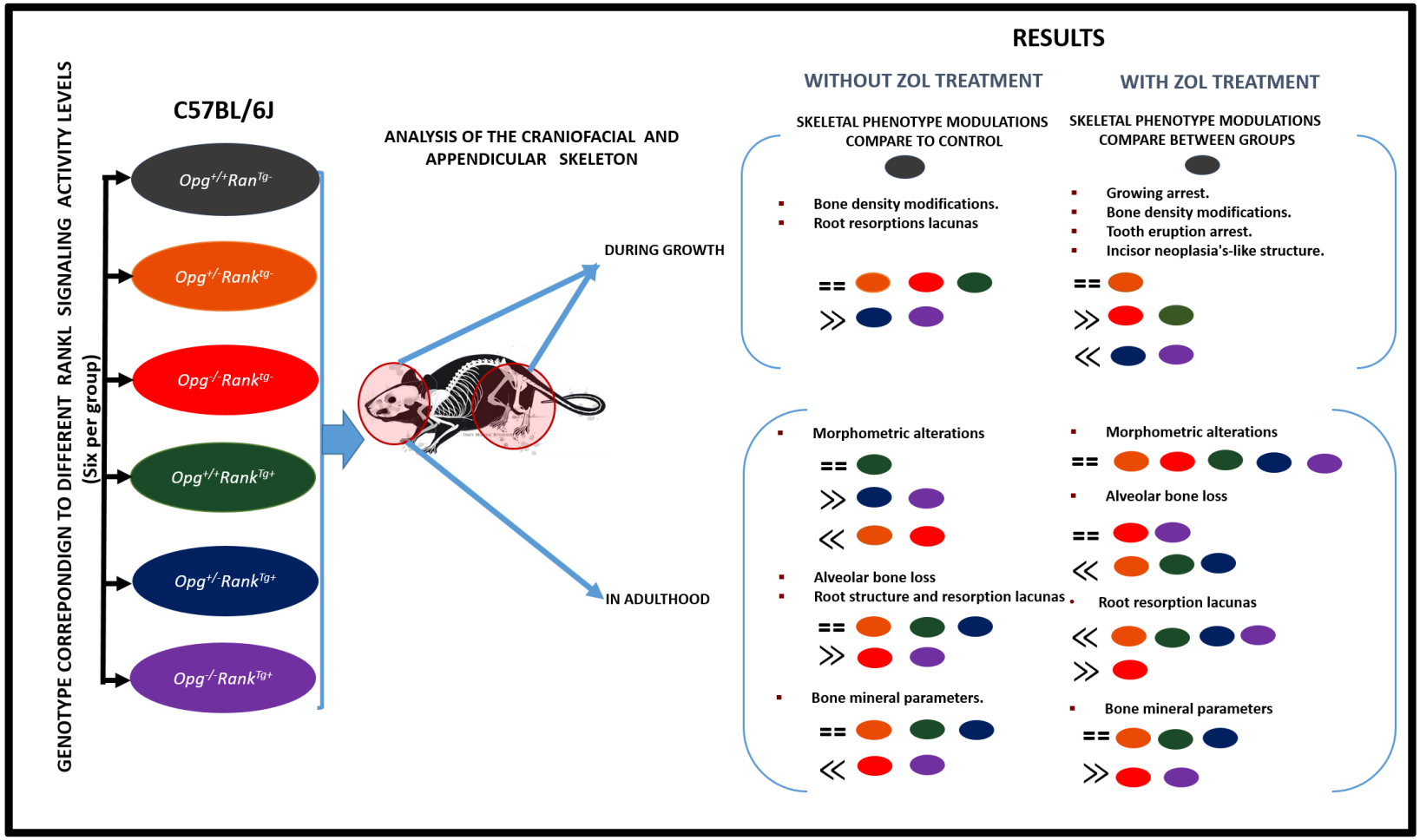


Figure 45. Summary of results: Comparative analysis of growing and adult mice with different genotype treated and no treated with zol

6. DISCUSSION

The importance of the RANKL/RANK/OPG signaling pathway in the bone modeling and remodeling, and specifically in the osteoclast cell differentiation and activity, was recognized 20 years ago. Due to its importance in the control of bone resorption, several studies were devoted to decipher the parts of this signaling pathway in the main pathologies corresponding to alterations of the bone modeling and remodeling as the osteoporosis (27,57), the osteopetrosis (14,329), the rheumatoid arthritis (56,330) the arthrosis, the periodontal disease (30,331) and the tumor-associated bone destruction. Mutations in the genes encoding elements for the RANKL/RANK/OPG signaling pathway (Table 2) or perturbations of the signaling pathway were reported for all these pathologies (14). The osteolytic pathologies are treated with bisphosphonates since 50 years and, surprisingly, following the discovery of the RANKL/RANK/OPG signaling pathway few studies have analyzed the impact of bisphosphonates on the expression of the elements of this pathway while no study has considered the impact of perturbations of this signaling pathway on the response to bisphosphonates. This appears all the most surprising that bisphosphonates are used in clinic to treat the pathologies associated to perturbations of the RANKL/RANK/OPG signaling. Interestingly, such a study was performed for another inhibitor of the bone resorption also used in the clinic, namely the strontium ranelate (332), showing that the absence of OPG highly modified the bone response to the treatment (332).

The aim of the works presented here, subjet of my doctorate, was to analyze the consequences of different levels of RANKL/RANK/OPG signaling activity, genetically achieved by mating OPG deficient mice and RANK overexpression mice, onto the response to ZOL treatment on the one hand during growth and on the other hand in adult.

This work will contribute to the analysis and implication of the RANKL signaling in different craniofacial pathologies and to elucidate its implication in N-BPs response. In this sense the comparative description of the phenotype of young (one month and a half of age) and adult (ten months of age) transgenic mice with different OPG/RANK genotypes (*Opg*$^{+/+}$*Rank*$^{tg-}$, *Opg*$^{+/-}$*Rank*$^{tg-}$, *Opg*$^{-/-}$*Rank*$^{tg-}$, *Opg*$^{+/+}$*Rank*$^{tg+}$, *Opg*$^{-/-}$*Rank*$^{tg+}$, *Opg*$^{-/-}$*Rank*$^{tg+}$) corresponding to a different RANKL signaling activity levels, treated or not with ZOL, was done. Finally, the relationship during growth (bone modeling) as in adulthood (bone remodeling) between the RANKL signaling activity level in the craniofacial skeleton and the response to ZOL was characterized.

6.1 OSTEOLYTIC MOUSE MODELS

A central aspect of this research was that by combining two transgenic mouse models -corresponding to an overexpression of RANK in the monocyte/macrophage lineage (*Rank*^{Tg}) and a global decrease in OPG expression (*Opg*^{KO}) made it possible to obtain a gradual series of osteolytic bone microenvironments. Thus it has been possible to obtain osteolytic models to identify the impact of RANKL signaling activity level onto the effects and side-effects of ZOL on growing and adult craniofacial skeleton. Such combinations of transgenic mice have never been realized before, so the first step was to characterize the skeleton phenotypes associated to the six different genotypes (*Opg*$^{+/+}$*Rank*$^{Tg-}$, *Opg*$^{+/+}$*Rank*$^{Tg+}$, *Opg*$^{+/-}$*Rank*$^{Tg-}$, *Opg*$^{+/-}$*Rank*$^{Tg+}$, *Opg*$^{-/-}$*Rank*$^{Tg-}$ and *Opg*$^{-/-}$*Rank*$^{Tg+}$) at the end of growth (one and a half months after birth) and in adulthood (ten months after birth). A gradual series of osteolytic microenvironments was obtained by this characterization and the phenotypic analyses let to conclude that the RANKL signaling activity level affects the craniofacial phenotype and induces alterations in either the bone, the periodontal and the dental structures during growth and adulthood.

6.1.1 Skeleton phenotypes associated with grade osteolytic genotypes at the end of growth. The bone morphometric and bone mineral parameters analyses performed for the different genotypes made it possible to obtain important unreported findings regarding the skeletal growth. Regardless the genotype, the long bones and craniofacial bones morphometric parameters were similar, meaning that the different *Opg* and *Rank* osteolytic genotypes (bone osteolytic microenvironments) did not affect the size of the bones at the end of growth. Interestingly this did not mean that the skeleton growth timing was the same, as an early alveolar bone modeling associated to tooth eruption and root elongation was previously described for $Rank^{Tg}$ (164) but that final sizes of bones were genetically determined and independent of the osteolytic status during growth, as previously suggested (325).

In contrast to bone morphometric parameters, certain bone mineral parameters (%BV/TV, Tb.N, and TMD) revealed differences at the end of growth (mainly in craniofacial bones), more specifically in the $Opg^{-/-}$ genotypes. For instance, the TMD in long bones and the %BV/TV in alveolar and basal bones, showed lower values for $Opg^{-/-}$ independently of the RANK expression. These observations demonstrated that despite no consequences on the bone morphometry, the different osteolytic genotypes had impacts on the bone mineral structure at the end of growth, what might influence the mechanical properties of bones and induced their premature wearing during adulthood and a high risk of fracture as visible for the $Opg^{-/-}\backslash Rank^{Tg+}$ mouse in Figure 25 A.

The histological analysis in craniofacial tissues showed a graded increase in the number of TRAP positive cells, proportional to the allelic reduction of *Opg* and the increase of *Rank*, validating the hypothesis of the existence of a grade osteolytic series associated to the different genotypes. This histological analysis also revealed that despite the normal eruption status of teeth observed regardless of the genotype, the dental root structure was affected specifically in the $Opg^{-/-}$ mice independently of

the *Rank* overexpression, with the presence of lacunas of resorption on the root cervical area. In addition, a reduction of the root diameter was present in the $Rank^{Tg}$ mice as previously reported (164,325). Altogether these data validated the importance of the RANKL signaling in the dental root formation through its implication in the communications between dental, periodontal and bone cells necessary to a harmonious and functional growth of the complex formed by the tooth and the alveolar bone (164,325,333,334). Interestingly, RANKL signaling was previously reported important later, in adulthood, for the homeostasis of the dental and periodontal tissues. Indeed, root resorptions, cementum mineralization reduction, dentine hypertrophy with decreased size of the pulp chamber and severe alveolar bone resorptions have been described in $Opg^{-/-}$ adult mice (28–30,33), while root and alveolar bone resorptions were reported in $Rank^{Tg}$ mice (32). In patients, idiopathic external resorption were reported in cases of Familial Expansile Osteolysis (14,335) and tooth loss and mandible deformities in early-onset familial Paget disease (335–338). Finally, the RANKL signaling appeared to be important at all stages of the life of the complex formed by the tooth and its associated alveolar bone, from the embryonic morphogenesis (326,339) through the growth (present work) to the adult homeostasis.

To summarize, the gradual allelic reduction of the *Opg* associated or not to the overexpression of *Rank* made it possible to generate a series of transgenic mice with grade levels of RANKL signalization activity in the bone microenvironment. The skeleton phenotype analysis of these different mice at the end of growth evidenced no difference concerning the bone morphometric parameters while the bone mineral parameters were reduced in absence of Opg ($Opg^{-/-}$) whatever the $Rank^{Tg}$ status. Concerning the complex formed by the tooth and its associated alveolar bone, the absence of *Opg* was allied to root resorptions and the overexpression of RANK to a reduction of the root diameter with surprisingly an apparent independency of these two effects.

6.1.2 Craniofacial skeleton phenotypes associated with grade osteolytic genotypes at adulthood.

6.1.2.1 Cortical and cancellous bones. Cortical and cancellous refer to different structural types of bone (340,341). Cortical bone (compact bone) is solidly filled with organic and inorganic salts, leaving only tiny spaces (lacunae) that contain the bone cells (6). Cortical bone is the primary component of diaphyses of the long and short bones of the extremities (6), and is also found surrounding the cancellous bone of the vertebral body, the metaphyses of the long bones, the iliac crest and the skull (6). On the contrary cancellous bone, which is found in areas of bone that are not subject to significant mechanical stress, is the main component of enlarged ends (epiphyses) of the long bones, ribs and flat bones of the skull, among others (6). The cancellous bone include numerous large spaces that give a spongy appearance (6). The structural element of cancellous bone is the trabeculae and the cancellous bone is so characterized by the number of trabeculae (Tb.N), the trabecular thickness (Tb.Th), and the trabecular separation (Tb.Sp). These factors determine the overall cancellous bone volume (341).

The impact of the RANKL signaling levels onto cortical and cancellous skull bones has been poorly documented (30). Using transgenic *Opg*$^{-/-}$ and RANKL over-expressing 8 weeks-old male mice, Koide et al reported the presence of a cortical porosity in long bones of *Opg*$^{-/-}$ but not of *Rankl*^{tg} mice. However, the decrease of the bone volume (BV) in femurs of both groups and spontaneous resorption in the cortical area of the alveolar bone of *Opg*$^{-/-}$ mice were observed (30). They conclude that the expression of OPG is an essential factor for the inhibition of bone resorption in the cortical areas of alveolar bone (30). No data regarding the impact of OPG or RANKL signaling levels in the cancellous skull bone have been reported in this work.

Clinical research publications have described radiographic findings in Hajdu-Cheney syndrome (OMIM #102500) (100,101) hyperphosphatasemia familial idiopathic also

known as Paget disease of bone 5 – juvenile onset (PDB5, OMIM #239000) (16), early-onset Paget disease or Paget disease of Bone 2 (PDB2, OMIM #602080) (16), familial expansile osteolysis and expansile skeletal hyperphosphatasia (FEO and ESH, OMIM #174810) (16) and other disorders associated to TNFRSF11A (RANK) (14). They reported, in those patients, marked expansion (undertubulation) of long bones, initially with osteopenic cortices (14), and in the cancellous bone, loss of the trabecular bone pattern and remarkable expansive defects (14).

We demonstrated here the importance of OPG in the homeostasis of the cortical and cancellous bones. We found structural alterations in basal cortical and cancellous bones, mainly in absence of OPG, with a gradual increase of the mandible dimensions in direct proportion with de depletion of Opg alleles (*Opg*$^{+/-}$,*Opg*$^{-/-}$) (Fig. 14 and 27). These findings were associated with an increase in the lacunes in cortical and cancellous bones, as shown by the histological analysis (Fig. 35). Interestingly, a decrease of the mineral parameters was evident mainly in the total absence of OPG and, as we showed (Fig. 28 and 29), the sole presence of one allele protects the mineral bone parameters, both in male and female. On the other hand, the RANK overexpression apparently does not affect the homeostasis of cortical nor cancellous bones. The histological Massons Trichrome and the TRAP staining (Fig. 33 and 35) enable to confirm the alteration in the cortical and cancellous bones and the dramatic increase of osteoclast multinucleated cells number associated with total Opg depletion, but not with the RANK overexpression. This finding is consistent with the mineral parameters levels in the cortical and cancellous bones presented in graphic 28 and 29. Our results, as well as genetic OPG and RANK disorders (14), demonstrate severe alterations of the cortical bone with noticeable expansion, loss of the trabecular bone pattern and significant bone defects in the cancellous bone. We have also identified the importance of the gender, only in adults, for in the mineral bone parameters, but not for morphometric bone parameters.

6.1.2.2 Periodontal alveolar bone. Several authors, including our group, have reported the implication of the RANKL/RANK/OPG system in periodontal disease and the homeostasis of the alveolar bone (30,32,33).

Indeed Koide and collaborators (30), established that *Opg*$^{-/-}$ mice spontaneously exhibit severe alveolar bone loss compared to *Rankl*^{Tg}. They report that the %BV/TV and Tb.Th of the first molar interradicular septum were significantly lower in *Opg*$^{-/-}$ comparing to *WT* and *Rankl*^{tg} mice. The conclusion of this study was that the RANKL/OPG ratio in serum constitutes an important indicator of bone resorption; therefore the most severe alveolar bone resorption of *Opg*$^{-/-}$ mice is due to a higher bone-resorption activity comparing with Rankl^{tg} mice (30). Sojod and collaborators, using 5 months-old *Rank*^{tg} females mice, demonstrated that RANK overexpression induces severe alveolar bone loss, with an increase of the TRAP positive cells number, and disorganization of the periodontal ligament (32). They identified by comparison with WT, a substantial reduction of alveolar bone height in the inter-proximal and the inter-radicular areas, and in buccal and lingual alveolar crests (32). Sheng and collaborators. used an *Opg*$^{-/-}$ 9-week-old female mice model to evaluate the long-term effects of OPG deprivation on the mineralization and morphology of the tooth (33). Their results evidenced that the mineralization of alveolar bone was decreased with the presence of a significantly lower bone mineral density in the alveolar bone of the incisor, the first molar and the third molar comparatively *WT* mice (33).

Our results are in agreement with those previous reports, but unlike to these studies, we performed our analyses in older animals (ten months-old mice). Additionally, an in-depth analysis of the interradicular alveolar bone was realized in order to compare not only the alveolar bone loss but also all the mineral parameters taking into account the sex differences. In our results, in addition to the reduction of the mineral parameters, the periodontal bone loss was strongest in the absence of OPG (Fig 30). We identify a slight difference in the %BV/TV comparing males and females.

Apparently females are less sensitive to the total absence of OPG. Indeed, the %BV/TV was higher in *Opg*$^{-/-}$ females (mean *Opg*$^{-/-}$*Rank*$^{tg-}$ 44.5 and *Opg*$^{-/-}$*Rank*$^{tg+}$ 42.4) than in males (mean *Opg*$^{-/-}$*Rank*$^{tg-}$ 33.8 and *Opg*$^{-/-}$*Rank*$^{tg+}$ 26.2). Interestingly, despite that the %BV/TV was lower in *Opg*$^{-/-}$ mice (Rank^{tg+} and Rank^{tg-}) the other mineral parameters (Tb.Th, Tb.N and Tb.Sp) did not show a statistically significant difference, except for *Opg*$^{-/-}$*Rank*$^{tg+}$. Comparatively to cancellous bone, which is dramatically affected in both structure and mineral parameters, the structure of periodontal alveolar bone appears more stable, regardless of the %BV/TV.

6.1.2.3 Root structure. The implication of the RANKL/RANK/OPG system in the dental root structure was probably one of the most important topics of analysis. Its study involves not only the impact on the development but also on the homeostasis in adult mice including root resorption due to physiologic or pathologic defects in either the cementum or the dentin (23,28,29,31,32,164,333,342).

The impact onto dental root length and diameter has been poorly studied. Castañeda and collaborators (164), using the *Rank*^{tg} mouse, have reported that RANK over-expression did not affect the root length, but significantly the root diameter with the presence of an important reduction. They concluded that the root length is determined by genetic factors while the root diameter is vulnerable to the alveolar bone resorption activity (164). Our study, at ten months of age, has confirmed that RANK over-expression did not affect the total length. Additionally, we observed that the absence of OPG increases slightly the total root length. Although not statistically significant comparatively to *WT*, a tendency to increase of the total length was observed for all groups, been higher in a total absence of OPG (*Opg*$^{-/-}$*Rank*$^{tg-}$ and *Opg*$^{-/-}$*Rank*$^{ktg+}$). However, this result can be associated to an increase of the cellular cementum formation in the apical region of the root (Fig. 35). Concerning the width, a tendency to increase in the sagittal plane was observed, for both males and females, only in absence of OPG (*Opg*$^{+/-}$*Rank*$^{tg-}$ *and Opg*$^{-/-}$*Rank*$^{tg+}$).Such increase was only significant in the apical region of the root, independently of the sex. The

histological and micro-CT images enable to confirm that this increase is also related to an expansion of cellular cementum in this region. We consider that the increase in the cellular cementum could be associated to the response to root resorption and the periodontal tissue repair, which was more severe in total deficiency of OPG.

Concerning the root resorption, several authors have demonstrated the importance of the OPG and RANK signaling (28,29,32). All of them have reported that, in young mice, the absence of OPG or an increase in RANK augments the resorptive cells number (osteoclast and odontoclasts), rising the risk of root resorption (28,29,32). Wang and collaborators in their study, of 26 and 54 weeks-old adult mice, observed the same characteristic (28). This phenotype has been also associated to morphological and quantitative damage of epithelial rests of Malassez (28,32). In the same way our results have clearly demonstrated that an imbalance in the OPG/RANK radio increases dramatically the presence of root resorption lacunas (Fig 33 and 34). However an in-depth histological analysis was still missing.

6.2 EFFECTS AND SIDE-EFFECTS OF ZOL ON GROWING SKELETON OF MICE WITH DIFFERENT OSTEOLYTIC GENOTYPES

In order to identify the impact of RANKL signaling activity levels onto effects and side effects of ZOL on a growing skeleton, a protocol that mimicked those used in onco-pediatric patients was applied to mice of the different genotypes and the skeleton phenotypes at the end of growth were established and compared. This experiment first evidenced that whatever the genotype considered ZOL impacted the growing skeletons (morphometric as well as mineral and dental parameters). However, variations were observed between the different genotypes confirming the hypothesis that genetically-achieved over-activation of RANKL signaling could deal with the effects and side effects of ZOL on a growing skeleton. Surprisingly, no linear relationship was observed between the grade osteolytic levels and the intensity of the effects and side-effects at the end of growth. Indeed, the two different ways of

increasing the RANKL signalization, namely the *Opg* gene invalidation and the *Rank* gene overexpression, seemed to differentially impact the effects and side-effects of ZOL. For bone morphometric parameters, the absence of *Opg* ($Opg^{-/-}$) was the only situation in which the effect of ZOL was prevented regardless the $Rank^{tg}$ status. For bone mineral parameters (BV/TV, Tb.N and Tb.Th), two situations were evidenced. First in absence of *Rank* overexpression ($Rank^{Tg-}$) the bone mineral parameters values were gradually decreasing with the *Opg* allelic reduction, close to zero observed in none treated mice. Secondly, in the *Rank*-overexpressing mice ($Rank^{Tg+}$), regardless of the *Opg* expression level, the bone mineral parameters were very high compared to non-treated mice. Altogether these results demonstrated that the genetically-achieved reduction in *Opg* expression was the only situation that enabled to limit or reverse the ZOL effects and side effects by the end of pediatric growth. Interestingly a similar consequence of *Opg* invalidation was reported for another inhibitor of the bone resorption also used in clinical practice, Strontium (332). The most surprising results in ZOL treated mice were the reverse than expected impacts of *Rank* overexpression on bone mineral parameters and the absence of effect on bone morphometric parameters. One explanation may come from the number of TRAP positive cells observed at the bone surface at the end of growth, which increased with *Opg* allelic loss while being low when *Rank* was overexpressed. This explanation, based on a more important bone resorption capacity during the month following the treatment (until the end of the growth), was supported by the observed relationship between the full tooth eruption and the unaffected sagittal growth of the skull, two independent processes highly sensitive to the osteoclast activity, but the underlying molecular mechanisms remain to be elucidated.

At least two hypotheses could be proposed to explain the differences in the effects and side-effects of ZOL on a growing skeleton between the *Opg* allelic reduction and the *Rank* overexpression. The first one was linked to the existence of a third receptor for RANKL called LGR4 (82). This receptor was expressed by the osteoclasts and

the osteoblasts (82,83,343–350) with reverse function regarding the differentiation of those cells. Osteoblastogenesis was effectively shown to be stimulated by LGR4 activation whereas osteoclastogenesis was inhibited (82,83,343–345,348). Interestingly, LGR4 expression was stimulated during the osteoclastogenesis (82), as a target of NFATC1, so in response to RANK stimulation by RANKL, suggesting the presence of an autoregulatory loop of RANKL effect on the osteoclast. Moreover, LGR4 activation was shown to control mature osteoclast survival by inducing apoptosis (82). In the *Opg*$^{-/-}$ mouse, the relative expression patterns of RANK and LGR4 during osteoclatogenesis until mature and functional osteoclast stage had no reason to be affected while in the *Rank*$^{Tg+}$ mouse it was undoubtedly the case. This may explain the difference in the response to ZOL observed between the two genetically achieved models of RANKL signalization over-activation.

The second hypothesis corresponded to a depletion of the osteoclast precursors in the *Rank*$^{Tg+}$ mouse following the treatment with ZOL. This transgenic mouse corresponds to an overexpression of *Rank* driven by the promotor of the Myeloid Related Protein 8 (MRP8 also known as S100A8) which induces in the bone marrow an important increase of the pre-monocyte-macrophage population (CD11b^{+} Gr1^{-}) over-expressing *Rank* (324,351). Physiologically, the osteoclastogenesis was achieved from the pre-monocyte-macrophage population (osteoclast precursors) by two successive inductions. The first corresponds to the binding of M-CSF on its receptor C-FMS expressed at the surface of the precursors which induces RANK expression and the second to the binding of RANKL to RANK enabling the fusion of the precursors into multinucleate osteoclastic cells (for review (19)). In the *Rank*$^{Tg+}$ mouse, the ectopic presence of RANK in the precursors made it possible to accelerate osteoclastogenesis in response to RANKL inducing the osteolytic phenotype previously described (164,325). According to the facts that N-BPs (as ZOL) induce an accumulation of giant osteoclasts unable to resorb the bone matrix at the bone surface (54,352–355), and a decrease of the number of macrophage

precursor in the bone marrow (356) a depletion of the RANK overexpressing precursors may result from the treatment with ZOL in the *Rank*$^{Tg+}$ mice.

Further studies will be necessary to validate or invalidate each of these hypotheses regarding the origin of the difference in the response to ZOL observed between the two genetically-achieved models of RANKL signaling over-activation.

To summarize, most of the ZOL effects and side-effects on mouse growing skeleton observed at the end of growth appeared to be contingent to the bone osteolytic levels associated with genetically achieved graded overactivation of the RANKL signaling. This was the case for the bone mineral parameters increase, blockage of tooth eruption and reduced appendicular and craniofacial bone growth. However, this dependence was not linear and two situations emerged. The grade *Opg* invalidation reduced the effects and side-effects of ZOL while *Rank* overexpression enforced them. Interestingly, when *Opg* invalidation was combined with the *Rank* over-expression, the effects and side-effects revealed different susceptibilities. The morphometric parameters and the tooth eruption were driven by the influence of *Opg* invalidation, and the bone mineral parameters by that of *Rank* overexpression. The basis of this dichotomy remains to be elucidated.

6.3 LONG-TERM STABILITY OF THE EFFECTS AND SIDE-EFFECTS OF ZOL ON GROWING SKELETONS

Analysis of the consequences on the skeleton of pediatric treatment with ZOL at the distance from the end of treatment (ten months of age) showed that most of the effects and side-effects of ZOL on long bones and craniofacial bones persisted into adulthood, although in some cases there were differences of intensity depending on the genotypes. The bone morphometric parameters were still lower in the treated mice regardless of the genotypes and the eruption defects were definitive as previously conclude (51,52). Surprisingly, the TMD was higher at ten months

compared to one and a half month in the treated groups regardless of the genotype, reaching values globally higher than in none treated animals (for instance in treated *Opg*$^{+/-}$*Rank*$^{Tg-}$ mice 5.0 at ten months versus 3.8±0.3 at one and half months whereas in untreated animal's values were respectively 4.65 and 4.5±0.4). However, the most remarkable results concerned the bone mineral parameters at teen months for which the dichotomy between *Opg* invalidation and RANK overexpression still seemed to be present. Despite the small number of mice that survived up to ten months due to the development of neoplasia-like structures in the apical part of the continuously growing incisors of treated mice, the BV/TV and the Tb.N increases induced by treatment with ZOL were close to reverse at ten months, in the absence of RANK overexpression, while maintained at high levels in presence of RANK overexpression.

To summarize, the consequences on the skeleton of ZOL treatment during growth were highly stable as could be seen after ten months. The differences in the skeletal phenotype associated with the different genotypes observed at the end of growth were also still present and often enforced, with *Opg* invalidation fostering normalization while the *Rank* overexpression maintaining or exacerbating the situation.

6.4 EFFECTS AND SIDE-EFFECTS OF ZOL ON ADULT SKELETON OF MICE WITH DIFFERENT OSTEOLYTIC GENOTYPES

Preclinical assays using animal models have supported the relationship between N-BPs and BRONJ, nevertheless until now none of these studies is conclusive and many questions remain unanswered (310,312,313,357,358). Surprising, before our study, no research has analyzed the specific relationship between the RANKL signaling activity level and the response to N-BPs, including the occurrence of BRONJ.

In our preclinical study, the relationship between the effects of ZOL in the craniofacial skeleton of adult mice and the RANKL activity level was questioned. Specifically, we wonder if the ZOL therapy, miming the therapy used for patients with ODs, could induce atraumatic necrotic effects on the jaw bone. Moreover we analyze the dental and periodontal phenotype of adult mice with different RANKL signaling activity levels treated with ZOL.

Different theories have been proposed concerning the pathophysiology of BRONJ, all reporting a multi-factorial origin (184,185,194,198,359). A genetic predisposition has been suggested by some authors (122,193), based on the fact that only certain patients with similar comorbidities and medical management develop BRONJ. Whatever all those theories support the necessary presence of local risk factors in the origin of BRONJ, like dento-alveolar surgery (especially extractions and implants, periapical surgery and periodontal surgery involving osseous injury), dental and periodontal infections and local anatomy (specially lower jawbone, torus, mylohyoid ridge), bone turnover level and micro-organisms of the oral cavity (124,183,185,186,189,360) However, some clinical reports have indicated the existence of the spontaneous appearance of osteonecrosis of the jaw, even in the absence of N-BPs therapy (203,204). In this sense, the hypothesis that the RANKL signaling imbalance in the craniofacial bone microenvironment could induce the spontaneous appearance of necrotic areas in the maxillary bone of mice treated with ZOL was explored here.

Based in our analyses it appears that the mentioned hypothesis was not confirmed, indeed no density modifications related to atraumatic necrotic areas in the craniofacial skeleton were observed, neither during the periodic scanner nor in the last ex-vivo scanner (figure 35 and 36). Moreover the analysis of basal bones (cortical and cancellous) and alveolar bone showed similar parameters (no significant variations) to those observed in untreated groups, with only minor modifications due to the effects of ZOL (Fig. 37 and 38). Although those results

cannot be considered conclusive (mainly due the small number of animals), they will have be to considered as an important point of reference for future studies, increasing the mice number and exploring the molecular events related to the RANKL signaling activity levels. Besides these not significant results, the decrease of the root resorption following the ZOL treatment, except in the *Opg*$^{-/-}$ mice, whatever *Rank*^{tg} status, is an important and significative result. Indeed; this validates the fact that the absence of OPG enables a rapid and important “rescue” of the bone resorption (osteoclast differentiation) following the end of the ZOL treatment with a resurgence of wrong effects of OPG deficiency.

7. GENERAL CONCLUSIONS AND PERSPECTIVES

N-BPs are considered the first line in the management of osteolytic diseases both in adults and children. Although their risk-benefit ratio in most cases significantly inclined towards the improvement of the patient's life, side effects have been reported, paradoxically affecting the skeleton in either pediatric or adult patients. In children, it has been reported that N-BPs induced a transient arrest of long bone and craniofacial bone growth, with an apparent graded rescue after the end of the treatment, and definitive retention of certain teeth, depending on the lapse-time of the treatment, the bisphosphonate used and its dosage. In adults, the main side effects are AFF and BRONJ. In those patients, extreme caution regarding the use of N-BPs is recommended, and follow-up by a dental practitioner throughout the treatment is required. However, the pathogenesis of these bisphosphonate-induced side effects is still non-elucidated and, for this reason, further studies will be necessary. The current limits of the research on bisphosphonate-induced side effects remain the absence of an "ideal" *in vivo* preclinical model mimicking the human version.

Different promising strategies are currently developed to minimize the appearance of such side effects in order to reach a safer use of N-BPs in the near future. Among those the implications of the RANKL/RANK/OPG triad in the etiology of ODs and their consequences in long bones and in craniofacial bones structures, as well as their implications in the response to N-BPs were poorly documented. Our preclinical research was done in order to explore the roles of this triad the craniofacial skeleton growth and adult homeostasis in the control or not of treatment with one of the most potent N-BPs, named ZOL.

The raised question was the existence of different individual sensibilities to ZOL treatment, taking into consideration the RANKL signaling activity level as the major parameter. In order to answer this question, mice deficient for *Opg* and over-

expressing *Rank* were mated to generate mice with grade levels of RANKL signaling activity. The skeleton phenotypes of these mice were analyzed at the end of the growth (one and half months of age) and at ten months of age, following or not treatment with ZOL.

In the growing skeleton, the results obtained demonstrated that the RANKL signaling activity levels had important repercussions on the effects and side-effects of ZOL on the skeleton, but that these repercussions were not proportional to the levels of activity but rather dependent to the way the RANKL signalization was boosted. *Opg* invalidation made it possible to reduce these effects and side-effects while the *Rank* overexpression enforced them. These repercussions appeared to be stable in time with however a noticeable amelioration in the mice with allelic reductions of *Opg* but not when *Rank* was over-expressed.

The analysis of RANKL signaling activity level in adult craniofacial skeleton demonstrated the importance of the OPG level for the homeostasis not only concerning the alveolar bone, as previous reported, but also regarding the cortical and cancellous bones. The absence of OPG impacts negatively the different skull structures, comparatively to the RANK overexpression. Concerning the ZOL effects in adult craniofacial skeleton, no density modifications related to atraumatic necrotic areas in the lower maxillary of adult mice treated with ZOL were observed and the analysis of basal bones (cortical and cancellous) and alveolar bone showed a similar phenotype to untreated groups. Due to the limited number of mice that reach the end of the experimental period, the analysis of the results cannot be conclusive and must be considered as an important point of reference to future studies.

This descriptive study, using different genetic background models, has demonstrated the implication of the RANKL signaling activity level in the homeostasis of the craniofacial skeleton. The relevance of the OPG in this triad was evidenced in this research, which enforces the previous analyses that have

demonstrated the higher affinity of the RANKL for OPG than for RANK, mainly link to the conformational difference between OPG and RANK. In this way, it is possible to consider that a fine regulation of OPG expression, increasing or decreasing its level, could help to improve the risk of occurrence of different clinical pathologies associated to the RANKL signaling activity level. However, in the future a molecular analysis using the transgenic mice presented here will be necessary to deeply understand the real implication of the RANKL system, and specifically the proper role of the OPG, in the homeostasis of craniofacial bones. On the other hand, the implications of RANKL signaling activity level in the adult response to ZOL remain unclear. Indeed, no implications in the appearance of MRONJ related to RANKL signaling activity level were found.

Add to the relevance of the OPG expression in the craniofacial morphology structures supported here, reproductive mouse models with severe osteopenic phenotypes have been proposed, named $Opg^{-/-}Rank^{tg-}$ and $Opg^{-/-}Rank^{tg+}$. The use of these models will help to better understand, at the molecular level, the etiology of the osteolytic disease involving genetic disorders like osteogenesis imperfecta syndrome and Paget disease (14), different bone pathologies like age-related osteoporosis, glucocorticoid-induced osteoporosis, skeletal metastases from multiple myeloma and other tumors. Specifically, in the craniofacial system, we consider that these mouse models will be important tools to better understand the etiology of dental diseases like periodontitis, osteonecrosis of the jaw and different causes of root resorption, like trauma, incorrect orthodontia force and periapical periodontitis. Finally, it will be of strategic help also to a better understanding of the drug response variabilities like those to antiresorptive therapies (bisphosphonates, Rankl-Antibody), ranelate strontium, corticosteroids, anabolic agents, among others

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ANNEXES

ARTICLES FIRST AUTHOR:

ARTICLE 1:

Paradoxical side effects of bisphosphonates on the skeleton: What do we know and what can we do?

Vargas-Franco JW, Castaneda B, Rédiní F, Gómez DF, Heymann D, Lézot F.
J Cell Physiol. 2018 Aug;233(8):5696-5715. doi: 10.1002/jcp.26465. Epub 2018 Mar 7. Review.
PMID: 29323712

ARTICLE 2:

Genetically-achieved disturbances to the expression levels of TNFSF11 receptors modulate the effects of zoledronic acid on growing mouse skeletons.

Vargas-Franco JW, Castaneda B, Gama A, Mueller CG, Heymann D, Rédini F, Lézot F.
Biochem Pharmacol. 2019 Oct;168:133-148. doi: 10.1016/j.bcp.2019.06.027. Epub 2019 Jun 28.
PMID: 31260659

SECOND AUTHOR:

Maternal RANKL Reduces the Osteopetrotic Phenotype of Null Mutant Mouse Pups.

Navet B, **Vargas-Franco** JW, Gama A, Amiaud J, Choi Y, Yagita H, Mueller CG, Rédini F, Heymann D, Castaneda B, Lézot F.
J Clin Med. 2018 Nov 8;7(11). pii: E426. doi: 10.3390/jcm7110426.
PMID: 30413057 Free PMC Article

The Intrinsic and Extrinsic Implications of RANKL/RANK Signaling in Osteosarcoma: From Tumor Initiation to Lung Metastases.

Navet B, Ando K, **Vargas-Franco** JW, Brion R, Amiaud J, Mori K, Yagita H, Mueller CG, Verrecchia F, Dumars C, Heymann MF, Heymann D, Lézot F.
Cancers (Basel). 2018 Oct 24;10(11). pii: E398. doi: 10.3390/cancers10110398.
PMID: 30355966 Free PMC Article

POSTER PRESENTATIONS CONGRESSES:

- 20èmes Journées Françaises de Biologie des Tissus Minéralisés. Société Française de Biologie des Tissus Minéralisés. Hôtel Novotel 21-23 Mars 2018, Monaco.
- Bisphosphonates2019. Celebrating 50 years. 15-17 July 2019 - Sheffield, UK

UNIVERSITE BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSIDAD DE ANTIOQUIA

UNIVERSITÉ DE NANTES

Titre : **Impacts on the growing and adult skeleton of different genetically-achieved RANKL activity levels, consequences on the response to zoledronic acid**

Key words : Craniofacial skeleton, N-BPs, Zoledronic acid, RANKL/RANK/OPG

Abstract:
Rational and hypothesis: Amino-bisphosphonates are powerful inhibitors of bone resorption. They are currently used in clinical practice to treat pediatric and adult osteolytic diseases. Variations between individuals in the intensity of their effects and side-effects have been reported with no clear explanation of their origins. The hypothesis that such variations could potentially be associated with different levels of activity in the RANKL signaling in bone during and after treatment was questioned here.
Objectives and methodology: A series of transgenic mice with graded levels of RANKL signaling was generated by mating mice invalidated for *Opg* and overexpressing *Rank*. These mice were subjected to zoledronic acid protocols mimicking those used in onco-pediatrics or in osteoporotic adult patients, and the skeleton phenotypes were compared one month after the last injection at the end of growth for the pediatric protocol and six months after the last injection at ten months of age for the adult protocol.
Results: The results validated the hypothesis with however one main surprise: the absence of a strict reverse correlation between the severity of the skeleton phenotypes and the increase in RANKL signaling activity. More precisely, the graded allelic reduction in *Opg* appeared to improve the skeleton phenotype observed both at the end of growth as in adult, while *Rank* overexpression made it worse.
Conclusion: In conclusion, the level of activity of RANKL signaling in the bone microenvironment was shown to be implicated in the modulation of the skeleton's phenotypic response to bisphosphonates, with differing impacts of *Opg* invalidation and *Rank* overexpression, whose molecular deciphering will form the next challenge.

Title : **Impacts sur le squelette adulte et en croissance de différents niveaux d'activité de RANKL obtenus génétiquement, conséquences sur la réponse à l'acide zolédronique**

Mots clé : Squelette craniofacial, N-BP, acide zolédronique, RANKL/RANK/OPG

Rationnel et hypothèse : Les amino-bisphosphonates sont de puissants inhibiteurs de la résorption osseuse. Ils sont actuellement utilisés en clinique pour traiter les maladies ostéolytiques de l'enfant et de l'adulte. Des variations entre les individus dans l'intensité de leurs effets et effets secondaires ont été signalés, sans explication claire de leurs origines. L'hypothèse selon laquelle de telles variations pourraient éventuellement être associées à différents niveaux d'activité de la signalisation RANKL dans l'os pendant et après le traitement a été posée ici.
Objectifs et méthodologie : Une série de souris transgéniques présentant des niveaux graduels de la signalisation RANKL a été générée par l'accouplement de souris invalidées pour l'*Opg* et sur-exprimant *Rank*. Ces souris ont été soumises à des protocoles d'acide zolédronique imitant ceux utilisés en oncopédiatrie ou chez les patients adultes ostéoporotiques, et les phénotypes squelettiques obtenus ont été comparés un mois après la dernière injection à la fin de la croissance pour le protocole pédiatrique et six mois après la dernière injection à l'âge de dix mois pour le protocole adulte.
Résultats : Les résultats obtenus ont validé l'hypothèse avec toutefois une surprise majeure : l'absence d'une stricte corrélation entre la sévérité des phénotypes squelettiques et l'augmentation de l'activité de signalisation RANKL. Plus précisément, la réduction allélique graduelle de l'*Opg* semblait améliorer le phénotype squelettique observé à la fin de la croissance comme chez l'adulte, tandis que la surexpression de *Rank* l'empirait.
Conclusion : En conclusion, il a été démontré que le niveau d'activité de la signalisation RANKL dans le microenvironnement osseux était impliqué dans la modulation de la réponse phénotypique du squelette aux bisphosphonates, avec des impacts différents de l'invalidation de l'*Opg* et de la surexpression de *Rank*, dont le décryptage moléculaire constituera le prochain défi.