Clinical and pre-clinical investigation of bisphosphonate-related osteonecrosis of the jaw (BRONJ)

ZAHER JABBOUR
DMD, MSc

Faculty of Dentistry
McGill University, Montreal, QC

March 2015

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Dental Sciences

Copyright © Zaher Jabbour 2015
# Table of Contents

Table of Contents.......................................................................................................................... ii  
Abstract ........................................................................................................................................ iv  
Résumé........................................................................................................................................... vi  
Acknowledgment .............................................................................................................................. viii  
Preface and Contribution of Authors ............................................................................................... ix  
List of Tables .................................................................................................................................... xii  
List of Figures .................................................................................................................................... xiii  
List of abbreviations .......................................................................................................................... xv  
Chapter one: Introduction ................................................................................................................ 1  
  1.1 Principles of bone physiology ................................................................................................. 2  
  1.2 Mechanism of action of bisphosphonates on bone ................................................................. 3  
  1.3 The use of bisphosphonates as therapeutic agents .................................................................. 5  
  1.4 Bisphosphonate-related osteonecrosis of the jaw (BRONJ) ................................................... 9  
    1.4.1 Definition and risk factors ............................................................................................... 9  
    1.4.2 Incidence of BRONJ ....................................................................................................... 11  
    1.4.3 Hypotheses for BRONJ ................................................................................................. 12  
    1.4.4 Management of BRONJ .............................................................................................. 16  
    1.4.5 Current animal models ................................................................................................. 19  
  1.5 Osteonecrosis of the jaws with other antiresorptive medications (denosumab) ................. 21  
Chapter two: Rationale, research hypothesis, and objectives ......................................................... 23  
  2.1 Rationale ................................................................................................................................. 24  
  2.2 Research hypothesis ............................................................................................................... 25  
  2.3 Objectives .............................................................................................................................. 25  
Chapter three: Assessment of treatment outcomes and oral microbiota in BRONJ patients ............ 27  
  3.1 Manuscript 1 .......................................................................................................................... 28  
  3.2 Manuscript 2 .......................................................................................................................... 45
Chapter four: Effect of bisphosphonates on the jaw bones and oral microbiota in a pre-clinical model of BRONJ ................................................................. 66
  4.1 Manuscript 3 .................................................................................. 67
  4.2 Manuscript 4 .................................................................................. 91
  4.3 Manuscript 5 .................................................................................. 108
Chapter five: General discussion ................................................................. 131
  5.1 BRONJ patients at the Montreal General Hospital ......................... 132
  5.2 Management of BRONJ .................................................................. 132
    5.2.1 Conservative and surgical management of BRONJ .................. 132
    5.2.2 Bisphosphonate withdrawal .................................................... 133
  5.3 Rat model to study jaw bone changes .......................................... 134
  5.4 Assessment of bacteria associated with BRONJ ......................... 136
    5.4.1 Study design in BRONJ patients ............................................ 136
    5.4.2 Study design in rats ................................................................. 137
    5.4.3 The use of the DNA checkerboard hybridization method ..... 138
  5.5 Limitations of the studies and future research .............................. 139
    5.5.1 Limitations of the studies ......................................................... 139
    5.5.2 Future clinical research ............................................................ 139
    5.5.3 Future translational research ................................................. 140
    5.5.4 Future microbial research ..................................................... 142
  5.6 Scientific contribution to clinical practice .................................... 142
Chapter six: Conclusions ......................................................................... 144
  Conclusions ......................................................................................... 145
References ............................................................................................... 146
Appendices .............................................................................................. 157
  CONSENT FORM ............................................................................ 158
  FORMULAIRE DE CONSENTEMENT ............................................. 163
  PATIENT EVALUATION FORM ....................................................... 168
Abstract

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is an oral complication found in patients receiving high doses of intravenous bisphosphonates for treatment of cancer or osteoporosis. BRONJ is clinically defined as an area of exposed bone in the maxillofacial region that has persisted for more than 8 weeks in patients undergoing, or with previous bisphosphonate treatment and no history of radiation therapy to the jaws.

Many hypotheses have been formulated to explain BRONJ based on a bacterial etiology or the direct effects of bisphosphonates on the jaw bone, soft tissue, or vascularization. Challenges in the management of BRONJ arise since the pathophysiology remains poorly defined. The American Association of Oral and Maxillofacial Surgeons recommend a conservative approach of treatment for BRONJ in its early stages, and surgical intervention for the more advanced disease. An interruption of bisphosphonates therapy also is suggested, depending on the patient’s overall condition. To determine the efficacy of different treatment interventions, we conducted a retrospective chart review of patients with BRONJ treated at the Montreal General Hospital. The study revealed that both conservative and surgical treatments were used for patients with BRONJ stage II, but that surgical treatment resulted in a faster resolution of the condition. Based on our chart review, we found that corticosteroids were frequently prescribed to BRONJ patients. Therefore, BRONJ-like disease was induced in rats by administering the bisphosphonate zoledronic acid and the corticosteroid dexamethasone. This pre-clinical model showed that bisphosphonate withdrawal might benefit the outcome by allowing bone remodeling to resume. In addition, this model showed that the combined administration of
bisphosphonate and corticosteroid increased the suppression of bone turnover compared to the administration of either drug alone.

To further explore the hypothesis that bacterial infection contributes to the development of BRONJ, we used the DNA checkerboard technique to determine whether bacterial probes prepared from human oral bacteria hybridized with the whole genome DNA extracted from the oral cavity of rats. This technique was then used to detect and quantify 43 microbial species relevant to human oral health in samples taken from the oral cavity of patients with BRONJ and rats with BRONJ-like disease. The results suggested that the microbiota of exposed bone in BRONJ patients had a different profile compared to the bacterial profile of other hard and soft tissues of the mouth. This unique microbial profile could have been caused by the medications the patients were taking, including chemotherapy and bisphosphonate. *E. corrodens*, *S. gordonii*, and *S. constellatus* were the three most dominant bacterial species in the exposed bone in addition to other non-pathogenic microbial species, and species linked to periodontal diseases and bone infection. As in the BRONJ patients, the exposed bone in rats treated with bisphosphonate and corticosteroid was colonized mainly by non-pathogenic bacteria. The most abundant species in the site of exposed bone in rats were *S. pasteuri*, *S. parasanguinis*, and *S. mitis*. 
Résumé

L'ostéonécrose des maxillaires associée aux bisphosphonates (BRONJ) est une complication dans la cavité buccale observée chez les patients recevant des doses élevées de bisphosphonates par voie intraveineuse pour le traitement du cancer ou de l'ostéoporose. BRONJ est cliniquement définie comme étant une exposition osseuse de la région maxillo-faciale persistante pendant plus de huit semaines chez un patient recevant ou ayant reçu un traitement de bisphosphonates sans avoir d’antécédent de radiothérapie aux mâchoires.

De nombreuses hypothèses ont été formulées pour expliquer BRONJ en se basant sur l'étiologie bactérienne ou les effets directs des bisphosphonates sur les tissus mous, l’os de la mâchoire ou la vascularisation. Les défis liés aux traitements BRONJ surviennent quand la pathophysiologie demeure inconnue. Une approche conservatrice pour le traitement de BRONJ est recommandée par l’American Association of Oral and Maxillofacial Surgeons pour les stades précoces avec un traitement chirurgical pour les stades avancés. L’interruption du traitement aux bisphosphonates a également été suggérée en fonction de l’état général du patient. Afin de déterminer l'intervention la plus appropriée pour traiter les patients souffrant de BRONJ, nous avons mené une étude rétrospective en révisant les dossiers des patients traités pour BRONJ à l'Hôpital général de Montréal. Cette étude a révélé que les traitements conservateurs et chirurgicaux ont été offerts aux patients ayant BRONJ stade II, alors que les traitements chirurgicaux entraînent une résolution plus rapide de la maladie. En se basant sur les résultats de cette étude, les corticostéroïdes ont été fréquemment prescrits aux patients ayant BRONJ. Pour
cela, une condition similaire à BRONJ (BRONJ-like) a été générer chez les rats par l'administration du bisphosphonate acide zolédronique et le corticostéroïde dexaméthasone. Ce modèle pré-clinique démontre que l’interruption du bisphosphonate peut être utile et permet de repartir le remodelage osseux. De plus, cette étude démontre que l'administration combinée de ces médicaments augmente la suppression du remodelage osseux en comparant à l’administration de chaque médicament séparément.

Pour explorer davantage l'hypothèse que l'infection bactérienne contribue au développement de BRONJ, nous avons d'abord utilisé la méthode de l'ADN en damier pour déterminer si les sondes préparées à partir de bactéries buccales humaines pourraient s’hybrider avec l'ADN génomique extrait de la cavité buccale des rats. La méthode a été ensuite appliquée pour détecter et quantifier 43 espèces bactériennes pertinentes à la santé buccale de l’être humain dans des échantillons prélevés de la cavité buccale des patients souffrants de BRONJ et des rats ayant BRONJ-like. Les résultats ont suggéré que le profil bactérien de l'os chez les patients ayant BRONJ est différent par rapport au profil bactérien d'autres tissus durs et mous de la bouche. Ce profil microbien est unique et pourrait être lié aux médicaments que les patients prennent, y compris la chimiothérapie et les bisphosphonates. *E. corrodens*, *S.gordonii* et *S. constellatus* étaient les trois espèces bactériennes dominantes dans l'os exposé, ainsi que d'autres espèces microbiennes non pathogènes et certaines espèces liées aux maladies parodontales et aux infections osseuses. Similairement aux patients ayant BRONJ, l'exposition osseuse chez les rats traités avec les bisphosphonates et corticostéroïdes a été colonisée par des bactéries principalement non pathogéniques. Les trois espèces bactériennes les plus dominantes dans le site d’exposition osseuse étaient *S.pasteuri*, *S.parasanguinis* et *S.mitis*. 
Acknowledgment

First and foremost, I would like to express sincere appreciation to my supervisors Dr Rubens Albuquerque and Dr Janet Henderson for their mentorship, leadership and patience. I learnt, both consciously and un-consciously, from their long experience in research. I appreciate all their contributions to this work, which would never have been completed without their support, enthusiastic and dedication.

I would further like to express my gratitude to Dr Michel El-Hakim and Dr Nicholas Makhoul who allowed me to learn from their clinical experience, to Dr Faleh Tamimi who always inspired me with new ideas, and to Dr Marc McKee for his support.

I would also like to thank all the past and present members of the Albuquerque, Henderson and Seguin laboratories for sharing their time, their assistance and cheerful interaction. I especially wish to thank Dr Cássio do Nascimento for his help with the DNA checkerboard method, Dr Chan Gao for showing me how to use the micro-CT and Ailian Li for assisting in the histological work. I also would like to thank all the people I worked and interacted with during my PhD training, particularly Dr Patricia Olivera, Dr Dominique Behrens and Dr Yongbiao Li.

To my family, I wish to express my warmest thanks for endless support.

This work was supported by McGill Faculty of Dentistry, McGill Faculty of Graduate and Postdoctoral Studies, the Fonds de recherche du Québec – santé, the Réseau de recherche en santé buccodentaire et osseuse, the Fondation de l'Ordre des dentistes du Québec and the Research Institute of the McGill University Health Centre.
Preface and Contribution of Authors

The present thesis includes five prepared or published manuscripts of which the candidate is the first author. Co-author contributions of each manuscript are described below:


- Jabbour Z designed and performed the study, analyzed the data and wrote the manuscript.
- El-Hakim M treated the patients, provided support and access to the patients’ charts, shared writing and reviewing the manuscript.
- Ardakani P was a summer student at the time of conducting the study and assisted in collecting the data and reviewing the charts.
- Henderson JE supervised the study and edited the manuscript.
- Albuquerque Junior RF supervised the study and edited the manuscript.


- Jabbour Z designed and performed the experiment, collected the samples, analyzed the data and wrote the manuscript.
• do Nascimento C analyzed the samples and shared writing and reviewing of the manuscript.

• El-Hakim M treated the patients, provided support and access to the patients, shared writing and reviewing the manuscript.

• Henderson JE supervised the study and edited the manuscript.

• Albuquerque Junior RF supervised the study and edited the manuscript.


  • Jabbour Z designed and performed the animal experiment, collected and analyzed the samples, analyzed the data and wrote the manuscript.

  • El-Hakim M shared writing and reviewing of the manuscript.

  • Henderson JE supervised the study and edited the manuscript.

  • Albuquerque Junior RF supervised the study and edited the manuscript.


  • Jabbour Z designed the study, analyzed the data and wrote the manuscript.

  • do Nascimento C conducted the rat experiment, collected and analyzed the samples.
• Kotake BG was a summer student at the time of conducting the study and assisted in conducting the experiment and analyzing the samples.

• El-Hakim M shared writing and reviewing of the manuscript.

• Henderson JE supervised the study and edited the manuscript.

• Albuquerque Junior RF supervised the study and edited the manuscript.


• Jabbour Z designed and performed the animal experiment, collected the samples, analyzed the data and wrote the manuscript.

• do Nascimento C analyzed the samples and shared writing and reviewing of the manuscript.

• El-Hakim M treated the patients, provided support and access to the patients, shared writing and reviewing the manuscript.

• Henderson JE supervised the study and edited the manuscript.

• Albuquerque RF supervised the study and edited the manuscript.
List of Tables

Chapter 3.1

Table 1: Patient characteristics at the time of admission.................................40

Table 2: The outcomes of conservative treatment of BRONJ at 3- and 6-month follow-up, and at last follow-up.................................................................41

Table 3: The outcomes of surgical treatment of BRONJ at 1-, 3- and 6-month follow-up, and at last follow-up.................................................................42

Chapter 3.2

Table 1: Human DNA microbial species used to prepare probes for cross-reaction with species extracted from the oral cavity of humans.................................61

Table 2: Patients’ characteristics at the time of bacteria collection.........................62

Table 3: The bacterial species per collection site and their detected frequencies ordered by decreasing mean proportions illustrated in Figure 1.................................63

Chapter 4.2

Table 1: Human DNA bacterial species used to prepare probes for cross-reaction with bacterial species from the oral cavity of rats.............................................103

Table 2: Median, lower and upper quartiles of bacterial genome counts (×104), extrapolated from cross-reaction signals released by the DNA checkerboard hybridization test in the control and alcohol-treated (AT) groups..................................104

Chapter 4.3

Table 1: Human DNA microbial species used to prepare probes for cross-reaction with species extracted from the oral cavity of rats.............................................123

Table 2: Target species per collection site ordered by decreasing mean proportion and their detected frequencies.................................................................124
List of Figures

Chapter 3.1
Figure 1: BRONJ staging after conservative or surgical treatments .......................... 43
Figure 2: Cumulative rate of sustained BRONJ stage 0 ................................. 44

Chapter 3.2
Figure 1: Mean proportions (% ± SEM) of the microbial species detected ............ 64
Figure 2: Scatter plots of the mean bacterial proportions .............................. 65

Chapter 4.1
Figure 1: (a) ROI of the maxilla ................................................................. 84
Figure 1: (b) ROI of the mandible .............................................................. 84
Figure 2: Change in rats weight from baseline ......................................... 85
Figure 3: Gross observation at the time of euthanasia .................................. 86
Figure 4: (a) Percentage of bone volume to total tissue volume (BV/TV) in the mandible and maxilla of the NON-EXO site ................................................... 87
Figure 4: (b) Cross-sectional micro-CT images of the mandible in the area of the non-extracted first molar .......................................................... 87
Figure 5: X-rays of the EXO sites ............................................................... 88
Figure 6: Cross-sectional micro-CT images of the mandible in the area of the extracted first molar .......................................................... 89
Figure 7: Quantitative micro-CT of the separated bone fragments from the first molar healing sites of the mandible and maxilla after tooth extraction ................. 90

Chapter 4.2
Figure 1: Mean signals released (±SEM) by bacterial genomes in the control and alcohol-treated (AT) groups ............................................................. 105
Figure 2: Percentages of bacterial-like species detected in the control group………..106

Figure 3: Percentages of bacterial-like species detected in the alcohol-treated (AT) group…………………………………………………………………………………………………………………….107

Chapter 4.3

Figure 1: Mean proportions (% ± SEM) of target species detected in rats……………..125

Figure 2: Scatter plots of the mean bacterial proportions (%)………………………………126

Figure 3: Images of the EXO sites in the maxilla and mandible of the experimental and control groups………………………………………………………………………………………………….127

Figure 4: (a) Seven measurements were taken and averaged to cover the site of the extracted first molar……………………………………………………………………………………………..128

Figure 4: (b) Box plots showing significantly larger alveolar bone height in the mandible with a similar trend in the maxilla of the ZA+DX group………………………………………..128

Figure 5: (a) Box plots show the results of micro-CT quantification of the non-resorbed bone fragments in the mandibular and maxillary extraction sites …………………….129

Figure 5: (b) Histological sections (VonKossa x10) of mandible and maxilla…………..129

Figure 6: (a) Plot box showing the percentage of bone volume to total tissue volume (%BV/TV) in the mandible and maxilla in the area of the non-extracted first molar….130

Figure 6: (b) Cross-sectional micro-CT images of the mandible in the area of the non-extracted first molar……………………………………………………………………………….130
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAOMS</td>
<td>American Association of Oral and Maxillofacial Surgeons</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BRONJ</td>
<td>Bisphosphonate-related osteonecrosis of the jaw</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl pyrophosphate synthase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Micro-CT</td>
<td>Micro-computer tomography</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone–related peptide</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NFkB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NFkB ligand</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>u-PA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
</tbody>
</table>
Chapter one: Introduction
1.1 Principles of bone physiology

The main function of the bony skeleton in vertebrates is to support muscles and body structures, and protect vital organs. The skeleton is able to continuously remodel in response to functional demands. In addition, it plays an important role in maintaining the ion balance in the body, especially calcium and phosphorus (Sommerfeldt and Rubin 2001).

Three major types of cells are dominant in the bony structure that allows the skeleton to undergo its function. These cells are the osteoblasts, the osteoclasts and the osteocytes. The osteoblasts, which have a cuboidal shape, differentiate from the pluripotent mesenchymal stem cells (Aubin 1998). The osteoblasts are located in the bone surface in which they lay down the bone extracellular matrix and regulate its mineralization process (Titorencu, Pruna et al. 2014). The osteocytes are originally osteoblasts that were trapped during the process of bone formation and mineralization. They are the most abundant cells in bone and have cellular processes that form a canalicular network, which connects them to adjacent cells (Curtis, Ashrafi et al. 1985). The osteoclasts, which are multinucleated cells able to resorb mineralized bone, are derived from hematopoietic stem cells (Teitelbaum 2000).

Bone remodeling is a continuous coordinated process between the osteoblasts and the osteoclasts through the receptor activator of the nuclear factor kappa-B (RANK), RANK-ligand (RANKL), and osteoprotegerin (OPG) systems. RANK is a transmembranous receptor located on osteoclast precursor cells. RANKL is expressed by osteoblasts in response to stimuli, such as mechanical forces (Sommerfeldt and Rubin 2001). The
interaction between RANK and RANKL induces the proliferation and differentiation of osteoclasts, leading to bone resorption. OPG is secreted by osteoblasts and also is expressed in other tissues. It interacts with RANKL to prevent it from binding to RANK, and thus protects bone from excessive resorption (Boyce and Xing 2008).

1.2 Mechanism of action of bisphosphonates on bone

Bisphosphonates are synthetic stable analogs of pyrophosphate (P–O–P), a physiological regulator of bone resorption (Fisher, Rogers et al. 1999). They are composed of two atoms of phosphate groups that share one atom of carbon (P-C-P) (Russell, Xia et al. 2007). Bisphosphonates are classified according to their mechanism of action into two groups: non-nitrogen containing bisphosphonates (e.g., clodronate, etidronate, tiludronate) and nitrogen-containing bisphosphonates (e.g., aldonate, ibandronate, pamidronate, risedronate, zoledronate) (Russell 2007).

Bisphosphonates have a poor absorption rate in the intestine. Therefore, the parenteral administration of bisphosphonates is more effective (Ezra and Golomb 2000). About one third to two thirds of bisphosphonates is absorbed by bone and bound to bone minerals. The bone uptake of bisphosphonates depends on the bone turnover rate (Sato, Grasser et al. 1991). The remainder of bisphosphonates is excreted unchanged in the urine by the kidneys (Russell, Xia et al. 2007).

The main function of bisphosphonates is to inhibit osteoclastic bone resorption through intracellular mechanisms. The deposition of bisphosphonates on the bone surface brings them into direct contact with osteoclasts (Azuma, Sato et al. 1995). To resorb bone, osteoclasts produce an acidic microenvironment (pH between 2-4) in the subosteoclastic
space by pumping protons on the peripheral border of the their membrane (Blair, Teitelbaum et al. 1989). This acidic microenvironment in the subcellular space activates secreted enzymes, such as a tartrate-resistant acid phosphatase, to dissolve hydroxyapatite, which leads to a bisphosphonates disassociation and exposure to the external bone surface (Russell, Xia et al. 2007). Bisphosphonate molecules are then taken up by osteoclastic endocytosis, which results in the localization of bisphosphonates into the endocytic vesicles and then into the cytosol (Sato, Grasser et al. 1991). The metabolism of the non-nitrogen containing bisphosphonates in the osteoclast cytosol results in ATP analogs that induce osteoclast apoptosis. The nitrogen-containing bisphosphonates inhibit the farnesyl pyrophosphate synthase (FPPS) enzyme and prevent the prenylation (addition of hydrophobic molecules) of the small GTPase proteins so that these proteins accumulate in their unprenylated form, which is incompatible with the function and survival of osteoclasts (Fleisch 1998).

Preliminary studies suggest that bisphosphonates might have a different mechanism of action on osteoblasts and osteocytes compared to osteoclasts, which preserves the vitality of both osteoblasts and osteocytes (Bellido and Plotkin 2011). Therefore, a bisphosphonate that does not affect osteoclasts could prevent the apoptosis of osteoblasts and osteocytes (Plotkin, Manolagas et al. 2006). The ability of bisphosphonates to prevent osteoblasts apoptosis also depends also on its concentration, which is lower than the range required to induce osteoclasts apoptosis. An inhibitory effect of bisphosphonates on osteoblasts is found at concentrations of $10^{-5} - 10^{-6}$M, whereas a prosurvival effect of bisphosphonates on osteoblasts is exerted at lower concentrations that range from $10^{-6}$M-$10^{-9}$M (Pozzi, Vallet et al. 2009). It appears that bisphosphonates
inhibit osteoblasts and osteocytes apoptosis by increasing the permeability of channels formed by connexin 43, and by the activation of extracellular signal-regulated kinases (ERKs) (Bellido and Plotkin 2011).

## 1.3 The use of bisphosphonates as therapeutic agents

Bisphosphonates were first introduced in the 1800s and used commercially in the 1960s. Initially, they were used in industry as corrosion inhibitors and detergent solutions for calcium and magnesium (Francis and Valent 2007). Bisphosphonates are used in medicine to inhibit bone resorption by regulating osteoclast function, particularly in the management of cancer, osteoporosis, and Paget disease (Siris 1997; Delmas 2005; Giordano, Fang et al. 2008; Saad and Hotte 2010).

**Cancer:** Bisphosphonates are frequently used in the context of cancer therapy, such as prostate cancer, breast cancer, and multiple myeloma (Hortobagyi, Theriault et al. 1996; Major 2002). Bone metastases can be osteoblastic, osteolytic, or mixed (Roodman 2004). In osteolytic lesions, a feature of multiple myeloma and breast cancer, excessive bone destruction occurs. In breast cancer, tumor cells produce peptides, such as parathyroid hormone, interleukin-6, prostaglandin E₂, tumor necrosis factor, and colony-stimulating factor that increase RANKL expression and stimulate osteoclast differentiation and activation, which results in bone resorption. The process of bone resorption releases factors that lead to an increased production of parathyroid hormone-related peptide, such as transforming growth factor b, insulin-like growth factors (IGFs), fibroblast growth factors, platelet-derived growth factor, and bone morphogenetic proteins. These factors
also increase tumor growth. This vicious circle leads to further increases of bone destruction and tumor growth (Yin, Pollock et al. 2005).

Other types of cancer, such as prostate cancer, could result in osteoblastic lesion and local bone formation. Current literature suggests that osteoblastic metastasis also can be a result of a vicious circle leading to increased osteoblast activation and increased tumor growth. The balance in osteoblastic lesion is tipped toward decreased bone resorption and increased bone formation. Decreased bone resorption in prostate cancer results from a cleavage of the parathyroid hormone–related peptide (PTHrP) at the N-terminal by Prostate Specific Antigen (PSA) that is released by the tumor cells (Roodman 2004). Increased bone formation results from tumor cells increased production of the urokinase-type plasminogen activator (u-PA), platelet-derived growth factor (PDGF), and Endothelin-1 (ET-1). These proteins stimulate bone metastasis and activate osteoblasts by releasing osteoblastic growth factors in the bone microenvironment, such as insulin-like growth factors I and II or transforming growth factor b. u-PA can cleave and activate TGF-β, which is produced in the bone microenvironment. The activation of TGF-β regulates osteoblast and osteoclast differentiation, and growth of tumor cells themselves, which suggests a vicious circle (Yin, Pollock et al. 2005).

Bisphosphonates have been used in the treatment of cancer to reduce the number of bone-related events. Depending on the type of tumor and the mechanism of action, bisphosphonates cause osteoclast apoptosis both in vitro and in vivo. Bisphosphonates reduce the number of osteoclasts on the bone surface and therefore reduce osteoclastic bone resorption, which helps to preserve bone integrity and breaks the vicious circle associated with tumor cells. In addition, some bisphosphonates have an effect on cells
other than osteoclasts. For example, zoledronic acid inhibits the adhesion, invasion, and proliferation of tumor cells, and activates T cells against tumor cells (Senaratne, Pirianov et al. 2000; Kabelitz, Wesch et al. 2007; Sun, Iqbal et al. 2010).

**Osteoporosis:** Osteoporosis is a systemic condition resulting from decreased bone mass, decreased bone strength, and increased susceptibility to fracture. Primary osteoporosis, the most common type, occurs more frequently in women than men. Secondary osteoporosis occurs as a result of certain medical conditions, such as long term exposure to glucocorticoid or hyperparathyroidism (Pietschmann, Rauner et al. 2009; Mosekilde, Vestergaard et al. 2013).

Primary osteoporosis is most common in postmenopausal women as a result of estrogen insufficiency. In men, up to 40% of osteoporosis cases are identified without secondary causes (Ebeling 2008). Osteoporotic bone fracture leads to pain, temporary disability, and increased mortality. It has been estimated that the lifetime risk for a woman to have a bone fracture at any skeletal site is 53.2% compared to 20.7% for a man (van Staa, Dennison et al. 2001). However, the mortality rate following hip fracture in men is 4 times higher compared to women (Pietschmann, Rauner et al. 2009). The majority of men who experience a hip fracture have bone mineral density assessments higher than women, which are not within the diagnostic osteoporotic range (Ebeling 2008).

Bisphosphonates are used successfully in the treatment of osteoporosis to reduce bone resorption and hypercalcemia, and to prevent pathologic bone fractures (Watts 2003; Delmas 2005; Iwata, Li et al. 2006; Russell, Xia et al. 2007). With the increase of life
expectancy, the use of bisphosphonates for osteoporosis is expected to grow, with a frequency of use in females six times higher than in males (CIHI 2009).

**Paget’s disease:** Paget’s disease, also known as osteitis deformans, is uncommon in younger ages and occurs in 3–4% of individuals over 40 years (Resnick 1988). It is a chronic progressive skeletal disease characterized by bone hypertrophy and osteosclerosis, which is caused by an excessive resorption and overproduction of poor quality bone (Meunier, Coindre et al. 1980). In its early stages, Paget’s disease is asymptomatic, and patients may have one or multiple bones affected (Britton and Walsh 2012). In late stages, it is associated with pain, irregular thickening, and enlargement or deformation of bone and skeleton elements (Theodorou, Theodorou et al. 2011). Oral and intravenous bisphosphonates have been used in the treatment of Paget’s disease to reduce bone resorption and abnormal remodeling (Cundy and Bolland 2008). Recent investigations suggest that 96.0% of patients receiving zoledronic acid had a therapeutic response at a 6 month follow up compared to 74.3% of patients receiving risedronate. The levels of alkaline phosphatase (ALP) in serum were normalized in 88.6% of patients receiving zoledronic acid compared to 57.9% of patients receiving risedronate (Reid, Miller et al. 2005).

**Osteogenesis imperfecta:** Osteogenesis imperfecta is a genetic condition with an autosomal dominant mode of inheritance that affects the extracellular matrix, which leads to a reduced quality and quantity of type 1 collagen fibers. The condition eventually leads to brittle fragile bone that is susceptible to fractures, vertebral compression, and brittle opalescent teeth (Rauch and Glorieux 2004). The severity of the disease ranges from very mild forms with little fractures to frequent bone fractures and death shortly after birth.
Early treatment of osteogenesis imperfecta with bisphosphonates has resulted in increases in bone mineral density; and reductions in fracture rates, chronic bone pain, and immobility (Phillipi, Remmington et al. 2008). Bisphosphonates also showed positive results in the treatment of types III and IV osteogenesis imperfecta (Letocha, Cintas et al. 2005).

Other uses for bisphosphonates: Bisphosphonates also are used to preserve bone in cases of hyperparathyroidism, prevent periprosthetic bone loss, improve the osseointegration of implants, and reduce bone loss in periodontal diseases. Bisphosphonates also were used, but with less frequency, in some cases of osteonecrosis of the hip, fibrous dysplasia, and calcinosis in juvenile dermatomyositis (Pyram, Mahajan et al. 2011; Silverman 2011).

1.4 Bisphosphonate-related osteonecrosis of the jaw (BRONJ)

1.4.1 Definition and risk factors

In the last few years, a dramatic increase has occurred in the number of reports describing cases of the necrosis of the jaw associated with the administration of bisphosphonates (Ruggiero, Mehrotra et al. 2004; Manfredi, Merigo et al. 2011). A recognition is growing that bisphosphonates, particularly nitrogen-containing bisphosphonates, may be associated with BRONJ. It has been noted that BRONJ can develop spontaneously or in association with dentoalveolar procedures, such as tooth extractions or wearing dentures (Khan, Sandor et al. 2008; Ruggiero, Dodson et al. 2009). BRONJ is clinically diagnosed, and has been defined by the American Association of Oral and Maxillofacial
Surgeons (AAOMS) as “an area of exposed bone in the maxillofacial region that did not heal within 8 weeks after identification by a health care provider, in a patient who was receiving or had been exposed to a bisphosphonate and had not had radiation therapy to the craniofacial region” (Ruggiero, Dodson et al. 2009). In 2007, a staging system that contained 4 stages based on clinical signs and corresponding treatment strategies were implemented. In brief, stage 0 represents nonspecific clinical findings and symptoms without evidence of exposed necrotic bone to the oral cavity. Stage 1 represents an area of exposed or necrotic bone without evidence of active infection. Stage 2 also represents an exposed or necrotic bone associated with infection, pain, and erythema. Stage 3 is the most severe stage and represents exposed or necrotic bone associated with infection, pain, and at least pathologic fracture, extra oral fistula, oral antral/oral nasal communication, or osteolysis.

The incidence of BRONJ in the population depends on the many risk factors identified in an AAOMS 2009 position paper (Ruggiero, Dodson et al. 2009) that include bisphosphonate potency, dose, route of administration, duration of treatment, presence of dentoalveolar surgeries, local anatomy such as mandibular or palatal tori, Caucasian race, and increased age. Gender and the type of malignancy were not identified as risk factors (Ruggiero, Dodson et al. 2009). Co-medications, such as chemotherapeutic and steroid agents, were thought to be risk factors, since they could significantly influence bone physiology (Zervas, Verrou et al. 2006; Jadu, Lee et al. 2007). However, other studies failed to confirm this association (Khamaisi, Regev et al. 2007; Wessel, Dodson et al. 2008).
1.4.2 Incidence of BRONJ

The incidence of BRONJ in patients treated with oral bisphosphonates for osteoporosis is relatively low, but not negligible (Edwards, Gounder et al. 2008; Manfredi, Merigo et al. 2011). It has been estimated that BRONJ develops in 0.1% of patients taking oral bisphosphonates for osteoporosis. This prevalence was assessed based on data from 1,005 survey respondents who reported relevant dental symptoms and underwent dental examination. Nine of these cases were identified as BRONJ (Lo, O'Ryan et al. 2010). A higher risk of BRONJ development was reported in patients taking intravenous bisphosphonates for osteoporosis (Hansen, Knirschke et al. 2013).

The prevalence of BRONJ among patients treated for Paget’s disease and osteogenesis imperfecta is largely undefined, but expected to be low. No cases of jaw bone necrosis were reported in previous clinical trials that assessed bisphosphonate efficacy for Paget’s disease (Reid, Miller et al. 2005; Langston, Campbell et al. 2010). Additionally, no cases of BRONJ were identified in a group of 64 patients who received intravenous disodium pamidronate for osteogenesis imperfecta even though more than a third of them had dental surgical procedures while undergoing bisphosphonate therapy (Malmgren, Astrom et al. 2008).

The incidence of BRONJ in cancer patients using bisphosphonates was estimated to be around 10%, which represents 94% of published BRONJ cases (Woo, Hellstein et al. 2006; Ruggiero, Dodson et al. 2009). Other systematic reviews estimated the overall prevalence of BRONJ to be 6.1%, with a range between 13.3% and 0.7% depending on the presence of follow-up and the quality of the reviewed studies (Migliorati, Woo et al.
No considerable difference was noted in the prevalence of BRONJ between malignancies (Reid and Cornish 2011). Depending on the overall health of the patients, the prevalence of BRONJ ranged between 2.5–18.6% among patients treated for prostate cancer, between 1–7% among patients treated for breast cancer, and between 2–11% among patients treated for multiple myeloma (Abu-Id, Warnke et al. 2008; Hoff, Toth et al. 2008; Walter, Al-Nawas et al. 2008; Fehm, Beck et al. 2009; Vahtsevanos, Kyrgidis et al. 2009).

1.4.3 Hypotheses for BRONJ

The pathophysiological process of BRONJ remains undefined, and no clear explanation exists as to why the osteonecrosis is restricted to the oral cavity (Allen and Burr 2009). Many hypotheses have tried to explain BRONJ, and four of them have been frequently reported in the literature. They are related to the effect of bisphosphonates on the jaw bones, mucosa, and other soft tissues of the jaws; the anti-angiogenesis effects of these drugs; and the role of bacteria in the development of BRONJ.

**Effect of bisphosphonates on the jaw bones:** Bisphosphonates inhibit osteoclasts activity and oversuppress bone remodeling (Fleisch 1997). It has been reported that the mandible and long bones have different responsiveness to similar stimulus (Mavropoulos, Rizzoli et al. 2007) and that the bone turnover rate in the mandible is higher than the rate in the long bones and maxilla (Huja, Fernandez et al. 2006). The jaw uptake of bisphosphonates was thought to oversuppress its remodeling capacity and, subsequently, makes it more sensitive to external trauma than other bones (Mashiba, Mori et al. 2005; Woo, Hellstein et al. 2006; Van den Wyngaert, Huizing et al. 2007). However, the
concentration of bisphosphonates in the jaw bone of rats was shown to be similar to other sites of the skeleton (Bauss, Pfister et al. 2008).

**Effect of bisphosphonates on soft tissues:** The toxicity of bisphosphonates to the endothelial and subcutaneous tissue has been previously demonstrated (Moreira, Katayama et al. 2005). For example, pamidronates have been shown to significantly inhibit the proliferation of the cells of the oral mucosa and wound healing *in vitro* (Landesberg, Cozin et al. 2008). Bisphosphonate administration has been shown to delay wound healing and bone formation in the extraction socket of rats treated with a local alendronate injection prior to tooth extractions (Hikita, Miyazawa et al. 2009). Recent evidence indicates that BRONJ may be associated with the incapacity of the soft tissues affected by bisphosphonates to heal (Landesberg, Cozin et al. 2008; Hikita, Miyazawa et al. 2009; Kobayashi, Hiraga et al. 2010). Damage to the alveolus initiated by trauma or other processes may result in the local release of bound bisphosphonates into surrounding tissue (Reid, Bolland et al. 2007). Although the quality of evidence is still poor, it has been hypothesized that the presence of dental intervention, such as tooth extraction or wearing dentures, may lead to trauma and a non-healing of soft tissues. As a result, bone becomes exposed to the oral cavity, which leads to bacterial infection and necrosis (Kyrgidis, Vahtsevanos et al. 2008; Landesberg, Cozin et al. 2008; Senel, Duman et al. 2010).

**Effect of bisphosphonates on the jaw bone vascularization:** Angiogenesis is a process of forming new vascular networks. The anti-angiogenesis effect of bisphosphonates on vascularization has been previously reported in the literature (Yamagishi, Abe et al. 2004). A decreased amount of new woven bone formation associated with a transient
reduction in the number of new blood vessels and the vascular area have been noted in rats following tooth extraction and a local injection of alendronate (Aguirre, Altman et al. 2010). Sonis et al. (Sonis, Watkins et al. 2009) used histological sections and pointed to differences in the vascular characteristics in the extraction socket of rats following the subcutaneous administration of zoledronic acid and dexamethasone. However, the difference reported in their results did not reach a statistically significant level, possibly because of the small number of animals in each group. Lopez-Jornet et al. (Lopez-Jornet, Camacho-Alonso et al. 2010) reported preliminary indications that the BRONJ generated in experimental rats could be linked to a reduced jaw bone vascularization. Other studies have demonstrated that bisphosphonates inhibit the vascular proliferation of endothelial cells taken from human umbilical veins in vitro (Fournier, Boissier et al. 2002). In another investigation, Yamashita, Koi et al. (2011) reported no effect of bisphosphonates on angiogenic markers in the bone marrow or soft tissue of rats. They concluded that bisphosphonates affect bone marrow remodeling by suppressing the genes associated with lymphangiogenesis.

**The role of oral biofilm in the development of BRONJ:** BRONJ has been noted to mainly occur in the jaw bones. A unique characteristic of the jaws that differentiates them from other skeletal bones is their close proximity to the external environment. Only thin layers of keratinized mucosa separate the jaw bones from the oral cavity, a reservoir of more than 700 bacterial species colonizing the teeth and mucosal surfaces of the mouth (Aas, Paster et al. 2005). The in vitro incubation of oral bacteria taken from healthy subjects with zoledronic acid resulted in an increase in the proliferation rate and number of white colonies after 24 hours (Kobayashi, Hiraga et al. 2010). In the same study, the
authors mentioned an increase in the rate of adhesion of *Streptococcus mutans* to hydroxyapatite. *In vivo* studies reported that the occurrence of BRONJ was related to the presence of infection (Hansen, Kunkel et al. 2006; Sedghizadeh, Kumar et al. 2008). It has been suggested that bisphosphonates may inhibit the immune response system against pathogenic bacteria such as *Actinomyces*, which may play a role in producing a non-healing inflammatory process that could lead to BRONJ (Hansen, Kunkel et al. 2006; Sedghizadeh, Kumar et al. 2008). However, in a study on experimentally induced BRONJ, *Actinomyces* was not found in all cases of necrotic exposed bone when Periodic Acid Schiff staining was used (Sonis, Watkins et al. 2009; Lopez-Jornet, Camacho-Alonso et al. 2010). In addition, Wei, Pushalkar et al. (2012) used a molecular technique and reported little presence of *Actinomyces* in their samples that were taken from BRONJ patients Lodi, Sardella et al. (Lodi, Sardella et al. 2010) reported minimal post-operation complications associated with 38 extractions in 23 patients undergoing bisphosphonate therapy and treated with amoxicillin (1 g, 3 times/day) 1 day before the dental intervention and continued for 17 days. Ferlito, Puzzo et al. (Ferlito, Puzzo et al. 2011) reported no signs of BRONJ following 102 tooth extractions in 43 patients exposed to intravenous zoledronate and treated with a prophylactic protocol of amoxicillin plus clavulanate (1g, 2 times/day) 2 days pre-operatively and 5 days post-operatively.

However, the role of bacteria in the development of BRONJ remains unclear, since BRONJ stage 0 develops spontaneously without bone exposure to the oral cavity. In addition, the histologically diagnosed osteonecrosis of the jaws in patients with a history of bisphosphonates treatment was not always associated with the presence of bone

1.4.4 Management of BRONJ

BRONJ is a significant clinical problem associated with pain, mucosa ulceration, infection, and bacteria colonization that leads to non-healing exposed bone. It dramatically affects the quality of life of patients. Treatment alternatives for BRONJ have been focused on conservative or surgical procedures, depending on the stage of the BRONJ. An interruption of bisphosphonates might be considered after consultation with the treating specialist and depending on patient health. Given the limited information about the pathophysiology of BRONJ, associated risk factors, and treatment alternatives, clinical guidelines might not be effective for the treatment and prevention of BRONJ. Consequently, no clear guidelines are available to define any preventive and therapeutic alternatives for patients who require dental interventions while being exposed to bisphosphonates.

**Conservative treatment:** According to the current guidelines (Ruggiero, Dodson et al. 2014), conservative treatment is recommended for early stages of BRONJ. This approach focuses on patient education and the non-operative management of symptoms, which includes the use of pain medication, antibiotics, antibacterial mouth rinse, maintenance of oral hygiene, regular clinical follow-up, and review of indications to continue bisphosphonate therapy. Several reports have described the successful treatment of BRONJ stage 1 and some cases of BRONJ stage 2 using conservative treatment (Van den Wyngaert, Claeys et al. 2009; Ferlito, Puzzo et al. 2012).
**Surgical treatment:** Surgical treatment in advanced BRONJ cases is proposed in the current guidelines (Ruggiero, Dodson et al. 2014). In addition to symptomatic treatment, a debridement of the area of necrosis is recommended to reduce trauma and relieve the irritation of the surrounding soft tissues. An extraction of symptomatic teeth in the necrotic area also should be considered. A surgical debridement and resection of the necrotic bone is advocated in severe advanced cases associated with extra-oral fistula or pathologic fracture (Ruggiero, Dodson et al. 2014). Several studies have demonstrated a resolution of advanced BRONJ following surgical treatment (Wilde, Heufelder et al. 2011; Rupel, Ottaviani et al. 2014). However, based on the current guidelines, a potential failure of bone grafting and reconstructive surgeries should be recognized, given the risk of necrosis, although recent reports have demonstrated a successful reconstruction using vascularized bone (Seth, Futran et al. 2010; Lemound, Eckardt et al. 2012).

**Interruption of bisphosphonates:** Bisphosphonates may remain in the bone matrix for an extended period of time after treatment cessation. The presence of bisphosphonates in the urine can be detected many months after bisphosphonate interruption (Allen and Burr 2011). Since bisphosphonates may stay in the bone matrix for an extended period of time, their inhibitory effect on bone remodeling may continue even after bisphosphonate interruption (Allen and Burr 2010). Therefore, it is unlikely that bisphosphonate discontinuation for a short period of time will allow bone to resume remodeling and significantly reduce the risk of developing BRONJ (Ruggiero, Dodson et al. 2009; Allen and Burr 2010). If the overall patient’s condition allows, an interruption or reduced exposure to bisphosphonates for individuals with severe oral complications or before dentoalveolar surgeries has been suggested (Ruggiero, Dodson et al. 2014). Although no
longitudinal prospective data exists to support the beneficial effects of bisphosphonate discontinuation in reducing the risk of BRONJ, the beneficial outcomes of interrupting or reducing bisphosphonate exposure prior to dental intervention have been described in the literature (Corso, Varettoni et al. 2007; Stanton and Balasanian 2009). In a previous case series, a successful surgical treatment of 33 patients with BRONJ was reported following a 2-month bisphosphonate holiday (Stanton and Balasanian 2009). Corso, Varettoni et al. (Corso, Varettoni et al. 2007) reported one case of BRONJ in a group of 55 patients treated with zelodronic acid once every 3 months compared to 6 cases in a group of 51 patients treated with monthly doses of zelodronic acid. However, several cases of BRONJ development were reported even after bisphosphonate discontinuation (Gallego and Junquera 2009; Del Conte, Bernardeschi et al. 2010). As a result, it is not clear yet how long bisphosphonate interruption or reduction is required to achieve a significant decrease in the risk of BRONJ development (Corso, Varettoni et al. 2007; Campisi, Fedele et al. 2009).

**Vascularized bone grafts:** The systemic administration of bisphosphonates results in the deposition of the drug not only in the jaw bones but also in the bonny skeleton. Many early reports provided arguments against the use of vascularized bone transfer in BRONJ patients. These opinions were based on the risk of transferring an autogenous bone graft that already has been exposed to the same antiresorptive medications (Ruggiero et al. 2004; Marx et al. 2005; Marx 2009). However, more recent case series have showed favorable results for the management of BRONJ using a vascularized bone flap (Engroff et al. 2007; Seth et al. 2010). Examples of the successful use of autogenous grafts include the reconstruction of mandibular bony defects with vascularized fibula flap (Seth et al.
2010; Lemound et al. 2012). Although reconstruction using vascular bone transfer seems to yield promising results, both clinicians and patients should be aware of the potential failure of these treatment modalities given the systemic inhibitory effect of the drugs on osteoclasts and the risk of transferring tissues containing the antiosteoclastic medications (Ruggiero, Dodson et al. 2014). The most recent consensus guidelines have favored the use of vascularized bone transfer for the management of the reconstruction of extensive jaw bone necrosis extending to the sinus or resulting in pathological fractures of the mandible (Khan, Morrison et al. 2015).

1.4.5 Current animal models

Previous studies have used rats to investigate the bony changes associated with bisphosphonate therapy. For example, Sonis, Watkins et al. (2009) created a rat model of BRONJ-like disease following the extraction of all the molars on one side of the mouth and the combined administration of zoledronic acid and dexamethasone. They demonstrated a high rate (80–100%) of presence of exposed bone in the area of extraction 2 and 4 weeks post-operatively. Removal of all molars could result in a significant trauma to the jaw and a defect that does not heal within the experimental timeline. In addition, the authors pointed to the potential association between reduced vascularization adjacent to the necrotic bone and the presence of exposed bone. However, the link between bone vascularization and the presence of necrosis has not been confirmed, most likely because of the small number of animals in the study groups (Sonis, Watkins et al. 2009). Other animal studies tested the combined administration of the bisphosphonate pamidronate and the corticosteroid dexamethasone in a larger number of animals (Lopez-
Jornet, Camacho-Alonso et al. (2010). They showed that the administration of this drug combination could also result in areas of exposed bone similar to BRONJ following tooth extraction. They also observed a decrease in the vascularization measured on the histological sections of the animals treated with this combination. Similarly, Bi, Gao et al. (2010) generated BRONJ-like disease in experimental mice following the combined administration of zoledronic acid, dexamethasone, and chemotherapy. They found a higher incidence of non-healing bone exposure in the group that received bisphosphonate combined with chemotherapy drugs compared to the group given only zoledronic acid and dexamethasone. However, the group treated with bisphosphonate had only three mice, and the incidence of the clinically diagnosed BRONJ-like condition was low when animals did not receive associations containing chemotherapy drugs (Bi, Gao et al. 2010). Another investigation linked the increased prevalence of a BRONJ-like condition with a vitamin D deficiency in rats (Hokugo, Christensen et al. 2010). Based on quantitative histological analysis only, Ali-Erdem, Burak-Cankaya et al. (2011) reported differences in bone formation, inflammation, and necrosis in the sites of tooth extractions of rats injected with combinations of zoledronic acid and dexamethasone, and non-treated rats. Senel, Duman et al. (2010) found that administering bisphosphonates can increase inflammatory responses in the jaw bones even without performing tooth extractions or any other dental procedure. Additional experimental limitations of other proposed models were lack of traumatic injury factor, use of local instead of systemic drug administration, short-term follow-up, different drug administration schedules and lack of analysis of residual bisphosphonate effect (Hikita, Miyazawa et al. 2009; Biasotto, Chiandussi et al. 2010; Senel, Duman et al. 2010).
Presently, no well-established pre-clinical model to study BRONJ exists (Sonis, Watkins et al. 2009; Bi, Gao et al. 2010; Lopez-Jornet, Camacho-Alonso et al. 2010). Therefore, the development of an animal model will help to evaluate the physiopathology of BRONJ and the potentially beneficial effect of bisphosphonate withdrawal. In addition, such a model will help to identify bacterial species that might be present in the area of exposed bone.

1.5 Osteonecrosis of the jaws with other antiresorptive medications (denosumab)

More recently, an osteonecrosis of the jaws has been reported in association with other antiresorptive medications, such as denosumab. Denosumab is a newly developed drug used to regulate osteoclasts and bone remodeling. It is a fully human monoclonal antibody with high affinity and specificity to RANKL (Lipton and Jacobs 2011). RANKL is a cytokine part of the tumor necrosis factor (TNF) family that has been shown to bind and activate RANK, a receptor located on the surface of the osteoclasts, which play an essential role in the differentiation, formation, function, and survival of osteoclasts (Boyle, Simonet et al. 2003). Denosumab binds to RANKL and prevents its interaction with the RANK receptor on the surface of osteoclast. As a result, denosumab inhibits bone remodeling by inhibiting the function of osteoclasts. The inhibition effect of denosumab has demonstrated similar results to intravenous bisphosphonate administration (Lipton and Jacobs 2011).

Patients treated with denosumab for cancer have shown mucosal ulceration and non-healing bone exposure with characteristics similar to BRONJ (Aghaloo, Felsenfeld et al.
Several clinical trials have reported that denosumab has an equivalent or higher inhibitory effect on bone remodeling than zoledronic acid. The administration of denosumab delayed or prevented skeletal-related events (SREs) in breast and prostate cancer patients (Stopeck, Lipton et al. 2010; Fizazi, Carducci et al. 2011; Henry, Costa et al. 2011). These trials also suggested that the incidence of an osteonecrosis of the jaws among patients treated with denosumab was similar or slightly higher than the incidence of the BRONJ found among patients treated with zoledronic acid (Lipton and Jacobs 2011).

Contrary to bisphosphonates, denosumab binds reversibly to the RANKL receptors located on the surface of osteoclasts (Kostenuik, Nguyen et al. 2009; Miller 2009). Therefore, an interruption of denosumab might result in a resumption of bone remodeling and a faster restoration of the osteoclastic function (Lipton and Jacobs 2011). This reversible binding is particularly important in cases where the restoration of bone remodeling is required, such as in bone fractures or the development of BRONJ.
Chapter two: Rationale, research hypothesis, and objectives
2.1 Rationale

As early as 2003, reports linking the use of anti-osteoclastic drugs to non-healing areas of the maxillofacial skeleton after surgical manipulation started to appear (Marx 2003). The risk of developing bone necrosis is a major clinical problem when patients treated with bisphosphonates undergo dentoalveolar surgical procedures, such as tooth extraction or the placement of dental implants. BRONJ is associated with pain; infection; bone loss; and difficulties chewing, swallowing, and breathing. Why BRONJ occurs only in the jaw is unknown. Challenges in the clinical management of BRONJ emerge as the etiology remains undetermined, and the outcomes of different treatment modalities are not well known. Therefore, a review of the treatment outcomes of BRONJ adds to the current knowledge, particularly in cases of BRONJ stage 2 for which conservative or surgical treatment is currently indicated.

Moreover, the effect of bisphosphonate withdrawal remains unknown. Bisphosphonates may stay in the bone for an extended period of time, so a short holiday might not have a significant impact on the risk of BRONJ development. Given the difficulties associated with addressing this issue clinically, we developed a pre-clinical model of BRONJ to evaluate the effect of bisphosphonates on the jaw bones and tested the beneficial effect of bisphosphonate withdrawal. In addition, we used this pre-clinical model to study the effect of bisphosphonates on the healing and remodeling of the jaw bones. This effect is not well described in previous experimental models in the literature that have attempted to explain BRONJ.
Although it is unlikely that infection is the primary etiology of BRONJ, oral bacteria can colonize an area of exposed bone and potentially lead to a non-healing lesion. Therefore, a description of the bacteria colonizing an area of exposed bone can contribute to the management and practice guidelines for BRONJ, and thus help with antibiotic selection. In addition determining whether bisphosphonate administration changes the profile of oral microbiota could be a new and relevant contribution to knowledge.

2.2 Research hypothesis

Our investigation of BRONJ includes treatment outcomes, bisphosphonate interruption, and the identification of bacteria colonizing the exposed bone.

Our research hypothesis is based on the controversial outcomes of the present therapeutic approach to treating BRONJ, particularly BRONJ stage 2 for which both conservative and surgical treatments are indicated. In addition, our hypothesis suggests that an interruption of bisphosphonate therapy could be beneficial, and that an identification of the bacterial species associated with the exposed bone could contribute to the management of BRONJ.

2.3 Objectives

1. To retrospectively assess the conservative and surgical treatment outcomes for BRONJ.

2. To assess the effect of bisphosphonates on the jaw bones and the benefit of bisphosphonate withdrawal.
3. To identify the bacterial species potentially associated with BRONJ, and to determine if bisphosphonate administration could change the microbiota profile of the oral cavity.
Chapter three: Assessment of treatment outcomes and oral microbiota in BRONJ patients
3.1 Manuscript 1

The outcomes of conservative and surgical treatment of stage 2 bisphosphonate-related osteonecrosis of the jaws: A case series.

Running title: Treatment outcomes of BRONJ stage 2.

Zaher Jabbour DMD, MSc¹, Michel El-Hakim DMD, MD, MSc², Pouya Mesbah-Ardakani BSc³, Janet E Henderson PhD⁴ and Rubens Albuquerque Junior BSc, DMD, MSc, PhD¹

¹ Division of Restorative Dentistry, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
² Division of Oral and Maxillofacial Surgery, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
³ Undergraduate student, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
⁴ Division of Orthopedic Surgery, Faculty of Medicine and JTN Wong Labs for Bone Engineering, McGill University, Montreal, QC, Canada.

This study was published in the International Journal of Oral and Maxillofacial Surgery, 2012 Nov;41(11):1404-9 (reprinted with permission from Elsevier).
Abstract

The purpose of the current retrospective chart review is to describe the outcomes of conservative or surgical treatment of stage 2 bisphosphonate-related osteonecrosis of the jaws (BRONJ). 14 charts (mean patient age 69.07 ± 10.37 years) describing 19 BRONJ stage 2 sites were identified. According to the treatment protocol, all patients received conservative treatment. Surgical treatment was delivered only to sites that did not respond to conservative treatment. Conservative treatment alone was delivered to 11 sites in 8 patients (mean postoperative follow-up 17.6 ± 9.4 months). Surgical treatment was delivered to 8 sites in 6 patients (mean postoperative follow-up 10.0 ± 6.1 months). Bisphosphonate exposure ranged from 1 to 8 years. In most cases, tooth extractions and wearing dentures were reported as triggers for BRONJ. At the last follow-up, BRONJ stage 0 was noted in 7 sites that received conservative treatment and 5 sites that received surgical treatment. Within the limitations of the current chart review, the results showed that although conservative treatment for BRONJ stage 2 can provide favourable outcomes, surgical treatment represents a suitable alternative in non-responsive case.

Key words: Bisphosphonates, necrosis, jaw bone, treatment, surgical, conservative.
Bisphosphonates and denosumab are drugs that have a strong influence on bone remodelling. Bisphosphonates inhibit osteoclast recruitment, differentiation and induce osteoclast apoptosis.¹

Bisphosphonates also have direct effects on other cells. They appear to prevent apoptosis of osteoblasts and osteocytes, and inhibit the growth of epithelial cells.² and ³ Oral bisphosphonates have been increasingly used in the management of osteoporosis, Paget's disease and osteogenesis imperfecta.⁴ and ⁵ The use of intravenous bisphosphonates is also common in the context of cancer therapy such as multiple myeloma and bone metastasis from solid tumours.⁶ and ⁷ Bisphosphonates associated with cancer therapy preserve bone integrity and reduce the risk of skeletal complications.⁸ They have direct antitumor effects by reducing the release of cancer growth factor, and inhibiting cancer cell adhesion and invasion.⁹ and ¹⁰

There is growing evidence supporting the link between bisphosphonate-related osteonecrosis of the jaws (BRONJ) and the administration of bisphosphonates.¹¹ It was estimated that up to 12% of patients treated with intravenous bisphosphonates for cancer develop BRONJ following dental intervention.¹² BRONJ among patients taking oral bisphosphonates was estimated at 0.1%.¹³ Spontaneous development of BRONJ with no history of dental treatment or trauma to the oral cavity was also observed.¹⁴

BRONJ stages and treatment alternatives are described in the current guidelines.¹² Accordingly, treatment for stages 0 and 1 is conservative and treatment for stage 3 is surgical resection of the necrotic bone. According to these guidelines, management of established BRONJ stage 2, which is typically associated with exposed
bone, erythema, ulceration of the soft tissues and pain with or without purulent drainage, is conservative, but recent case series showed positive outcomes after surgical therapy. The present retrospective chart review aimed to describe the outcomes of BRONJ stage 2 in a cohort of patients who received conservative therapy alone or followed by surgical treatment.

**Materials and methods**

This study was approved by the McGill University Health Center Research Ethics Board. All patients diagnosed and treated for BRONJ stage 2 between July 2008 and July 2011 were identified. The inclusion criteria were: male and female patients aged 18 years and older; patients receiving oral and/or intravenous bisphosphonates; patients diagnosed with BRONJ stage 2 at the time of admission to the Oral and Maxillofacial Surgery Clinic; patients treated according to the protocol for BRONJ stage 2 at the Montreal General Hospital; and patients with minimum 6-month follow-up.

Patients with history of radiation to the head and neck area were excluded from the study. According to the treatment protocol for BRONJ stage 2 at the Montreal General Hospital, all patients received conservative therapy. Conservative treatment focused on reinforcing oral hygiene, regular dental follow-up, mouthrinse administration (chlorhexidine 0.12%, twice per day) and superficial necrotic bone debridement with topical anaesthesia. Patients who did not show signs of improvement after 8 weeks of conservative treatment associated with appropriate antibiotic coverage and when the area of radiolucency measured more than 1 cm in thickness on panoramic X-ray were offered surgical treatment after discussion with their treating specialist.
Temporary bisphosphonate discontinuation was also discussed with the treating specialist. Patients who accepted the surgical treatment underwent surgical debridement and sequestrectomy of the necrotic bone. As indicated by the treatment protocol for cases with large areas of necrotic bone, general anaesthesia was used. After induction of general anaesthesia, the face and neck were prepped and draped. The gingiva over the affected area was incised and full thickness flaps were elevated on the buccal and lingual aspects. The necrotic bone was removed using a high speed drill until bleeding bone was present in the surgical site. Care was taken to preserve vital structures when possible (e.g. inferior alveolar nerve). The site was irrigated and closed primarily with 3.0 vicryl sutures. When tension was noted in the flaps, releasing incisions and scoring of the periosteum were performed. All surgical procedures were performed by the same surgeon. The following parameters were extracted from the charts: age, gender, indication of bisphosphonate therapy, type and duration of bisphosphonate therapy, locations of BRONJ, co-medications, dental and medical history. BRONJ was diagnosed and staged according to the American Association of Oral and Maxillofacial Surgeons position paper (AAOMS). Descriptive analysis was used in the current case series.

Results

19 sites in 14 patients (males 4, females 10, mean age 69.07 ± 10.37 years) were identified (Table 1). Exposure to bisphosphonates ranged from 1 to 8 years. The mean postoperative follow-up for both treatments was 12.67 ± 9.02 months. Intravenous bisphosphonates were prescribed to 11 patients for the treatment of: metastatic breast cancer (n = 5); metastatic prostate cancer (n = 2); kidney cancer (n = 2); multiple myloma (n = 1); and osteoporosis (n = 1). Oral bisphosphonates were administered in 3 patients
for the treatment of osteoporosis and osteopenia. The corticosteroid prednisone was used in 4 patients (Table 1).

BRONJ was more frequent in the mandible than in the maxilla (4 in maxilla; 15 in mandible). In most cases, patients reported that the lesions were initiated in association with dental procedures such as tooth or root extractions ($n = 8$) or wearing dentures ($n = 4$). One patient reported spontaneous BRONJ. The trigger was not documented in 1 case.

During the course of BRONJ therapy, none of the treated sites progressed to stage 3 BRONJ. At the last follow-up, none of the patients died or had their health status apparently changed due to bisphosphonate therapy discontinuation (Table 1).

Conservative treatment was delivered to 11 sites in 8 patients (mean postoperative follow-up $17.6 \pm 9.4$ months). At the 3-month follow-up, BRONJ stage 0 was not noted in any site (Fig. 1 and Fig. 2). At the last follow-up, BRONJ stage 0 was present in 7 sites, stage 1 was present in 2 sites and stage 2 was present in 2 sites (Table 2).

Surgical intervention was delivered to 8 sites in 6 patients (mean postoperative follow-up $10.0 \pm 6.1$ months). The presence of bone necrosis in these patients was confirmed by the pathology report. At the 1-month follow-up, 6 sites were characterized by BRONJ stage 0 (Fig. 1 and Fig. 2). At the last follow-up, BRONJ stage 0 was present in 5 sites, stage 1 was present in 1 site and stage 2 was present in 2 sites (Table 3).
Discussion

BRONJ is an important complication of bisphosphonate therapy that dramatically influences the patient's quality of life and requires immediate intervention. Patients with BRONJ suffer from various symptoms such as exposed necrotic bones, mucosal ulceration, pain, infection and tooth loss. The authors reported the follow-up results of a group of patients who received surgical and/or conservative treatment. In the present study, conservative treatment alone resulted in BRONJ stage 0 in certain cases. Surgical treatment, although not the treatment of choice, seems to yield positive outcomes in cases not responding to conventional therapy.\textsuperscript{16, 17 and 18} In the present study, surgical treatment appeared to provide faster resolution of the symptoms associated with BRONJ stage 2 (Fig. 2). The faster response in the surgical cases could be due to the removal of the large necrotic bone sequestrum which is colonized by bacteria and could not be penetrated by systemic antibiotics. Surgical treatment was also helpful to reduce the overall burden of BRONJ, and therefore, could improve the quality of life in severely ill patients non-responsive to conservative treatment. A previous case series reported successful surgical treatment with platelet-rich plasma in 25 patients of BRONJ who did not respond to conservative treatment.\textsuperscript{16}

Successful surgical treatment of 33 patients with BRONJ was also reported following a 2-month bisphosphonate holiday.\textsuperscript{17} In the current series, there was no standard protocol for bisphosphonate interruption. Temporary bisphosphonate discontinuation was considered in certain cases after discussion with the treating specialist (Table 1). Bisphosphonates stay in the jawbones for an extended period of time after bisphosphonate interruption.\textsuperscript{19} Although interrupting or reducing bisphosphonate
exposure might have beneficial effects on the development of BRONJ, it is unlikely that bisphosphonate discontinuation for a short period of time will allow significant reduction in the risk of developing BRONJ. As a result, it is not clear how long bisphosphonate interruption or reduction is required to achieve significant decrease in the risk of development of BRONJ.

In the present case series, the majority of patients received intravenous bisphosphonates for cancer and 3 patients received oral bisphosphonates. Most patients developed BRONJ following dental interventions, such as tooth extraction or wearing dentures. Previous case series described BRONJ following dental interventions in patients treated mainly with intravenous bisphosphonates for cancer or with oral bisphosphonates for osteoporosis. In comparison with these reports, the present cohort of patients represented similar characteristics to those previously described. BRONJ-like disease was also reported in patients treated with other bone antiresorptive agents, such as denosumab, a fully human monoclonal antibody with high affinity and specificity to the receptor activator of nuclear factor-κB ligand (RANKL). In addition to bone antiresorptive agents, cancer patients are usually exposed to a combination of drugs that significantly influence the bone and its vascularisation, such as the corticosteroid prednisone and different chemotherapeutic agents (Table 1). Steroid use was reported to produce avascular necrosis in the femoral head in humans and rats. Additional studies are needed to clarify the complex pathophysiology of BRONJ and the potential synergic effects of bisphosphonates with other drugs such as corticosteroids.

In the current study, patients were referred for BRONJ treatment after the disease was fully developed. There was no standard protocol for antibiotic administration prior to the
dental procedures that could have triggered BRONJ. Although bacteria might have accelerated the development of BRONJ, it is probably not the primary aetiology for the condition since BRONJ was reported spontaneously without prior bone exposure to oral microbiota.\textsuperscript{12}

Prophylaxis protocols before dental intervention for patients at risk of developing BRONJ could have a significant beneficial effect in the prevention of secondary infection associated with BRONJ. Lodi et al.\textsuperscript{30} reported minimal post operation complications associated with 38 extractions in 23 patients undergoing bisphosphonate therapy and treated with amoxicillin (1 g, 3 times/day) 1 day before dental intervention and continued for 17 days. Ferlito et al.\textsuperscript{31} reported no signs of BRONJ following 102 tooth extractions in 43 patients exposed to intravenous zoledronate and treated with prophylactic amoxicillin plus clavulanate (1 g, 2 times/day), 2 days preoperatively and for 5 days postoperatively. Once BRONJ is developed, systemic antibiotics are unable to reach the bacteria in the necrotic bone sequestrum, so they have limited if any impact on the treatment of advanced stages of BRONJ although they can be beneficial in the management of the associated cellulitis.\textsuperscript{32} Identification of the bacterial species involved in the development of BRONJ and the beneficial use of selective antibiotic in the prevention and management of BRONJ is critical and should receive more attention.

The present retrospective study included a small number of patients and was designed to describe the outcomes of conservative and surgical treatment of BRONJ stage 2. Well controlled prospective clinical trials are difficult to conduct given the relatively low incidence of BRONJ, the bioethical considerations and the complicated medical history of the patients. Therefore, retrospective studies are still important tools that add to the
pool of patients described in the literature and help to better understand the physiopathology of BRONJ and its treatment outcomes.

In conclusion, although conservative treatment for BRONJ stage 2 might result in favourable outcomes, surgical intervention represents a suitable alternative in non-responsive cases.

**Funding**

None.

**Competing interests**

None declared.

**Ethical approval**

This chart review study was approved by McGill University Health Center Research Ethics Board (11-619 GEN).
References

19. Allen MR, Burr DB. Bisphosphonate effects on bone turnover, microdamage, and mechanical properties: what we think we know and what we know that we don’t know. Bone 2011;49:56–65.
Table 1. Patient characteristics at the time of admission.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Indication for bisphosphonate therapy</th>
<th>Bisphosphonates</th>
<th>Temporary Bisphosphonate discontinuation</th>
<th>Related co-medications</th>
<th>Reported Duration of Bisphosphonate therapy (months)</th>
<th>Reported trigger</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>F</td>
<td>Osteoporosis</td>
<td>Alendronate (PO)</td>
<td>Not available</td>
<td>Not available</td>
<td>Not available</td>
<td>Denture</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>F</td>
<td>Osteoporosis</td>
<td>Alendronate (PO)</td>
<td>Yes</td>
<td>Prednisone</td>
<td>36</td>
<td>Denture</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>F</td>
<td>Kidney cancer</td>
<td>Pamidronate (IV)Zoledronic acid (IV)</td>
<td>Yes</td>
<td>-</td>
<td>6 10</td>
<td>Denture</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
<td>F</td>
<td>Multiple myeloma</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Prednisone, melphalan</td>
<td>10</td>
<td>Tooth came out on its own</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>F</td>
<td>Breast cancer</td>
<td>Pamidronate (IV)</td>
<td>Yes</td>
<td>Capecitabine</td>
<td>&gt; 84</td>
<td>Denture</td>
</tr>
<tr>
<td>6</td>
<td>78</td>
<td>M</td>
<td>Prostate cancer</td>
<td>Zoledronic acid (IV)</td>
<td>No</td>
<td>Prednisone</td>
<td>24</td>
<td>Remaining root extractions</td>
</tr>
<tr>
<td>7</td>
<td>69</td>
<td>M</td>
<td>Kidney cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Sunitinib</td>
<td>24</td>
<td>Spontaneous</td>
</tr>
<tr>
<td>8</td>
<td>87</td>
<td>F</td>
<td>Osteopenia</td>
<td>Alendronate (PO)</td>
<td>Yes</td>
<td>-</td>
<td>Not available</td>
<td>Tooth extractions</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
<td>F</td>
<td>Breast cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Exemestane</td>
<td>60</td>
<td>Not available</td>
</tr>
<tr>
<td>10</td>
<td>66</td>
<td>M</td>
<td>Prostate cancer</td>
<td>Zoledronic acid (IV)</td>
<td>No</td>
<td>Prednisone, docetaxel, vinorelbine, abiraterone acetate, sunitinib</td>
<td>12</td>
<td>Tooth extractions</td>
</tr>
<tr>
<td>11</td>
<td>68</td>
<td>F</td>
<td>Breast cancer</td>
<td>Zoledronic acid (IV)</td>
<td>No</td>
<td>Exemestane</td>
<td>72</td>
<td>Tooth extractions</td>
</tr>
<tr>
<td>12</td>
<td>79</td>
<td>M</td>
<td>Osteoporosis</td>
<td>Alendronate (IV)</td>
<td>No</td>
<td>-</td>
<td>96</td>
<td>Tooth extractions</td>
</tr>
<tr>
<td>13</td>
<td>59</td>
<td>F</td>
<td>Breast cancer</td>
<td>Pamidronate (IV)</td>
<td>Yes</td>
<td>Tomoxilen, trastuzumab</td>
<td>17</td>
<td>Tooth extractions</td>
</tr>
<tr>
<td>14</td>
<td>76</td>
<td>F</td>
<td>Breast cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Sunitinib</td>
<td>24</td>
<td>Tooth extractions</td>
</tr>
</tbody>
</table>
Table 2. The outcomes of conservative treatment of BRONJ at 3- and 6-month follow-up, and at last follow-up.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site of necrosis</th>
<th>Conservative treatment</th>
<th>BRONJ at 3 month follow-up</th>
<th>BRONJ at 6 month follow-up</th>
<th>BRONJ at the last follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Left maxilla</td>
<td>Mouthwash</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (27)</td>
</tr>
<tr>
<td>1</td>
<td>Right posterior maxilla</td>
<td>Mouthwash</td>
<td>(+)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (27)</td>
</tr>
<tr>
<td>2</td>
<td>Left mandible</td>
<td>Mouthwash</td>
<td>Not available</td>
<td>(+)B (-)P (-)I</td>
<td>(-)B (+)P (-)I (24)</td>
</tr>
<tr>
<td>2</td>
<td>Right mandible</td>
<td>Mouthwash</td>
<td>Not available</td>
<td>(+)B (+)P (-)I</td>
<td>(-)B (+)P (-)I (24)</td>
</tr>
<tr>
<td>3</td>
<td>Left anterior maxilla</td>
<td>Mouthwash</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (+)P (+)I (6)</td>
</tr>
<tr>
<td>4</td>
<td>Left posterior mandible</td>
<td>Mouthwash</td>
<td>Not available</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (+)P (+)I (7)</td>
</tr>
<tr>
<td>5</td>
<td>Mylohyoid ridge of left mandible</td>
<td>Conservative debridement and mouthwash</td>
<td>(+)B (+)P (+)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (8)</td>
</tr>
<tr>
<td>5</td>
<td>Mylohyoid ridge of right mandible</td>
<td>Conservative debridement and mouthwash</td>
<td>(+)B (+)P (+)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (8)</td>
</tr>
<tr>
<td>6</td>
<td>Left posterior mandibular</td>
<td>Surgical incision and drainage</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I (27)</td>
</tr>
<tr>
<td>7</td>
<td>Right posterior mandible</td>
<td>Superficial debridement</td>
<td>(+)B (-)P (+)I</td>
<td>(+)B (-)P (+)I</td>
<td>(+)B (-)P (-)I (10)</td>
</tr>
<tr>
<td>8</td>
<td>Left posterior mandible</td>
<td>Mouthwash</td>
<td>(+)B (-)P (+)I</td>
<td>(+)B (-)P (+)I</td>
<td>(-)B (-)P (-)I (25)</td>
</tr>
</tbody>
</table>

Table 3. The outcomes of surgical treatment of BRONJ at 1-, 3- and 6-month follow-up, and at last follow-up.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site of necrosis</th>
<th>Duration between diagnosis and surgical treatment (months)</th>
<th>Surgical treatment</th>
<th>Antibiotic coverage</th>
<th>BRONJ at 1 month follow-up</th>
<th>BRONJ at 3 month follow-up</th>
<th>BRONJ at 6 month follow-up</th>
<th>BRONJ at the last follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Right mandible</td>
<td>36</td>
<td>Debridement/squestectomy</td>
<td>Amoxicillin + Clavulanate</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (+)P (-)I (17)</td>
</tr>
<tr>
<td>9</td>
<td>Right maxilla</td>
<td>84</td>
<td>Debridement/squestectomy</td>
<td>Amoxicillin + Clavulanate</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (17)</td>
</tr>
<tr>
<td>10</td>
<td>Right mandible</td>
<td>36</td>
<td>Debridement/squestectomy</td>
<td>Amoxicillin + Clavulanate</td>
<td>(-)B (-)P (+)I</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (-)P (+)I (15)</td>
</tr>
<tr>
<td>11</td>
<td>Anterior mandible</td>
<td>7</td>
<td>Debridement/squestectomy</td>
<td>Amoxicillin + Clavulanate</td>
<td>(-)B (+)P (-)I</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I (13)</td>
</tr>
<tr>
<td>12</td>
<td>Left mandible</td>
<td>11</td>
<td>Debridement/squestectomy</td>
<td>Penicillin VK</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (6)</td>
</tr>
<tr>
<td>12</td>
<td>Right mandible</td>
<td>11</td>
<td>Debridement/squestectomy</td>
<td>Penicillin VK</td>
<td>(+)B (+)P (+)I</td>
<td>(+)B (+)P (+)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (+)P (-)I (6)</td>
</tr>
<tr>
<td>13</td>
<td>Left mandible</td>
<td>2</td>
<td>Debridement/squestectomy</td>
<td>Amoxicillin + Clavulanate</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I</td>
<td>(-)B (-)P (-)I (6)</td>
</tr>
<tr>
<td>14</td>
<td>Left posterior mandible</td>
<td>3</td>
<td>Debridement/squestectomy</td>
<td>Penicillin VK</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (-)I</td>
<td>(+)B (-)P (+)I</td>
<td>(+)B (-)P (+)I (6)</td>
</tr>
</tbody>
</table>

Fig. 1. BRONJ staging after conservative or surgical treatments.
Fig. 2. Cumulative rate of sustained BRONJ stage 0.
3.2 Manuscript 2

Unique profile of bacteria colonizing the exposed bone in patients with bisphosphonate-related osteonecrosis of the jaws: A clinical survey

Running title: Microbiota associated with bisphosphonate osteonecrosis

Zaher Jabbour\textsuperscript{a,d}, Cássio do Nascimento\textsuperscript{b}, Michel El-Hakim\textsuperscript{c}, Janet E Henderson\textsuperscript{d,e} and Rubens F. de Albuquerque Junior\textsuperscript{a,b}.

\textsuperscript{a}Division of Restorative Dentistry, Faculty of Dentistry, McGill University, Montreal, QC, Canada.

\textsuperscript{b}Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of Sao Paulo, Brazil.

\textsuperscript{c}Division of Oral and Maxillofacial Surgery, Faculty of Dentistry, McGill University, Montreal, QC, Canada.

\textsuperscript{d}Bone Engineering Labs, Research Institute, McGill University Health Center, Montreal, QC, Canada.

\textsuperscript{e}Department of Medicine and Surgery, Faculty of Medicine, McGill University, Montreal, QC, Canada.
Abstract

**Background:** Microbial etiology of bisphosphonate-related osteonecrosis of the jaw (BRONJ) remains uncertain. This study aimed to investigate the oral microbiota of BRONJ patients.

**Materials and methods:** Microbiota samples of 10 BRONJ patients were collected from the exposed bone, immediately adjacent teeth, contra-lateral teeth and the tongue. Microbiota analysis was performed using DNA checkerboard hybridization with probes from human genomic DNA of 38 bacterial and 5 candida species. Frequencies and mean proportion for each bacterial species were used for descriptive, comparative and correlation analyses.

**Results:** Strong significant correlations were found between the mean bacterial proportions colonizing the adjacent teeth, contra-lateral teeth and tongue. No correlation was found between the bacteria colonizing the exposed bone and other evaluated sites. *E. corrodens* was the only species detected in all BRONJ sites. *E. corrodens, S. gordonii* and *S. constellatus* were the three most dominant bacterial species in the exposed bone.

**Conclusions:** Within the study limitations, it was concluded that microbiota of the exposed bone in BRONJ patients has different profile compared to the bacterial profile of other hard and soft tissues of the mouth. Non-pathogenic microbial species, and species linked to periodontal diseases and bone infection were prevalent in the sites of exposed bone.

**Key words:** Bisphosphonates, jaw, osteonecrosis, bacteria biofilm, DNA checkerboard, cancer.
Introduction

Bisphosphonates are a class of drug commonly used to inhibit bone resorption in the management of hematologic cancers like multiple myeloma, metastatic lesion from solid tumors such as breast and prostate, as well as in the treatment of metabolic bone disease like osteoporosis (Coleman et al., 2011). Osteonecrosis of the jaw has been recognized as a serious complication of anti-resorptive (e.g., bisphosphonates) and anti-angiogenic (e.g., sunitinib) drugs (MRONJ) (Ruggiero et al., 2014). Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been defined as the presence of persistent, exposed, non-healing bone for more than 8 weeks in the maxillofacial region in patients treated with bisphosphonates but with no history of radiation to the head and neck region (Ruggiero et al., 2009). The etiology of BRONJ remains largely un-defined (Otto et al., 2010).

The human oral cavity represents a reservoir of more than 700 bacterial species that colonize the teeth and mucosal surfaces of the mouth (Aas et al., 2005). Some of these species have been implicated in the pathogenesis of periodontitis and in life threatening diseases like pneumonia, endocarditis, and osteomyelitis (Shay, 2002). Wei et al. (2012), using molecular techniques to characterize the microbiota associated with BRONJ, have identified Streptococcus, Eubacterium, and Pseudoramibacter as the three top genera in BRONJ patients compared to Parvimonas, Streptococcus, and Fusobacterium in control cancer patients with oral infection but no history of bisphosphonate exposure. In another investigation, Ji et al. (2012) reported that oral antibiotics had limited effect on the bacterial population colonizing the sites of bone exposure in BRONJ patients. To date, no conclusive investigation has determined whether the site of exposed bone in patients with
BRONJ has a different microbial profile than other sites of the mouth. Therefore, the aim of the current survey was to describe the profile of the bacterial species colonizing the teeth, tongue, and exposed bone in BRONJ patients who were treated with bisphosphonates for cancer or osteoporosis.

**Materials and methods**

**BRONJ patients**

This study was approved by the McGill University Health Center Research Ethics Board. Patients diagnosed and treated for BRONJ at the Oral and Maxillofacial Clinic, Montreal General Hospital were identified and signed an informed consent form to participate in the study. The inclusion criteria were the following:

1. Male and/or female patients aged 18 years and older
2. Patients diagnosed and treated for bone exposure associated with BRONJ stage 1 or 2 between April and September 2012
3. Patients receiving oral and/or intravenous bisphosphonates

Patients with a history of radiation to the head and neck area were excluded from the study. Details about patient age, gender, bisphosphonates, co-medications, periodontal health, main complaint, and site of the bone necrosis were documented.

**Microbial sample collection**

Microbial samples were collected using a microbrush (Microbrush International, Grafton, WI, USA) by rubbing it till saturation for 30 seconds. The use of a microbrush was shown to be a suitable method for microbial biofilm collection (do Nascimento et al.,
Given the possible inaccuracy of measuring the weight of the samples, we used standard-sized microbrush tips that are capable of absorbing a volume of about 6 µL. Bacteria biofilm samples were collected from the exposed necrotic bone, the supragingival part of the immediately adjacent teeth, the contra-lateral teeth, and the dorsal surface of the tongue. Immediately after collection, samples were individually disposed into a microtube containing 150 µl of TE (10Mm Tris-HCl, 1Mm EDTA pH 7.6), and then 150 µl of 0.5M sodium hydroxide was added to cause cell lysis and expose genetic material. Samples were stored at 4°C until the DNA checkerboard hybridization was performed. Sample collection did not cause any pain or discomfort to the patients and was performed without local anaesthesia.

**DNA checkerboard hybridization**

Bacterial and fungal DNA hybridization was performed using the checkerboard method as described previously (do Nascimento et al., 2010; Jabbour et al., 2013). Hybridization probes were prepared by direct labeling (Amersham Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare, Buckinghamshire, UK) of the genomic DNA of the 38 bacterial and 5 candida species listed in Table 1. Probe selection was based on the relevance of the species to human oral health and their pathogenicity. The suitable specificity and sensitivity of these probes were already tested and optimized for the detection of $10^4$ cells (Socransky et al., 2004). The bacterial and fungal whole-genomic DNA extracted from the biofilm samples of patients were denatured, precipitated, and blotted onto the hybridization membrane (Hybond N+, Amersham Biosciences, Buckinghamshire, UK) using a MiniSlot 30 apparatus (Immunetics, MA, USA). As reference samples, defined amounts of genomic DNA corresponding to either
the $10^5$ or $10^6$ cells of each analyzed target species were mixed in a single tube, denatured, precipitated, and applied to the membranes. Samples were fixed on the hybridization membrane using a vacuum and baked for 2 hours at 80 degrees C. Membranes were then pre-hybridized using an oven shaker (Amersham Biosciences) to control for temperature and humidity. Labeled probes were then introduced using a Miniblotter 45 device (Immunetics, Immunetics, Cambridge, MA, USA), and the membranes hybridized overnight at a controlled temperature and humidity under gentle agitation. After washing, hybridization signals were detected by chemiluminescence using a CDP-Star reagent (GE Healthcare), and the membranes were exposed to ECL Hyperfilm-MP for 60 minutes (GE Healthcare). Hyperfilm images were digitized and quantified using TotalLab Quant analysis software (TotalLab Life Science Analysis Essentials, Newcastle upon Tyne, UK).

**Statistical analyses**

To adjust for any potential variation in the volume or weight of the samples, the mean proportion was used by calculating the percentage of each detected microbial species within each sample, and then averaging it for each collection site. Multiple sites of exposed bone in the same patient were averaged and considered one sample. A Pairwise Wilcoxon signed rank test was used for within-group comparisons of the mean bacterial proportions colonizing the adjacent and contra-lateral teeth. A Spearman non-parametric correlation was used to assess the relationship between the proportions of the bacterial species colonizing the exposed bone, adjacent teeth, contra-lateral teeth, and tongue. All the statistical analyses were performed using SPSS 19 with a significance level $p<0.05$. 

50
Results

Patients’ characteristics

BRONJ was noted clinically as exposed bone surrounded by inflamed soft tissues. A total of 10 BRONJ patients (male = 5, female = 5, mean age = 73.2 ± 10.8 years) were identified. These patients’ characteristics are presented in Table 2. Exposure to bisphosphonates ranged from 1 to 8 years; intravenous bisphosphonates were prescribed for 7 patients as adjuvant cancer medication, and oral bisphosphonates for 3 patients to treat osteoporosis; prednisone was the most frequent co-medication, which was prescribed to four patients; seven patients had taken antibiotics in the 6 months prior to the sample collection for systemic and/or oral infection control; only 2 patients were totally edentulous; multiple sites of bone exposure were noted in 3 patients.

Microbiological results

All probes hybridized with the microbial whole genome extracted from the oral samples of BRONJ patients. \textit{E. corrodens} was the only bacterial species detected in all sites of exposed bone. \textit{T. forsythia}, \textit{E. faecalis}, and \textit{N. mucosa} were detected in 9 BRONJ sites (Table 3). The most dominant bacterial species colonizing the teeth were \textit{V. parvula}, \textit{T. forsythia}, \textit{S. gordonii}, \textit{S. oralis}, \textit{S. mitis}, and \textit{T. denticola}. The tongue was mainly colonized by \textit{V. parvula}, \textit{S. mitis}, \textit{S. gordonii}, \textit{S. parasanguinis}, and \textit{S. oralis} (Table 3). The mean proportions of bacteria per collection site are shown in Figure 1.

The mean proportions of \textit{C. krusei}, \textit{E. faecalis}, \textit{K. pneumonia}, and \textit{N. mucosa} tended to be higher or significantly higher in the contra-lateral teeth when compared to the teeth
adjacent to the exposed bone (Figure 1). However, only the mean proportion of *S. mutans* was significantly higher in the teeth adjacent to the exposed bone compared to the contra-lateral teeth (Figure 1).

Significantly strong correlations were found when assessing the relationships between the mean proportions of the bacterial species colonizing the adjacent teeth, contra-lateral teeth, and tongue (Figure 2). However, no significant correlation was found between the mean proportions of bacterial species colonizing the exposed bone and any other collection site in the mouths of BRONJ patients (Figure 2).

**Discussion**

Only a few studies in the literature have investigated the role of oral microbiota in the development of BRONJ (Ji et al., 2012; Wei et al., 2012). In the present investigation, to investigate the oral microbiota collected from BRONJ patients, we employed a DNA checkerboard technique using 43 genomic DNA probes prepared from human oral bacteria and fungi relevant to human health.

In the current study, strong significant correlations were noted between the mean proportions of the bacterial species colonizing adjacent teeth to exposed bone, contra-lateral teeth to exposed bone, and the tongue of BRONJ patients. These results suggest a similar profile for the microbiota colonizing the teeth and tongues of the patients. Moreover, the exposed bone exhibited a distinguishable bacterial profile from the other sites of the oral cavity. The differences noted in the proportions of the species colonizing the exposed bone, tongue, and the supragingival dental biofilm could be attributed to the distinct surface characteristics of each, and the inherent ability of specific microbial
species to selectively colonize them. In the current investigation, although the teeth and tongue have different surface characteristics and are expected to have different bacterial profiles, a strong significant correlation was found between the bacterial profiles colonizing their surfaces. These strong correlations suggest a different effect of the prescribed medications on the bone compared to the teeth and tongue.

Although the solid phase of bone and teeth is composed of calcium-phosphates, the difference in their protein composition could contribute to the unique profile of the bacterial species colonizing the exposed bone. Enamel has little collagen, whereas bone is rich in collagen. *S. aureus* contains collagen receptors (Holderbaum et al., 1987), which could explain its high incidence as the 6th most dominant species in the area of exposed bone. In addition, its high incidence on exposed bone also could suggest that bisphosphonates enhance the adhesion of *S. aureus* to bone hydroxyapatite (Kobayashi et al., 2010; Kos et al., 2013). However, the remarkable presence of *E. corrodens* in all sites of BRONJ, and the role of this species in the development of the necrosis, is unclear. *E. corrodens* is a Gram-negative facultative anaerobic bacillus that could be present in dentoalveolar abscess and is possibility implicated in chronic periodontitis (Kolenbrander et al., 2002). The ability of *E. corrodens* to adhere to bone is not described in the literature. *E. corrodens* has a specific lectin-like substance that facilitates its adherence to other bacterial species such as *S. oralis* and *S.sanguis*. These species did not have a high incidence in our samples collected from exposed bone. The lectin-like substance of *E. corrodens* also could mediate its adherence to other cell surfaces such as macrophages and buccal epithelial cells (Yamazaki et al., 1981; Miki et al., 1987). The debris from
these cells could be present in necrotic exposed bone, which may help to explain the high detection of *E. corrodens* in our samples.

A particular feature of bone infections by the *S. aureus* and *E. corrodens* species is the destruction of mineralized tissue that is caused by the ability of the *S. aureus* protein A to activate the nuclear factor kappa B, which in turn activates osteoclastic bone resorption in the area of the infection (Claro et al., 2013). *E. corrodens* also has surface-associated material that stimulates the synthesis of the Tumor Necrosis Factor alpha (TNFα), which leads to potent bone resorption (Meghji et al., 1994; Meghji et al., 1995). Although the current investigation did not measure bone loss clinically or radiographically, bone loss was not visually observed in our cohort of patients. It is known that bisphosphonates inhibit osteoclastic activity and inhibit bone resorption. Therefore, the absence of bone loss in the area of necrotic bone could be the direct result of bisphosphonate administration.

It has been suggested that the non-shedding, hard surface of teeth could facilitate the adhesion of certain bacteria such as *Streptococci* (Marcotte et al., 1998). In the present investigation, species from this genus were frequently found in high proportions on the surface of the adjacent and contra-lateral teeth.

On the other hand, quantifiable amounts of DNA from pathogenic species were found colonizing the tongue dorsum of BRONJ patients, for example, *T. forsythia, A. actinomycetemcomitans, F. nucleatum*, and *P. gingivalis*. This finding is supported by studies showing that the dorsum of the tongue houses an organized biofilm that could act as an important reservoir for the re-colonization of oral surfaces, which could make its
microbiota less sensitive to medications and to changes in systemic conditions (Mager et al., 2003; Faveri et al., 2006).

Although significant, the differences in the mean proportions of *C. krusei*, *E. faecalis*, *K. pneumonia*, *N. mucosa*, and *S. mutans* colonizing the adjacent and contra-lateral teeth were small (Figure 1). In addition, the frequent presence of these species in the adjacent and contra-lateral teeth was almost similar (Table 3). As a result, these small differences might not be directly related to the development of BRONJ when compared to the overall difference observed.

While several *Streptococcus* and *Staphylococcus* species were observed in high proportions in the area of exposed bone, pathogenic species such as *C. rectus*, *C. gingivalis*, *B. fragilis*, and *A. actinomycetemcomitans* were rarely noted. This observation is consistent with Wei et al. (2012) who reported a high incidence of *Streptococcus* in the area of necrotic bone and a low incidence of *Actinomyces* in their samples when using molecular techniques to identify microorganisms in patients with BRONJ. However, Hansen et al. (2006, 2007) described the presence of *Actinomyces* in the histologic sections of samples taken from patients with jaw osteoradionecrosis or bisphosphonate-induced osteonecrosis. Although *A. actinomycetemcomitans* often has been associated with periodontal diseases (Slots et al., 1999; Amano, 2010; Henderson et al., 2010), its sporadic presence in the area of exposed bone in our study suggests a minor role for this species in the development of BRONJ.

The presence of the non-pathogenic microbial species colonizing exposed bone could be explained by some inhibitory effects of the antibiotics the patients were taking. However,
Ji et al. (2012) reported a limited effect of antibiotic administration on the population of bacteria colonizing the bone. Antibiotics were prescribed to the majority of patients in our cohort by the referring dentist or the treating specialist to control the systemic and/or local infection associated with the original illness.

Our results also support those of Wei et al. (2012) who reported a low incidence of the *Veillonella* and *Lactobacillus* species in the area of exposed bone in BRONJ patients. However, we demonstrated a higher proportion of these species on the teeth of BRONJ patients, which is in line with Napenas et al. (2010) who reported that chemotherapy administration could increase the proportion, number, and diversity of certain bacterial species, including *Veillonella, Lactobacillus, Peptostreptococcus, Aggregatibacter,* and *Bacteroidetes.*

In future studies, a match including medical history, prescribed medications, periodontal status (probing depth, attachment level, and bleeding on probing), age range, gender, and race could be considered for participants serving as the control. In addition, the change in the oral microbiota of the patients taking only bisphosphonates should be evaluated. Although the cross-sectional design of the current study provided information about the microbial species associated with BRONJ, it does not provide information about the sequence of events that led to BRONJ and whether BRONJ is the result of microbial infection or microorganisms colonizing the bone after its exposure to the oral cavity. Longitudinal studies with a larger number of patients are needed to better understand the changes of oral microbiota in cancer patients and whether other factors such as medications, gender, or ethnicity could play a role in the development of BRONJ.
**Conclusion**

Within the limitations of this study, it was concluded that the area of exposed bone of BRONJ patients is predominantly contaminated by non-pathogenic opportunistic microbial species, such as *S. gordonii* and *S. constellatus*. Species linked to periodontal diseases and bone infections such as *E. corrodens* and *S. aureus* also were prevalent. This bacterial profile is unique to exposed bone and does not correlate with the bacterial profile of the teeth and soft tissues of the oral cavity of BRONJ patients.

**Acknowledgements**

Zaher Jabbour received awards from the Fonds de recherche en santé du Québec (FRSQ), the FRSQ-Réseaux de recherche en santé buccodentaire et osseuse (RSBO), and the Fondation de l'Ordre des dentistes du Québec (FODQ). The study was made possible by operating funds from FODQ and Synthes Canada, and infrastructure support from the FRQ-S-RSBO and the Jo Miller Orthopaedic Research Fund.

**Conflict of Interest**

None.
References


Table 1: Human DNA microbial species used to prepare probes for cross-reaction with species extracted from the oral cavity of humans.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregatibacter actinomycetemcomitans a</td>
<td>ATCC 29523</td>
</tr>
<tr>
<td>Aggregatibacter actinomycetemcomitans b</td>
<td>ATCC 29522</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>ATCC 25285</td>
</tr>
<tr>
<td>Campylobacter rectus</td>
<td>ATCC 33238</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>ATCC MYA 646</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>ATCC 90030</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>ATCC 6258</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>ATCC 750</td>
</tr>
<tr>
<td>Capnocytophaga gingivalis</td>
<td>ATCC 33624</td>
</tr>
<tr>
<td>Eikenella corrodenes</td>
<td>ATCC 23834</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC 51299</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC 10798</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>ATCC 25586</td>
</tr>
<tr>
<td>Fusobacterium periodonticium</td>
<td>ATCC 33693</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC 700721</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>ATCC 393</td>
</tr>
<tr>
<td>Mycoplasma salivarium</td>
<td>ATCC 23064</td>
</tr>
<tr>
<td>Neisseria mucosa</td>
<td>ATCC 25996</td>
</tr>
<tr>
<td>Parvimonas micra</td>
<td>ATCC 33270</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>ATCC 49031</td>
</tr>
<tr>
<td>Porphyromonas endodontalis</td>
<td>ATCC 35406</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>ATCC 25611</td>
</tr>
<tr>
<td>Prevotella melaninogenica</td>
<td>ATCC 25845</td>
</tr>
<tr>
<td>Prevotella nigrescens</td>
<td>ATCC 33563</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 27853</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>ATCC 12633</td>
</tr>
<tr>
<td>Solobacterium moorei</td>
<td>CCUG 39336</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>Staphylococcus pasteuri</td>
<td>ATCC 51129</td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>ATCC 27823</td>
</tr>
<tr>
<td>Streptococcus gordonii</td>
<td>ATCC 10558</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>ATCC 49456</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>ATCC 25175</td>
</tr>
<tr>
<td>Streptococcus oralis</td>
<td>ATCC 35037</td>
</tr>
<tr>
<td>Streptococcus parasanguinis</td>
<td>ATCC 15911</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>ATCC 25975</td>
</tr>
<tr>
<td>Streptococcus sanguinis</td>
<td>ATCC 10556</td>
</tr>
<tr>
<td>Streptococcus sobrinus</td>
<td>ATCC 27352</td>
</tr>
<tr>
<td>Tannerella forsythia</td>
<td>ATCC 43037</td>
</tr>
<tr>
<td>Treponema denticola</td>
<td>ATCC 35405</td>
</tr>
<tr>
<td>Veillonella parvula</td>
<td>ATCC 10790</td>
</tr>
</tbody>
</table>
Table 2: Patients’ characteristics at the time of bacteria collection.

<table>
<thead>
<tr>
<th>N</th>
<th>ID</th>
<th>Gender</th>
<th>Age</th>
<th>Original illness</th>
<th>BPs</th>
<th>BPstopped</th>
<th>Co-medications (last 6 months)</th>
<th>Antibiotics (last 6 months)</th>
<th>Site of BRONJ (stage)</th>
<th>Plaque (adjacent teeth)</th>
<th>Plaque (Contra-lateral teeth)</th>
<th>Presence of denture</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRONJ1</td>
<td>MP02</td>
<td>M</td>
<td>66</td>
<td>Prostate cancer</td>
<td>Zoledronic acid (IV)</td>
<td>No</td>
<td>Prednisone, Abraterone, Sanitininb</td>
<td>Amoxicillin + clavulanic acid, Moxifloxacin</td>
<td>44, 43 lingual ridge (II)</td>
<td>3</td>
<td>3</td>
<td>Partial</td>
</tr>
<tr>
<td>BRONJ2</td>
<td>RF18</td>
<td>F</td>
<td>76</td>
<td>Breast cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Sunitinib</td>
<td>-</td>
<td>37, 38 (I) 47 (I)</td>
<td>-</td>
<td>-</td>
<td>Complete</td>
</tr>
<tr>
<td>BRONJ3</td>
<td>DP17</td>
<td>M</td>
<td>78</td>
<td>Prostate cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Prednisone, Clindamycin</td>
<td>37, 38 lingual ridge (I)</td>
<td>2</td>
<td>1</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>BRONJ4</td>
<td>CV24</td>
<td>F</td>
<td>62</td>
<td>Osteoporosis</td>
<td>Risedronate (PO)</td>
<td>No</td>
<td>Prednisone, Moxifloxacin</td>
<td>46 (II)</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BRONJ5</td>
<td>KN35</td>
<td>F</td>
<td>73</td>
<td>Osteoporosis</td>
<td>Alendronate (PO)</td>
<td>Yes</td>
<td>-</td>
<td>37 (I)</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BRONJ6</td>
<td>LJ31</td>
<td>M</td>
<td>85</td>
<td>Prostate cancer</td>
<td>Denosumab (SC)</td>
<td>No</td>
<td>Prednisone</td>
<td>-</td>
<td>36 (I) 15 (I)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BRONJ7</td>
<td>RR30</td>
<td>M</td>
<td>85</td>
<td>Osteoporosis</td>
<td>Alendronate (PO)</td>
<td>No</td>
<td>-</td>
<td>Penicillin VK</td>
<td>47, 46, 45, 44 (II)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BRONJ8</td>
<td>QT26</td>
<td>M</td>
<td>56</td>
<td>Neuro-endocrine carcinoma</td>
<td>Pamidronate (IV)</td>
<td>No</td>
<td>Sunitinib, Teva-amoxicillin Metranidazole</td>
<td>12 buccal ridge (I) 23, 24, 25 (I) 47 (II)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BRONJ9</td>
<td>DF25</td>
<td>F</td>
<td>87</td>
<td>Breast cancer</td>
<td>Alendronate (PO), Pamidronate (IV)</td>
<td>No</td>
<td>Tamoxifen, Aramasin</td>
<td>36, 37 lingual ridge (I)</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BRONJ10</td>
<td>GM01</td>
<td>F</td>
<td>64</td>
<td>Breast cancer</td>
<td>Zoledronic acid (IV)</td>
<td>Yes</td>
<td>Fulvestrant, Amoxicillin + clavulanic acid Ciprofloxacin</td>
<td>47, 48 (II)</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

BPs = Bisphosphonates, IV= Intra-vascular, PO= Oral.

Periodontal score: 0 = Clean tooth surface, 1 = Plaque detected on the gingival third with explorer, 2 = Plaque visible, 3 = Tooth covered with abundant plaque.
Table 3: The bacterial species per collection site and their detected frequencies ordered by decreasing mean proportions illustrated in Figure 1.

<table>
<thead>
<tr>
<th>Exposed bone</th>
<th>Adjacent teeth</th>
<th>Contra-lateral teeth</th>
<th>Tongue</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Number of sites = 10)</td>
<td>(Number of sites = 8)</td>
<td>(Number of sites = 8)</td>
<td>(Number of sites = 10)</td>
</tr>
<tr>
<td>E. corrodens (10)</td>
<td>V. parvula (8)</td>
<td>V. parvula (8)</td>
<td>V. parvula (10)</td>
</tr>
<tr>
<td>S. gordonii (8)</td>
<td>T. forsythia (8)</td>
<td>S. gordonii (8)</td>
<td>S. gordonii (8)</td>
</tr>
<tr>
<td>S. constelatus (8)</td>
<td>S. gordonii (8)</td>
<td>S. oralis (8)</td>
<td>S. oralis (8)</td>
</tr>
<tr>
<td>F. nucleatum (8)</td>
<td>S. mitis (8)</td>
<td>T. denticola (7)</td>
<td>S. parasanguinis (10)</td>
</tr>
<tr>
<td>E. faecalis (9)</td>
<td>S. oralis (8)</td>
<td>T. forsythia (8)</td>
<td>S. oralis (10)</td>
</tr>
<tr>
<td>S. aureus (8)</td>
<td>S. mutans (8)</td>
<td>S. mitis (8)</td>
<td>S. aureus (10)</td>
</tr>
<tr>
<td>S. mitis (8)</td>
<td>S. sanguinis (8)</td>
<td>S. sanguinis (8)</td>
<td>P. intermedia (7)</td>
</tr>
<tr>
<td>N. mucosa (9)</td>
<td>P. intermedia (6)</td>
<td>P. intermedia (6)</td>
<td>S. mutans (10)</td>
</tr>
<tr>
<td>P. gingivalis (9)</td>
<td>S. mutans (8)</td>
<td>S. sanguinis (10)</td>
<td>S. sanguinis (10)</td>
</tr>
<tr>
<td>E. corrodens (6)</td>
<td>T. denticola (6)</td>
<td>P. melaninogenica (7)</td>
<td>E. corrodens (9)</td>
</tr>
<tr>
<td>S. sobrinus (8)</td>
<td>P. gingivalis (7)</td>
<td>C. gingivalis (7)</td>
<td>Aa_a (10)</td>
</tr>
<tr>
<td>P. gingivalis (7)</td>
<td>S. parasanguinis (8)</td>
<td>F. nucleatum (8)</td>
<td>P. gingivalis (7)</td>
</tr>
<tr>
<td>P. aeruginosa (8)</td>
<td>P. melaninogenica (6)</td>
<td>S. pasteurii (8)</td>
<td>P. melaninogenica (7)</td>
</tr>
<tr>
<td>K. pneumonia (7)</td>
<td>S. constelatus (8)</td>
<td>P. micra (8)</td>
<td>T. forsythia (10)</td>
</tr>
<tr>
<td>S. parasanguinis (5)</td>
<td>F. nucleatum (7)</td>
<td>S. sobrinus (8)</td>
<td>S. pasteurii (9)</td>
</tr>
<tr>
<td>P. endodontalis (8)</td>
<td>S. pasteurii (7)</td>
<td>Aa_a (8)</td>
<td>M. salivarium (10)</td>
</tr>
<tr>
<td>P. intermedia (6)</td>
<td>S. salivarius (8)</td>
<td>S. aureus (8)</td>
<td>C. gingivalis (8)</td>
</tr>
<tr>
<td>P. anaerobios (8)</td>
<td>S. sobrinus (8)</td>
<td>P. sanguinis (8)</td>
<td>Aa_b (7)</td>
</tr>
<tr>
<td>S. moroei (6)</td>
<td>C. gingivalis (6)</td>
<td>P. gingivalis (8)</td>
<td>L. casei (10)</td>
</tr>
<tr>
<td>C. kru sei (7)</td>
<td>P. nigrescens (7)</td>
<td>P. nigrescens (8)</td>
<td>L. casei (10)</td>
</tr>
<tr>
<td>F. periodonticum (7)</td>
<td>P. intermedia (4)</td>
<td>E. corrodens (8)</td>
<td>T. denticola (7)</td>
</tr>
<tr>
<td>M. salivarium (7)</td>
<td>C. albicans (6)</td>
<td>S. moroei (8)</td>
<td>P. nigrescens (7)</td>
</tr>
<tr>
<td>S. pasteurii (6)</td>
<td>P. putida (8)</td>
<td>C. rectus (7)</td>
<td>P. putida (8)</td>
</tr>
<tr>
<td>C. glabrata (7)</td>
<td>P. micra (6)</td>
<td>F. periodonticum (8)</td>
<td>S. moroei (8)</td>
</tr>
<tr>
<td>C. albicans (7)</td>
<td>L. casei (7)</td>
<td>S. salivarius (8)</td>
<td>C. rectus (6)</td>
</tr>
<tr>
<td>C. dubliniensis (2)</td>
<td>S. moroei (7)</td>
<td>L. casei (8)</td>
<td>F. nucleatum (7)</td>
</tr>
<tr>
<td>S. oralis (6)</td>
<td>C. rectus (5)</td>
<td>P. putida (8)</td>
<td>E. faecalis (9)</td>
</tr>
<tr>
<td>P. melaninogenica (6)</td>
<td>M. salivarium (7)</td>
<td>M. salivarium (8)</td>
<td>K. pneumonia (8)</td>
</tr>
<tr>
<td>P. micra (7)</td>
<td>C. glabrata (7)</td>
<td>S. constelatus (8)</td>
<td>P. micra (7)</td>
</tr>
<tr>
<td>L. casei (6)</td>
<td>F. periodonticum (6)</td>
<td>P. anaerobios (8)</td>
<td>S. salivarius (7)</td>
</tr>
<tr>
<td>P. putida (5)</td>
<td>E. corrodens (7)</td>
<td>K. pneumonia (8)</td>
<td>N. mucosa (4)</td>
</tr>
<tr>
<td>S. salivarius (6)</td>
<td>P. anaerobios (7)</td>
<td>B. fragilis (6)</td>
<td>C. albicans (4)</td>
</tr>
<tr>
<td>C. tropicalis (5)</td>
<td>P. endodontalis (7)</td>
<td>C. glabrata (8)</td>
<td>S. constelatus (2)</td>
</tr>
<tr>
<td>S. sanguinis (4)</td>
<td>E. coli (6)</td>
<td>E. faecalis (8)</td>
<td>E. coli (3)</td>
</tr>
<tr>
<td>V. parvula (4)</td>
<td>K. pneumonia (7)</td>
<td>Aa_b (6)</td>
<td>P. endodontalis (3)</td>
</tr>
<tr>
<td>T. denticola (5)</td>
<td>B. fragilis (5)</td>
<td>P. endodontalis (8)</td>
<td>P. anaerobios (3)</td>
</tr>
<tr>
<td>S. mutans (4)</td>
<td>C. dubliniensis (6)</td>
<td>N. mucosa (8)</td>
<td>B. fragilis (2)</td>
</tr>
<tr>
<td>P. nigrescens (5)</td>
<td>E. faecalis (7)</td>
<td>E. coli (6)</td>
<td>F. periodonticum (1)</td>
</tr>
<tr>
<td>Aa_a (1)</td>
<td>Aa_a (4)</td>
<td>C. albicans (6)</td>
<td>P. aeruginosa (1)</td>
</tr>
<tr>
<td>C. gingivalis (0)</td>
<td>N. mucosa (7)</td>
<td>P. aeruginosa (8)</td>
<td>C. tropicalis (1)</td>
</tr>
<tr>
<td>Aa_b (0)</td>
<td>P. aeruginosa (6)</td>
<td>C. tropicalis (8)</td>
<td>C. glabrata (0)</td>
</tr>
<tr>
<td>C. rectus (0)</td>
<td>C. tropicalis (6)</td>
<td>C. dubliniensis (6)</td>
<td>C. dubliniensis (0)</td>
</tr>
<tr>
<td>B. fragilis (0)</td>
<td>C. kru sei (6)</td>
<td>C. kru sei (6)</td>
<td>C. kru sei (0)</td>
</tr>
</tbody>
</table>
Figure 1: Mean proportions (% ± SEM) of the microbial species detected. Statistically significant difference between the mean bacterial species colonizing the adjacent and contra-lateral teeth (Pairwise Wilcoxon signed rank test *p ≤0.05, **p<0.01, ***p=0.07).
Figure 2: Scatter plots of the mean bacterial proportions (%) showing significant strong correlations between the bacteria colonizing the adjacent teeth, contra-lateral teeth, and tongue. No correlation was found between the bacteria colonizing the exposed bone and other evaluated sites of the mouth.
Chapter four: Effect of bisphosphonates on the jaw bones and oral microbiota in a pre-clinical model of BRONJ.
4.1 Manuscript 3

Bisphosphonates inhibit bone remodeling in the jaw bones of rats and delay healing following tooth extractions

Running title: Bisphosphonates inhibit jaw bone remodeling in rats

Zaher Jabbour\textsuperscript{1,3}, Michel El-Hakim\textsuperscript{2}, Janet E Henderson\textsuperscript{3,4} and Rubens F. de Albuquerque Junior\textsuperscript{1,5}.

\textsuperscript{1}Division of Restorative Dentistry, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
\textsuperscript{2}Division of Oral and Maxillofacial Surgery, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
\textsuperscript{3}Bone Engineering Labs, Research Institute, McGill University Health Center, Montreal, QC, Canada.
\textsuperscript{4}Department of Medicine and Surgery, Faculty of Medicine, McGill University, Montreal, QC, Canada.
\textsuperscript{5}Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of Sao Paulo, Brazil.

This study was published in the Journal of Oral Oncology, 2014 May;50(5):485-90 (reprinted with permission from Elsevier).
Abstract

Objective: This study aimed to evaluate the impact of concurrent administration of clinically relevant doses of zoledronic acid (ZA) and dexamethasone (DX) on bone healing after tooth extraction (EXO).

Materials and Methods: Forty-four Sprague–Dawley rats (6–8 month old) were randomized into five groups: ZA + DX = weekly injection of ZA with DX for 7 weeks; WD = ZA with DX for 3 weeks then DX alone for 4 weeks; C = control saline for 7 weeks; ZA = ZA alone for 7 weeks and DX = DX alone for 7 weeks. ZA was administered at 0.13 mg/kg/week and DX at 3.8 mg/kg/week and body weights recorded at the time of injection. All rats underwent extraction (EXO) of the mandibular and maxillary first molars at 3 weeks and were euthanized at 7 weeks. The extracted and non-extracted sides of both jaws were harvested for micro-CT analyses.

Results: All rats, particularly those injected with ZA, exhibited weight gain till EXO followed by decline then recovery. ZA + DX group demonstrated highest fractional bone to tissue volume (BV/TV) in the non-extracted side. ZA + DX rats exhibited also highest volume and surface of sequestra. Only sequestra volume was statistically higher in the WD group compared to C group.

Conclusion: Combined treatment with ZA and DX over a prolonged period inhibits bone remodeling and increased sequestra formation to a greater extent than either drug alone. Trauma caused by these sequestra cutting through the mucosa could play a key role in the development of BRONJ by potentially facilitating infection. ZA withdrawal may promote bone-remodeling reactivation following EXO.
Key words: Bisphosphonates; Corticosteroid; Jaw bone; Necrosis; Tooth extraction; Sequestra; Cancer treatment.
Bisphosphonates were proven to be effective drugs in the treatment of osteoporosis and cancer [1]. Intravenous bisphosphonates, such as zoledronic acid and pamidronate are given to reduce the skeletal-related events associated with cancer [2], [3] and [4].

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) was defined in 2006 as an area of exposed bone in the maxillofacial region that does not heal within 8 weeks in a patient under bisphosphonates treatment without history of radiation to the craniofacial region [5].

Bisphosphonates inhibit osteoclast recruitment, differentiation and induce osteoclast apoptosis, which could lead to change in bone microarchitecture and increase the percentage of bone volume to tissue volume [1] and [6]. Although the pathophysiology of BRONJ remains unknown, previous rat models described in the literature reported that the administration of bisphosphonates could change the jaw bone vascularization [7], [8], [9] and [10]. In addition, zoledronic acid administration in rats reported to suppress genes associated with lymphoangiogenesis and tissue remodeling, such as VEGF-C and MMP-13 [11]. Another investigation linked increased prevalence of BRONJ with vitamin D deficiency in rats [12]. Based on quantitative histological analysis only, Ali-Erdem et al. reported differences in bone formation, inflammation and necrosis in sites of tooth extractions between rats injected with combinations of zoledronic acid and dexamethasone and a control group [13]. Senel et al. found that the administering bisphosphonates can increase inflammatory responses of the jaw bone without performing tooth extractions or any other dental procedure [14]. However, the effect of bisphosphonates on the microarchitecture and healing of the jaw bones is not well described in previous experimental models. In addition, the effect of bisphosphonates
withdrawal remains unknown. Bisphosphonates might stay in the bone for an extended period of time and a short holiday might not have a significant impact on the risk of development of BRONJ [1] and [5].

The bisphosphonate zoledronic acid (ZA) and the glucocorticoid dexamethasone (DX) are commonly used together in the treatment of cancer patients. The combined treatment was reported to induce a BRONJ-like disease in rodents [8] and [9]. Using a rat model, this study was therefore aimed at the evaluation of the effect of combined administration of ZA and DX on the microarchitecture of jaw bones and the effect of bisphosphonate withdrawal on healing following tooth extractions.

**Materials and methods**

**Drug administration**

ZA (0.13 mg/kg/week body weight) and DX (3.8 mg/kg/week body weight) were administered by intra-peritoneal (IP) injections. These doses were converted from human doses of ZA (4 mg/person/3 weeks) and DX (40 mg/person/week) according to the National Institute of Health (NIH) guidelines [15]. Forty-four skeletally mature Sprague–Dawley rats aged 6–8 months were prospectively and randomly divided into the following groups: ZA + DX (n = 10) received combined treatment with the drugs throughout the 7-week experiment; WD (n = 10) received ZA + DX for 3 weeks prior to EXO, when ZA was withdrawn and DX continued for an additional 4 weeks; C (n = 8) was injected with physiological saline solution throughout the experiment to serve as a negative control for ZA + DX therapy; ZA (n = 8) and DX (n = 8) were treated with the respective drugs alone throughout the experiment as positive controls. Throughout the
study, body weights were recorded once a week, at the time of drug administration. All animals underwent extractions (EXO) of the upper and lower left first molars three weeks after starting drug administration and were euthanized 4 weeks post-EXO based on literature indicating bone density in the extraction socket is stable after about 30 days [16].

**Tooth extraction (EXO)**

As described previously [8], [16] and [17], animals were anesthetized with a single IP injection of rodent anesthetic cocktail (1 mL/kg) composed of 5.0 mL ketamine (100 mg/mL), 2.5 mL xylazine (20 mg/mL), 1.0 mL acepromazine (25 mg/mL) in 1.5 mL saline. The first molars were removed from the left side of the maxilla and mandible using a dental explorer No. 5, adapted elevators and cotton pliers while the tongue was retracted to one side of the mouth using a mosquito hemostat and sutures. Teeth selected for extractions were first loosened by running the tip of the explorer around the cervical portion and then extracted by luxation with a cotton plier. The extraction sockets were cleaned of residual roots using a medium straight headpiece (5100-015-250) and round fluted SST bur with diameter of 1.0 mm (1608-006-155, Stryker Canada). Following extraction, the bone ridge was shaped and smoothed using surgical rongeurs. Only one upper and one lower molar tooth was extracted in each animal to minimally interfere with the normal eating habits and result in minor post-operative complications [9]. To reduce pain and discomfort, rats were injected with carprofen (10 mg/kg) before the intervention and at 1 and 2 days post-EXO. Wet food was also offered to the animals to ensure regular nutritional intake. Rats were observed twice/week throughout the post-operative period till euthanasia.
Radiologic imaging

X-rays were captured (Kubtec, Milford, CT) at the time of EXO and 3 weeks post-EXO. Although serial X-rays cannot provide precise quantitative information, they were favored over in vivo micro-CT images to reduce the amount of irradiation to the soft and hard tissues surrounding the area of EXO, which could significantly interfere with the healing process. Following euthanasia using CO2 inhalation, both, the EXO and NON-EXO sides of the mandible and maxilla were excised, cleaned, fixed overnight at 4 °C in 4% paraformaldehyde, rinsed thoroughly in three changes of sterile phosphate buffered saline (PBS) and stored at 4 °C while waiting for micro-computed tomography (micro-CT) scan and analysis (Skyscan 1172, Kontich, Belgium). Specimens were scanned at 8.9-μm resolution with exposure time 550 ms, electric voltage 59 kV and current 167 μA adjusted to allow maximum differentiation between mineralized and non-mineralized tissues and using a 0.5 mm thickness aluminum filter. The instrument was equipped with a 1.3 MP camera to capture high-resolution 2D images that were assembled into 3D reconstructions using NRecon software supplied with the instrument. In the NON-EXO side, a region of interest (ROI) covering the area extending from the mesial aspect of the second molar to the mesial aspect of the first molar, including both the cortical and trabecular bone and excluding the first molar itself, was selected for quantitative analyses (Fig. 1). In the mandible, the ROI extended towards the lower border of the mandible to cover the bone till the beginning of the incisor’s enamel, which provided a clear anatomical reference (Fig. 1b). Since visual and threshold separations between trabecular and cortical bones in the mandible and maxilla were not reliably reproducible, the outlined ROI included both the cortical and trabecular bones (Fig. 1). In the EXO side,
the ROI was extended 3.0 mm mesially from the mesial surface of the second molar to cover the EXO area including the separated bone fragments (sequestra) excluding the alveolar ridge. The percentage of bone volume to tissues volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf) and bone structure model index (SMI) were quantified using the CTAn Skyscan software. The number, volume and surface area of the isolated bone fragments in the EXO sites were also measured.

**Statistical analysis**

The number of rats used for this study was based on our pilot investigations and on previous studies [8] and [9]. A total of 10 rats in the ZA + DX group and 8 rats in the C group were estimated to detect a 6% difference in the BV/TV in the NON-EXO side at the site of the first mandibular molar. This sample size was calculated based on a t-test, a standard deviation of 4.2%, 80% power and type 1 error of 0.05, with an increase of 15% to accommodate for the asymptotic relative efficiency of the Mann–Whitney-U test. All groups were compared individually to group C to determine the effect of drug administration on the jaw bone micro-architecture. Wilcoxon signed rank test was used for a within-group comparison of animal weights. Statistically significant difference was set at a p value less than 0.05.

**Results**

**Animal weight**
All rats exhibited weight gain for the first 2–3 weeks with weight loss after EXO. Gradual recovery and weight gain was noted in the post-EXO period in all study groups. Statistically significant increase in the animals’ weight was noted at day 15 in the ZA and ZA + DX groups (Fig. 2). The animals’ weight at the time of euthanasia was about the same as day 15 in all groups except WD, in which statistically significant decrease was noted at the time of euthanasia compared to day 15. There was statistically significant increase in the animals’ weight of groups ZA and ZA + DX at the time of euthanasia compared to day 1 (Fig. 2).

**Gross observation at the site of EXO**

Signs of healing of the EXO sites were noted in the samples from the group C animals at the time of euthanasia (Fig. 3). Inflammation associated with small areas of open wounds and exposed bone, indicating delayed healing at the EXO sites, was noted in five of eight animals in the ZA alone group, but only one of eight animals in the DX alone group. Nine out of ten animals in each of the ZA + DX and WD groups demonstrated clear bone exposure, open wounds and inflammation in the area of EXO in either or both jaws (Fig. 3).

**Micro-CT analysis of the NON-EXO side**

Animals exposed to both ZA + DX demonstrated highest fractional bone volume to tissue volume (BV/TV) in the mandible of the NON-EXO side (Fig. 4). The differences between the C and DX groups in the BV/TV and other bony parameters were not significant. The mandibular BV/TV of the NON-EXO sides in the ZA group was considered marginally significantly different from the C group (p < 0.06). When
compared to group C, animals in the WD and ZA + DX groups showed statistically significant higher BV/TV. Groups exposed to ZA demonstrated narrower bone marrow space in the body of the mandible and between the roots of the first molar (Fig. 4b). No significant differences in the BV/TV in the maxilla, and most other bone parameters in the NON-EXO side of the mandible and maxilla were noted.

**Radiographic images and micro-CT analysis of the EXO side**

Representative images of serial X-rays of bone healing in the different treatment groups are shown in Fig. 5. X-rays of the mandible showed signs of healing in the EXO sites in the C group, including bone remodeling in the first 2 weeks post-EXO and bone formation 3–4 weeks post-EXO (Fig. 5). Most animals in the DX group showed similar signs of gradual healing in the site of EXO. Images from animals of the ZA group demonstrated signs of reduced bone remodeling and unchanged radiopacity at the EXO site by the end of the experiment (Fig. 5). Certain rats exposed to both ZA and DX showed images with bone fragments separated from the mandible, suggesting formation of sequestra at the site of EXO starting at 1 week post-EXO. Resorption of these sequestra in several WD animals was noted 2 weeks post-EXO. However, these bone fragments persisted throughout the study till the time of euthanasia in the ZA + DX rats (Fig. 5).

Three dimensional micro-CT images of the EXO sites in the mandible revealed bone remodeling and minimum amount of separated bone fragments in the C group (Fig. 6). Animals from the DX group showed some signs of delayed alveolar bone resorption. Rats injected with ZA alone demonstrated noticeable reduction in bone remodeling, indicated
by the unchanged shape of the alveolar bone, exposed trabeculae and significantly higher number of un-resorbed bone fragments in the sites of EXO at the time of euthanasia. Animals in the WD and ZA + DX groups presented also un-resorbed bone fragments. Larger bone fragments with high surface area were found in animals of the ZA + DX group. Quantitative micro-CT analysis of the mandibular EXO sites showed statistically significant increase in the volume of separated bone fragments in groups ZA + DX and WD compared to group C (Fig. 7). The difference in the surface area of the bone fragments separated from the mandible was significant only between the ZA + DX and C groups. Animals in the ZA group showed the highest number of separated bone fragments in the maxilla and mandible, which was statistically significant when compared to group C (Fig. 7).

Discussion

Many animal models of BRONJ and its incidence have been described in the literature [7], [8], [9], [10], [11], [12], [13] and [14]. In the current study, we aimed to evaluate the effect of bisphosphonates and corticosteroid on the rat jaw bone microarchitecture and healing of the extraction socket. The effect of bisphosphonates appears to inhibit the remodeling of the jaw bones and be associated with formation of sequestra.

All animals in the current study lost weight immediately following EXO. Gradual recovery was noted in the post-EXO period. This pattern of change in body weight was noted in a previous investigation [18], although only one upper and lower molar per animal were extracted in our study, to minimize the traumatic destruction of the soft and hard tissues and to maintain the normal diet intake of the rats as suggested by others [9].
The weight of the rats in C group at day 15 was similar to their weight at the time of euthanasia showing positive signs of recovery in these animals. Animals injected with DX alone showed a similar pattern of weight gain and recovery as animals from group C, and reminiscent of patients with treated with high dose glucocorticoids. Rats exposed to ZA alone showed a significantly faster rate of weight gain, similar to that seen in rats that received combined ZA + DX therapy. This is consistent with reports indicating a rapid weight gain in patients after IV administration of ZA [19] but diverges from data reported by Matsunaga et al. [20] who found no significant change in the animal weight after 8 weeks of ZA administration, but with a small study group with a high variation. We also suggest that the WD rats did not gain weight following EXO in our experiment due to ZA withdrawal.

The ZA and DX regimens used in the present study are equivalent to 4 mg/person/3 weeks and 40 mg/person/week, respectively, and are commonly used to treat cancer patients. These dosages were calculated for rats based on equivalent surface area conversion factors between humans, rats and other species [15]. It appeared that the combined administration of ZA and DX at these doses had a higher impact on healing in the jaw bone and leads to higher suppression of bone remodeling when compared to the effect of the same doses of ZA or DX alone. The higher incidence of mandibular sequestra associated with higher bone volume and surface area in the ZA + DX group compared to the other groups could have been related to suppression of bone remodeling. Administration of DX or ZA alone seemed to reduce the jaw bone remodeling to a lesser extent, as it did not lead to statistical differences in the area or volume of sequestra, or in the BV/TV levels. Administration of ZA alone resulted in numerous small sequestra,
which suggested osteoclast activity was less suppressed in rats treated with ZA alone compared with those treated with the combination of DX and ZA.

In agreement with observations made by Sonis et al. [8], our data suggests a minor independent effect of DX alone on the healing of the extraction socket. The inhibitory effect of DX on osteoblasts is well documented [21]. Moreover, previous reports indicated that corticosteroid could inhibit the bone-resorbing activity of mature osteoclasts [22] and [23], which in turn could potentiate the inhibitory effect of ZA on the osteoclasts and lead to stronger suppression of the jaw bone remodeling. In addition, the fact that DX is an immunosuppressant could reduce the host reaction to the presence of non-resorbed bone fragments colonized with bacteria and increasing the chances of bone infection and bone exposure similar to BRONJ. These fragments, which sometimes were observed projecting through the mucosal external surface, can be promptly colonized by bacteria in the oral environment and lead to a non-healing bone infection and bone exposure. Repeated damage to the epithelial barrier caused by repeated penetration of un-resorbed sequestra cutting through the mucosa in this study was considered a potential key factor to the onset and development of BRONJ.

Bisphosphonates like ZA bind to hydroxyapatite crystals in bone and are thus retained after administration ceases and continue to inhibit osteoclast activity. This study is the first to investigate the effect of bisphosphonate withdrawal at the time of tooth extraction on subsequent remodeling of the jaw bones and sequestra formation. Serial X-rays of the mandible taken at timed intervals post-EXO revealed sequestra formation 1 week post-EXO in the ZA + DX group, which indicated a low rate of remodeling in this group. These sequestra persisted throughout the study in the ZA + DX group. In the WD group,
resorption of the sequestra on serial X-rays appeared 2 weeks post-EXO (Fig. 5), which suggests resumption bone remodeling following ZA withdrawal. In addition, since there was no statistically significant difference in the surface of mandibular sequestra at the end of experiment between the WD and C groups (Fig. 7), this could indicate a beneficial effect of bisphosphonate withdrawal on resuming bone remodeling. However, the volume of sequestra remained significantly higher in the WD group when compared to the C group (Fig. 7), indicating that longer periods for ZA withdrawal should be further investigated.

In conclusion, we found that the administration of clinically relevant doses of ZA leads to reduced jaw bone remodeling and is associated with increased sequestra formation. Bisphosphonate withdrawal might lead to the resumption of bone remodeling and reduce sequestra formation. The mechanism of chronic epithelial injury associated with sequestra and bacterial infection, and their relationship in the development of BRONJ should be further investigated.

**Conflict of interest statement**

None declared.

**Acknowledgments**

Zaher Jabbour received awards from the Fonds de recherche du Québec – Santé (FRQ-S), the FRQ-S-sponsored Réseau de recherche en santé buccodentaire et osseuse (RSBO) and the Fondation de l’Ordre des dentistes du Québec (FODQ). The study was performed
at the FRQ-S-funded Research Institute of the McGill University Health Centre, with peer-reviewed funding from the FODQ.
References


Fig. 1. (a) ROI of the maxilla and (b) ROI of the mandible.
Fig. 2. Change in rats weight from baseline expressed as mean difference from day 1 ± standard error (SEM) measured weekly at the time of drug administration till euthanasia (Euth). *Statistically significant difference from day 1 (p < 0.05). **Statistically significant difference from day 15 (p < 0.05).
Fig. 3. Gross observation at the time of euthanasia: groups C and DX showing healing of the site of EXO covered by mucosa (white arrows). Group ZA showing delayed healing and small areas of open wounds and exposed bone (outlined arrows). Groups WD and ZA + DX showing clear open wounds and bone exposure associated with inflammation in the area of EXO (black arrows). Mx: maxillary jaw bone. Mn: mandibular jaw bone.
Fig. 4. (a) Percentage of bone volume to total tissue volume (BV/TV) in the mandible and maxilla of the NON-EXO site. Study groups were compared to the C group. *p < 0.05, **p = 0.06. □ = C, □ = DX, □ = ZA, □ = WD, □ = ZA+DX. (b) Cross-sectional micro-CT images of the mandible in the area of the non-extracted first molar showing reduced bone marrow space in the body of the mandible and between the roots of the first molar associated with increased BV/TV in the groups exposed to ZA.
Fig. 5. X-rays of the EXO sites suggesting bone remodeling and formation in groups C and DX (wide short arrows), unchanged bone opacity in group ZA (wide long arrows), sequestra formation (outline long arrows) and resorption (outlined short arrows) in group WD, and persisted sequestra (long narrow arrows) in the ZA + DX group.
Fig. 6. Cross-sectional micro-CT images of the mandible in the area of the extracted first molar. Group C showed round alveolar ridge associated with normal healing and bone remodeling of the EXO site (solid long arrow). Group DX showed delayed bone remodeling as demonstrated by the residual alveolar ridge (solid short arrow). Group ZA showed un-resorbed alveolar ridge, exposed trabeculae and high number of small sequestra (outlined long arrows). Groups WD and ZA + DX also showed sequestra, and the highest volume and surface area noted in the ZA + DX group (outlined short arrows).
Fig. 7. Quantitative micro-CT of the separated bone fragments from the first molar healing sites of the mandible and maxilla after tooth extraction. Study groups were compared to group C. *p < 0.05.
4.2 Manuscript 4

Assessing the oral microbiota of healthy and alcohol-treated rats using whole-genome DNA probes from human bacteria.


Zaher Jabbour¹, Cássio do Nascimento², Bruna Gabriela dos Santos Kotake², Michel El-Hakim³, Janet E Henderson⁴ and Rubens Albuquerque¹,².

¹Division of Restorative Dentistry, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
²Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Brazil.
³Division of Oral and Maxillofacial Surgery, Faculty of Dentistry, McGill University, Montreal, QC, Canada.
⁴Division of Orthopedic Surgery, Faculty of Medicine, McGill University, Montreal, QC, Canada.

This study was published in the Archives of Oral Biology, 2013 Mar;58(3):317-23. (reprinted with permission from Elsevier).
Abstract

Objective: This study aimed to evaluate the capacity of whole-genome DNA probes prepared from human oral bacteria to cross-react with bacteria from the oral cavity of rats, and to assess the influence of alcohol ingestion on the animals’ oral biofilm.

Design: Twenty four mature Wistar rats were equally divided in two groups. One group (control) was fed balanced diet of rat pellets and water. The alcohol-treated group (AT) received the same diet and 20% ethanol solution. Upon euthanasia after 30 days, bacterial samples from the oral biofilm covering the animals’ teeth were collected using microbrushes. Bacteria identification and quantification were performed using the DNA checkerboard hybridization method with 33 probes prepared from human oral bacteria. Signals corresponding to bacterial genome counts and percentages were compared using a Mann–Whitney U test with a significance level <0.05.

Results: Cross-reaction for all targeted species, except Streptococcus mutans and Streptococcus mitis-like species, occurred in the control group. Escherichia coli, Pseudomonas aeruginosa, Porphyromonas endodontalis, and Veillonella parvula-like species only produced detectable signals in the AT group. Significantly more signals were detected in the control group compared to the AT group ($p = 0.001$). The percentage of $E.\ coli$-like species was highest in both groups.

Conclusions: Whole-genome DNA probes prepared from human oral bacteria can cross-react with rats’ oral bacterial species. Alcohol consumption is associated with lower levels and diversity of bacterial species in the oral cavity of rats.

Keywords: DNA checkerboard hybridization; Rats; Alcohol; Oral bacteria.
1. Introduction

The use of genetic molecular methods for identification and quantification of oral bacterial is increasing. These methods are considered faster and offer higher accuracy compared to traditional bacterial culture methods. The DNA checkerboard hybridization method was well described in 1994 by Socransky. It uses whole-genome DNA probes to allow the simultaneous screening of up to 45 bacterial species in 28 samples.

Alcohol consumption has well documented effects on the oral cavity in humans. It was directly linked to pharyngeal cancer, presence of fewer teeth, periodontal disease, horizontal bone loss and higher prevalence of caries. The influence of alcohol ingestion on the oral microbiota is not fully described in the literature. It has been reported that alcohol might decrease the salivary flow leading to an increase in the concentration and number of gram-positive bacteria, and that production of acetaldehyde from oral yeasts and bacteria may result in inhibition of certain species. However, information about the effect of alcohol ingestion on oral bacteria remains scarce and limited to a small number of species isolated in culture media. More analyses using molecular methods are still lacking.

Similarities in the oral microbiota between humans and other mammalians were reported previously. Gram-positive cocci, such as *Streptococcus*, *Staphylococcus* and *Enterococcus*, and gram-positive rods, such as *Lactobacillus*, were found in the oral cavity of humans and rats. Considering handling and culturing difficulties associated with traditional methods, particularly in relation to fastidious and anaerobic-strict species,
and the high sensitivity of molecular methods, it would be beneficial to verify the applicability of molecular methods in animals. More recently, DNA probes prepared from human bacteria were found to cross-react with DNA from bacteria of the oral cavity of dogs and rats with ligature-induced periodontitis. However, these probes failed to produce detectable signals for samples taken from healthy sites of rats’ mouth. As a result, it is still unclear whether the DNA hybridization method could be used to detect bacteria from the oral cavity of healthy rats. Therefore, the aim of this study was to evaluate the potential of whole-genome DNA probes prepared from human oral bacteria to cross-react with oral bacterial species from healthy rats, and to assess the influence of alcohol consumption on their oral microbiota using the DNA checkerboard hybridization method.

2. Methods

2.1. Animals

Twenty four mature Wistar rats were housed for 30 days in groups of six per cage, kept in a temperature-controlled room (23–25 °C), with a light/dark cycle of 12/12 h and free access to food and water. Animals were divided in two groups: one group was fed regular solid diet of rat pellets and water (control group). Rats in the alcohol-treated group (AT group) received the same diet and 20% ethanol solution. The study was approved by the Animal Ethics Committee at University São Paulo. After euthanasia by CO₂, samples of oral biofilm were collected by rubbing a sterile microbrush on the surface of the teeth for 30 s. The use of microbrush was shown to be a suitable method for microbial biofilm collection. Standard-sized microbrush tips are capable to absorb a
volume of about 6 μL. Each sample was placed in individual microtubes containing 150 μL of TE (10 mM Tris–HCl, 1 mM EDTA pH 7.6), followed by the addition of 150 μL of 0.5 M sodium hydroxide. Samples were boiled for 5 min for DNA denaturation and then neutralized using 800 μL of ammonium acetate. The tubes were stored at 4 °C until laboratory processing.

2.2. Microbiological evaluation

Bacterial hybridization was performed using the DNA–DNA checkerboard method, according to do Nascimento et al.\textsuperscript{22} and\textsuperscript{23} Validation of the method in humans is well reported.\textsuperscript{6} Thirty three probes prepared from bacteria whole-genome DNA taken from human oral cavity were tested (Table 1). Bacterial species selection was based on the relevance to the human oral health. Following DNA denaturation, biofilm samples were individually inserted in the extended slots of the MiniSlot 30 apparatus (Immunetics, MA, USA) and fixed on the hybridization membrane (Hybond N+, Amersham Biosciences, Buckinghamshire, UK) using vacuum and baked during 2 h at 80 °C. Next, samples were pre-hybridized using oven shaker (Amersham Biosciences) to control for temperature and humidity. Labelled human probes (Amersham Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare, Buckinghamshire, UK) were applied on the membrane using Miniblotter 45 device (Immunetics, Immunetics, Cambridge, MA, USA). Chemoluminescent signals from the DNA hybridization process were registered onto a Hyperfilm (Amersham Biosciences), digitized and quantified using Image Quant TL software (GE Healthcare, UK).
2.3. Statistical analyses

Percentages of each detected bacterial-like species were calculated and averaged for each group. Between-group comparisons of the released signals were performed using Mann–Whitney U test and SPSS 18 statistical software with significance level <0.05.

3. Results

At the end of the experiment, animals mean weight was 316.3 g and mean age was 4 months. Cross-reaction signals from all targeted bacteria were detected in the control group, with exception of *Streptococcus mutans* and *Streptococcus mitis*-like species. Only signals corresponding to *Escherichia coli*, *Pseudomonas aeruginosa*, *Porphyromonas endodontalis* and *Veillonella parvula*-like species were detected in the AT group (Table 2, Fig. 1). Significantly lower bacterial genome counts were found in the AT group compared to the control group \((p = 0.001)\). The percentage of *E. coli*-like was the highest in both groups with significantly lower bacterial genome counts in the AT group (Table 2, Fig. 2 and Fig. 3). The levels of *P. aeruginosa*-like tended to be lower in the AT compared to the control group \((p = 0.068)\). Signals from *Streptococcus oralis*-like were only detected from two animals of the control group in lowest percentages (Table 2, Fig. 2). *Prevotella melaninogenica*-like was detected in one animal only, and *Bacteroides fragilis*-like was detected in another animal only (Table 2). In the present investigation, cross-reaction signals from *E. coli* and *Lactobacillus casei* probes presented quantification accuracy problems, respectively in seven and five animals, and were thus excluded from the analysis (Table 2).
4. Discussion

This study was designed to test the capability of whole-genome DNA probes prepared from human oral bacteria to cross-react with bacteria from the oral cavity of rats, and to evaluate possible changes of the oral microbiota associated with alcohol ingestion. The positive hybridization reactions observed in the current study are in agreement with previous studies indicating that DNA whole-genome probes prepared from human bacteria can cross-react with bacterial species from the oral cavity of rats.\(^\text{19}\)

In the current study, \textit{E. coli}-like was the most prevalent specie in both groups. Although \textit{E. coli} species can be found in the human mouth, their most common habitat is the gastrointestinal track.\(^\text{24, 25}\) The high prevalence of \textit{E. coli}-like found in both groups was possibly associated with the continuous oral contamination by rats’ faeces (coprophagia habit), since all animals were housed in collective cages without faeces collection. Conversely, \textit{S. mutans} and \textit{S. mitis}-like species were not detected in the current study. A possible explanation for this fact is that all animals in the control and AT groups were free of caries and periodontal diseases. It is well known that these two species are frequently associated with tooth cavities in humans.\(^\text{26}\) However, as these two species have already been detected in rats’ mouth with selective culture media,\(^\text{16}\) it is possible that they were present in both groups, but not in detectable quantities by the DNA checkerboard method. Since the sensitivity of the DNA method was adjusted to allow the detection of \(10^4\) bacterial cells, chemoluminescent signals from positive reactions below this threshold were not detected yet bacterial genomes could have been present in low quantities. Both the visibly small amount of bacterial biofilm collected from the teeth’ surfaces and potential DNA degradation are consistent with the absence of
signals observed in several samples. This is in accordance with findings by Duarte et al., who were not able to detect signals in non-periodontitis sites using the same method. Further calibration of the probe concentration to increase the test sensitivity could have prevented these detection failures to occur. However, previous investigations by Socransky et al. revealed that the adjustment of sensitivity can impair the ability of the method to accurately quantify larger numbers of cells and also amplify nonspecific background signals. Moreover, when probe concentration is increased, nonspecific binding of either the chemoluminescent agent or the antibody conjugate to non-DNA debris may occur potentially adding up to cross-reactions between different species.

Presence of stains on the membranes might also lead to difficulties regarding bacterial cell quantitation. Certain cross-reaction signals from E. coli and L. casei probes were not included with the current analysis since they presented reading difficulties.

From 33 bacterial species targeted in the current study, 31 were detected in the control group and four in the AT group, indicating a significant effect of alcohol ingestion on the oral microbiota of rats. Ethanol 20% caused a considerable decline on bacterial genome counts and the diversity of species. Contrastingly, Kantorski et al. reported an increased number of colony forming units of S. mutans in rats treated with 20% ethanol for 56 days. However, the author reported that the difference did not reach statistical significance. The inhibitory effect of alcohol on oral microorganisms observed in the present study is consistent with findings from Sissons et al., who also observed reduction of bacterial growth in microcosm plaques from saliva treated with ethanol. Nonetheless, this comparison should also be interpreted with caution, since Sissons et al. examined bacteria in vitro using “artificial mouth” model with human saliva.
Since cross-reactivity is known to occur with the DNA checkerboard method at the species level in humans, the term “like” was used in the current study as suggested by Rober et al.\textsuperscript{20} It is also recognized that similar species from different hosts may show different genotypes\textsuperscript{28} and that cross-reactivity may occur due to limitations associated to temperature optimisation during membrane processing and varying G + C content.\textsuperscript{29} Therefore, one of the limitations of this study is that cross-reactions might have occurred with other closely related species.

Within the limitations of the present study, we concluded that whole-genome DNA probes prepared from human oral bacteria can cross-react with bacterial genomes from the oral cavity of rats, and that alcohol ingestion reduces their oral bacteria levels and species diversity.

**Funding**

This study was funded by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

**Competing interests**

None declared.

**Ethical approval**

The study was approved by the Animal Ethics Committee at University São Paulo.

**Acknowledgement**
This study was funded by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).
References

Table 1. Human DNA bacterial species used to prepare probes for cross-reaction with bacterial species from the oral cavity of rats.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 53323</td>
</tr>
<tr>
<td><em>Streptococcus mitis</em></td>
<td>ATCC 49456</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>ATCC 10558</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
</tr>
<tr>
<td><em>Neisseria mucosa</em></td>
<td>ATCC 25996</td>
</tr>
<tr>
<td><em>Parvimonas micra</em></td>
<td>ATCC 33270</td>
</tr>
<tr>
<td><em>Porphyromonas endodontalis</em></td>
<td>ATCC 35406</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>ATCC 393</td>
</tr>
<tr>
<td><em>Fusobacterium periodonticum</em></td>
<td>ATCC 33693</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>ATCC 25586</td>
</tr>
<tr>
<td><em>Staphylococcus pasteurii</em></td>
<td>ATCC 51129</td>
</tr>
<tr>
<td><em>Veillonella parvula</em></td>
<td>ATCC 10790</td>
</tr>
<tr>
<td><em>Tannerella forsythia</em></td>
<td>ATCC 43037</td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td>ATCC 35405</td>
</tr>
<tr>
<td><em>Solobacterium moorei</em></td>
<td>CCUG 39336</td>
</tr>
<tr>
<td><em>Streptococcus parasanguinis</em></td>
<td>ATCC 15911</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>ATCC 25975</td>
</tr>
<tr>
<td><em>Streptococcus sobrinus</em></td>
<td>ATCC 27352</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td>ATCC 10556</td>
</tr>
<tr>
<td><em>Streptococcus oralis</em></td>
<td>ATCC 35037</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>ATCC 25175</td>
</tr>
<tr>
<td><em>Streptococcus constellatus</em></td>
<td>ATCC 27823</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 25923</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>ATCC 12633</td>
</tr>
<tr>
<td><em>Prevotella intermedia</em></td>
<td>ATCC 25611</td>
</tr>
<tr>
<td><em>Prevotella melaninogenica</em></td>
<td>ATCC 25845</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>ATCC 33277</td>
</tr>
<tr>
<td><em>Eikenella corrodens</em></td>
<td>ATCC 23834</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 51299</td>
</tr>
<tr>
<td><em>Capnocytophaga gingivalis</em></td>
<td>ATCC 33624</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>ATCC 25285</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
<td>ATCC 29523</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
<td>ATCC 29522</td>
</tr>
</tbody>
</table>
Table 2. Median, lower and upper quartiles of bacterial genome counts (×104), extrapolated from cross-reaction signals released by the DNA checkerboard hybridization test in the control and alcohol-treated (AT) groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>AT group</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Lower quartile</td>
<td>Median</td>
<td>Upper quartile</td>
<td>N</td>
<td>Lower quartile</td>
<td>Median</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td>6/12</td>
<td>0</td>
<td>0.16</td>
<td>1.44</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>3/5 †</td>
<td>0</td>
<td>4.03</td>
<td>7.53</td>
<td>0/5 †</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. pasteurii</em></td>
<td>6/12</td>
<td>0</td>
<td>1.25</td>
<td>5.32</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. parasanguinis</em></td>
<td>8/12</td>
<td>0</td>
<td>0.33</td>
<td>4.46</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. corrodens</em></td>
<td>7/12</td>
<td>0</td>
<td>1.13</td>
<td>4.32</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>6/12</td>
<td>0</td>
<td>0.48</td>
<td>4.56</td>
<td>1/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>N. mucosa</em></td>
<td>6/12</td>
<td>0</td>
<td>0.54</td>
<td>3.53</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>7/7 ††</td>
<td>1.19</td>
<td>1.53</td>
<td>2.00</td>
<td>3/7 ††</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. actinomycetemcomitans a</td>
<td>4/12</td>
<td>0</td>
<td>0</td>
<td>1.60</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>5/12</td>
<td>0</td>
<td>0</td>
<td>1.48</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. endodontalis</em></td>
<td>7/12</td>
<td>0</td>
<td>0.50</td>
<td>1.58</td>
<td>4/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. moorei</em></td>
<td>7/12</td>
<td>0</td>
<td>0.22</td>
<td>2.23</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. constellatus</em></td>
<td>8/12</td>
<td>0</td>
<td>0.83</td>
<td>2.27</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>7/12</td>
<td>0</td>
<td>0.94</td>
<td>1.69</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3/12</td>
<td>0</td>
<td>0</td>
<td>1.03</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. intermedia</em></td>
<td>4/12</td>
<td>0</td>
<td>0</td>
<td>1.17</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>F. periodonticum</em></td>
<td>3/12</td>
<td>0</td>
<td>0</td>
<td>0.87</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. gingivalis</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. sobrinus</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. forsythia</em></td>
<td>5/12</td>
<td>0</td>
<td>0</td>
<td>0.44</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td>5/12</td>
<td>0</td>
<td>0</td>
<td>0.66</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. gordonii</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
<td>3/12</td>
<td>0</td>
<td>0</td>
<td>0.59</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. micra</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. actinomycetemcomitans b</td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. melaninogenica</em></td>
<td>1/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. sanguinis</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>V. parvula</em></td>
<td>2/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>1/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>0/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>0/12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.21</strong></td>
<td><strong>27.32</strong></td>
<td><strong>39.42</strong></td>
<td><strong>0</strong></td>
<td><strong>1.05</strong></td>
<td><strong>2.92</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

Difference in the total signals detected between the control and AT groups (*p < 0.05, Mann-Whitney U test).
†Seven and ††five probes were excluded due to quantification accuracy.
Fig. 1. Mean signals released (±SEM) by bacterial genomes in the control and alcohol-treated (AT) groups.
Fig. 2. Percentages of bacterial-like species detected in the control group.
Fig. 3. Percentages of bacterial-like species detected in the alcohol-treated (AT) group.
4.3 Manuscript 5

Bacterial profile and bone healing in a pre-clinical model of bisphosphonate-related osteonecrosis of the jaw (BRONJ)

Running title: Characterization of BRONJ-like disease in rats

Zaher Jabbour\textsuperscript{a,d}, Cássio do Nascimento\textsuperscript{b}, Michel El-Hakim\textsuperscript{c}, Janet E Henderson\textsuperscript{d,e} and Rubens F. de Albuquerque Junior\textsuperscript{a,b}.

\textsuperscript{a}Division of Restorative Dentistry, Faculty of Dentistry, McGill University, Montreal, QC, Canada.

\textsuperscript{b}Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of Sao Paulo, Brazil.

\textsuperscript{c}Division of Oral and Maxillofacial Surgery, Faculty of Dentistry, McGill University, Montreal, QC, Canada.

\textsuperscript{d}Bone Engineering Labs, Research Institute, McGill University Health Center, Montreal, QC, Canada.

\textsuperscript{e}Department of Medicine and Surgery, Faculty of Medicine, McGill University, Montreal, QC, Canada.
Abstract

The microbial etiology of bisphosphonate-related osteonecrosis of the jaw (BRONJ) remains undefined. This study aimed to characterize the oral microbiota and socket healing after tooth extraction in a pre-clinical model of BRONJ. First, upper and lower molars were extracted in eight adult rats injected with zoledronic acid and dexamethasone for 3 weeks. Rats were euthanized after 9 weeks. Oral microbiota and bone healing were compared with six control rats. Probes prepared from the whole-genomic DNA of 38 bacterial and 5 candida species were hybridized to DNA extracted from the rats oral cavity. Bone healing was evaluated using micro-computed tomography. The eight experimental rats exhibited impaired bone healing characteristic of BRONJ. The top three species colonizing BRONJ-like lesions hybridized to S.pasteuri, S.parasanguini, and S.mitis probes. Significant between-group differences were noted in several Staphylococcus and Streptococcus species colonizing the teeth. A significant correlation was found between the mean proportions of species colonizing the BRONJ-like lesions and the teeth of the experimental rats. Micro-computed tomography revealed increased residual bone in the mandibular and maxillary tooth sockets of the experimental rats. BRONJ-like lesions were colonized mainly by non-pathogenic bacteria. The rats oral microbiota changed with the administration of bisphosphonates and corticosteroids. Drug administration was associated with impaired bone healing with no bone loss in the sockets of extracted teeth.

Key words: Bisphosphonates, oral bacteria biofilm, DNA checkerboard, tooth extraction, bone repair.
Introduction

The use of anti-resorptive and anti-angiogenic drugs (e.g., bisphosphonates and sunitinib, respectively) has been linked to the osteonecrosis of the jaw, which is a major clinical problem associated with pain, infection, and bone loss (1). The bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been defined as the presence of persistent, exposed, non-healing bone for more than 8 weeks in the maxillofacial region in patients treated with bisphosphonates but with no history of radiation to the head and neck region (2).

Bisphosphonates, particularly nitrogen containing bisphosphonates, are drugs used to inhibit osteoclast activities and decrease bone resorption. These drugs are prescribed to decrease skeletal complications in the management of metastatic lesion from solid tumors, such as breast and prostate cancers, and to treat metabolic bone diseases like osteoporosis (3).

Although a direct cause/effect relationship between bacterial infection and the development of BRONJ has not been demonstrated, bacterial infection has been proposed as an etiological factor for BRONJ (4). A previous study of BRONJ patients showed that the jaw bone affected by BRONJ was heavily colonized by bacteria (5). The investigator found that Streptococcus, Eubacterium, and Pseudoramibacter were the three top genera colonizing the necrotic bone. A number of pre-clinical studies have used bisphosphonates to develop BRONJ-like disease in rodents but did not provide information about the bacteria colonizing the teeth or the area of exposed bone (6-9). Previous work by Mawardi et al. noted that the infection of the extraction sockets with Fusobacterium
nucleatum in mice treated with high dose bisphosphonates resulted in delayed wound healing that left exposed bone (10). The BRONJ-like lesions were thought to have arisen from the reduced proliferation and increased death of gingival fibroblasts induced by down-regulation (10). To date, no conclusive studies exist to define the bacterial profile of the oral cavity of rodents with BRONJ-like disease. Therefore, the current study investigates the bacteria colonizing the exposed bone and teeth of rats with BRONJ-like lesion, and assesses the exposed bone response to these bacteria.

Materials and methods

Rats with BRONJ-like disease

The Animal Use Protocol was approved by the McGill University Animal Care Committee to perform the current experiment according to the Principles of Laboratory Animal Care established by the Canadian Council on Animal Care (CCAC). A total of 14 retired breeder female Sprague-Dawley rats (4–6 months old) were randomly divided into two groups: experimental and control. The control group (n=6) did not receive any medications. The experimental group (n=8) received a combination of zoledronic acid (ZA: 125 µg/kg, twice/week) and dexamethasone (DX: 5 mg/kg body weight, weekly). These doses were converted from human doses of ZA (8 mg/person/3 weeks) and DX (55mg/person/week) according to National Institute of Health (NIH) guidelines (11). These doses, although higher than those typically prescribed to cancer patients, were within the limit described in the literature (12, 13), and were used to ensure the development of BRONJ to its full extent. After 3 weeks of drug administration, all the rats had their left maxillary and mandibular first molars extracted (EXO). Drug
administration was continued for 9 additional weeks until euthanasia. Immediately after CO₂ inhalation, samples of biofilm were collected from the supragingival part of the teeth and the upper and lower site of exposed bone by rubbing a microbrush (Microbrush International, Grafton, WI, USA) for 30 seconds till saturation. The use of the microbrush was shown to be a suitable method for oral microbial biofilm collection (14). Given the possible inaccuracy in measuring the weight of the samples, we used standard-sized microbrush tips that are capable of absorbing a volume of about 6 µL. Immediately after collection, the samples were individually disposed into a microtube containing 150 µl of TE (10Mm Tris-HCl, 1Mm EDTA pH 7.6); then 150 µl of 0.5M sodium hydroxide was added to cause cell lysis and expose genetic material. Samples were stored at 4°C until the DNA checkerboard hybridization was performed.

**DNA checkerboard hybridization**

Bacterial and fungal DNA hybridization were performed using the checkerboard method as described previously (15, 16). Hybridization probes were prepared from the whole-genomic DNA of 38 bacterial and 5 candida species (Table 1). Probe selection was based on the relevance of the species to human oral health and their pathogenicity. The suitable specificity and sensitivity of these probes already had been tested and optimized for the detection of $10^4$ cells (17, 18). The bacterial and fungal whole-genomic DNA extracted from the biofilm samples of the rats were denatured, precipitated, and blotted onto the hybridization membrane (Hybond N+, Amersham Biosciences, Buckinghamshire, UK) using a MiniSlot 30 apparatus (Immunetics, MA, USA). As reference samples, defined amounts of genomic DNA corresponding to the $10^5$ or $10^6$ cells of each of the analyzed
target species were mixed in a single tube, denatured, precipitated, and applied to the
membranes. Samples were fixed on the hybridization membrane using a vacuum and
baked for 2 hours at 80 degrees C. Membranes were then pre-hybridized using an oven
shaker (Amersham Biosciences) to control for temperature and humidity. Labelled probes
were then introduced using a Miniblotter 45 device (Immunetics, Immunetics,
Cambridge, MA, USA), and the membranes hybridized overnight at controlled
temperature and humidity under gentle agitation. After washing, hybridization signals
were detected by chemiluminescence using a CDP-Star reagent (GE Healthcare), and the
membranes were exposed to ECL Hyperfilm-MP for 60 minutes (GE Healthcare). The
hyperfilm images were digitized and quantified using TotalLab Quant analysis software
(TotalLab Life Science Analysis Essentials, Newcastle upon Tyne, UK).

**Micro computed tomographic (micro CT) analysis of rat jaw bones**

Following euthanasia and the collection of the biofilm samples, the EXO and non-EXO
sides of both the upper and lower jaws were removed, cleaned of muscle attachments and
soft tissues, fixed overnight at 4°C in 4% paraformaldehyde, rinsed thoroughly in three
changes of sterile phosphate buffered saline (PBS), and stored at 4°C. Micro-CT scans
were captured on a Skyscan 1172 instrument (Kontich, Belgium) at a 8.9 micron
resolution with 550 milliseconds of exposure time, 59 kV and 167, mA adjusted to allow
maximum differentiation between mineralized and non-mineralized tissues, and using a
0.5 mm thickness aluminum filter (9). An average of seven measurements (mm)
separated by 0.5 mm and spanning the extraction sites in each jaw were taken to calculate
the height of the residual exposed bone.
The region of interest (ROI) was outlined as described previously (9). Briefly, in the non-EXO side, the ROI extended from the mesial aspect of the second molar to the mesial aspect of the first molar, which included both the cortical and trabecular bone and excluded the first molar itself. In the mandible, the ROI extended towards the lower border of the mandible to cover the bone till the beginning of the incisor’s enamel, which provided a clear anatomical reference. On the EXO side, the ROI was extended 3.0 mm from the mesial surface of the second molar to cover the EXO area, which included the separated bone fragments (sequestra) and excluded the alveolar ridge (9). The bone volume to tissues volume (BV/TV) fractions of the non-EXO sides, and the number, volume, and surface area of the isolated bone fragments of the extraction sites were quantified using CTAn Skyscan software.

Following the micro-CT scanning, undecalcified samples were embedded in polymethylmethacrylate (MMA), sectioned serially (5-micron), and dried for 4 days before staining for bone mineral (Von Kossa).

**Statistical analyses**

To adjust for any potential variation in the volume or weight of the samples, mean proportions were used by averaging the percentages of the target species detected within each sample. A Mann–Whitney U test was employed for between-group comparisons of bacterial proportions, bone height, and micro-CT outcomes. A non-parametric spearman correlation was used to assess the within-group relationship of the proportions of species colonizing the BRONJ-like lesion and teeth. All statistical analyses were performed using SPSS 19 with a significance level of <0.05.
Results

Microbiological results

A total of 39 different microbial species were detected in the exposed bone, 26 on the teeth of the experimental rats, and 40 on the teeth of the control group. The S.pasteuri, S.parasanguinis, S.mitis, S.gordonii, and S.oralis species had the highest mean proportions. Only S.pasteuri, S.parasanguinis, and S.mitis were detected in all BRONJ-like lesions in the experimental group. No hybridization signals were quantified for the B. fragilis and C. rectus probes. Targeted species and their frequencies ordered by the decreased mean proportions per collection site are shown in Table 2.

The mean proportions of S. pasteuri, S. parasanguinis, S. oralis, S. mutans, S. moorei, S. mitis, and S. gordonii were significantly higher on the teeth of the ZA+DX group compared to the control group. On the other hand, the mean proportions of P. melaninogenica, P. intermedia, P. gingivalis, and P. endodontalis were higher on the teeth of the control group compared to the ZA+DX group (Figure 1).

A significant non-parametric correlation was found between the mean proportions of the microbial species colonizing the BRONJ-like lesions and teeth of the ZA+DX rats (p<0.0001, r=0.818) (Figure 2).

Bone results in rats

In comparison with the normal healing of the control rats, all the rats treated with ZA+DX showed clinical signs of mucosal inflammation and fenestration, which are characteristic of BRONJ-like disease. BRONJ-like disease was noted in all rats, with
bone exposure in 7 mandibular and 5 maxillary EXO sites at the time of euthanasia (Figure 3).

The residual alveolar bone of the mandibular sockets was significantly higher in the ZA+DX group compared to the control group, with a similar trend in the maxilla (Figure 4).

Quantitative micro-CT data (Figure 5a) revealed significant differences in volume, surface area, and the number of non-resorbed bone fragments in the mandibular, but not in the maxillary, EXO sites of the experimental group compared to the control group. The histological sections of the mandible and maxilla stained with Von Kossa/Toluidine blue also demonstrated non-resorbed mineralized tissue embedded in the mucosa of the EXO sites in the ZA+DX group only (Figure 5b).

Quantitative micro-CT images of the mandibular non-EXO side showed a statistically higher BV/TV ratio for the ZA+DX group compared to the control rats. This result was associated with a reduction in the bone marrow in the body of the mandible and between the roots of the first molar (Figure 6).

**Discussion**

The combined administration of the ZA+DX associated with a trauma to the jaw bone, caused by tooth extractions, resulted in BRONJ-like disease in all the experimental rats. We employed a DNA checkerboard technique using 43 genomic DNA probes prepared from human oral bacteria and fungi relevant to human health to detect the changes in the bacteria species colonizing the teeth of the ZA+DX rats. We also noted a similarity in the
bacterial profile of exposed bone and teeth, and no loss of alveolar bone height in the EXO sites of the experimental rats.

Although it is recognized that the use of rodent models to study the oral microbiota of humans is debatable and might not necessarily provide an accurate representation (19), the oral cavity of humans and rodents shares some common microbial species (15). In the current investigation, three non-pathogenic species *S. mitis, S. parasanguinis*, and *S. pasteurii* had the highest mean proportions and were detected in all sites of exposed bones of the ZA+DX rats. These species have considerable presence in the exposed bone of patients with BRONJ (5, 20). In addition, the rare presence or absence of pathogenic bacteria such as the *B. fragilis, C. rectus, C. gingivalis*, and *Actinomyces* species is consistent with human observation and suggests a limited role for these bacterial species in the development of BRONJ (20). Although it was proposed that *F. nucleatum* could play a role in the development of BRONJ (10), chemiluminescent signals from the *F. nucleatum* targeted species were detected only in one rat with a BRONJ-like condition (Table 2). In our experiment, the sensitivity of the DNA method was adjusted to allow the detection of $10^4$ bacterial cells. Therefore, any chemiluminescent signals below this threshold could not be detected, although bacterial genomes could be present in low quantities. In addition, the visibly small amount of bacterial biofilm collected from the oral cavity of rats and a potential DNA degradation could result in an absence of chemiluminescent signals (15).

In the present study, the combined administration of ZA+DX resulted in a change in the proportion of some *Streptococcus* and *Staphylococcus* species compared to the control rats. This result suggests that a systemic administration of bisphosphonates and
corticosteroids can change oral microbiota. This change in the bacterial profile of the
ZA+DX rats contributed to the significant within-group correlation between the mean
proportions of bacterial species colonizing the exposed bone and teeth. The ability of
bacteria to adhere to bone or teeth depends on their surface characteristics (21). Although
bone and teeth are composed of calcium-phosphates, enamel has little collagen, whereas
bone is rich in collagen. In a previous investigation of patients treated for BRONJ, the
bacterial profile of exposed bone was different from the bacterial profile of teeth (20).
However, the majority of these patients were receiving other treatments for cancer, such
as chemotherapy drugs.

It has been suggested that bisphosphonates could enhance the adhesion of oral bacteria
such as S. aureus to hydroxyapatite (22, 23). In the current investigation, S. aureus had a
considerable presence in the exposed bone of the ZA+DX rats. A particular feature of the
bone infections of this species is the destruction of the mineralized tissue that results from
the ability of the S. aureus protein A to activate the nuclear factor kappa B, which in turn
activates osteoclastic bone resorption in the area of the infection (24). However, the
alveolar bone in the mandible and to a lesser extent in the maxilla of the ZA+DX rats was
significantly higher than that of the control animals. This novel finding is consistent with
the lack of bone loss observed clinically in our experimental animals in the area of
exposed bone, and suggests a direct inhibitory effect of ZA on jaw bone remodeling, even
under the presence of bone-destroying bacteria. Moreover, a delay in the resorption of
bone sequestra and alveolar ridge healing is probably a consequence of the ZA-induced
inhibition of osteoclast activity at the site of exposed bone (Figure 5). The reduced
activity of osteoclasts is supported by quantitative micro-CT scans and Von Kossa
stained sections of bone, which show an increased amount of non-resorbed mineralized tissue embedded in the mucosa, and a higher BV/TV value in the non-EXO sites of rats with BRONJ-like disease. In turn, the reduced ability of the bone to remodel results in a higher incidence of sequestra cutting through the mucosa and delayed bone healing. This result also was noted in our previous investigation (9). The higher volume, surface area, or number of sequestra observed in the experimental group could have been related to an increase in microbial colonization, which suggests a higher risk of bone infection by bacteria and fungi, and a potential role for bacteria in aggravating BRONJ. However the relatively small number of animals used in the current investigation and the lack of detection of some chemiluminescence signals prevented an exploratory analysis between the sequestra parameters and colonizing microorganisms. A larger number of animals could be used to further explore this relationship. Moreover, an analysis of biofilm samples in the same rats before and after drug administration could help to better assess the change in the bacterial profile associated with BRONJ-like disease.

Within the limitations of this study, we concluded that the exposed bone in rats with BRONJ-like disease is mainly colonized by non-pathogenic bacterial species. The oral microbiota of rats changes with BRONJ-like disease. A systemic administration of bisphosphonates results in a reduced loss of the alveolar bone height typically observed in the presence of bone-destroying bacteria.

**Acknowledgements**

Zaher Jabbour received awards from the Fonds de recherche en santé du Québec (FRSQ), the FRSQ-Réseau de recherche en santé buccodentaire et osseuse (RSBO), and the
Fondation de l'Ordre des dentistes du Québec (FODQ). The study was made possible through operating funds from FODQ and Synthes Canada, and infrastructure support from the FRQ-S-RSBO and the Jo Miller Orthopaedic Research Fund. Technical assistance provided by Ailian Li for histological staining was greatly appreciated.

**Conflict of Interest**

None.
References


Table 1: Human DNA microbial species used to prepare probes for cross-reaction with species extracted from the oral cavity of rats.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em> a</td>
<td>ATCC 29523</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em> b</td>
<td>ATCC 29522</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>ATCC 25285</td>
</tr>
<tr>
<td>Campylobacter rectus</td>
<td>ATCC 33238</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>ATCC MYA 646</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>ATCC 90030</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>ATCC 6258</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>ATCC 750</td>
</tr>
<tr>
<td>Capnocytophaga gingivalis</td>
<td>ATCC 33624</td>
</tr>
<tr>
<td>Eikenella corrodens</td>
<td>ATCC 23834</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC 51299</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC 10798</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>ATCC 25586</td>
</tr>
<tr>
<td><em>Fusobacterium periodonticum</em></td>
<td>ATCC 33693</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC 700721</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>ATCC 393</td>
</tr>
<tr>
<td>Mycoplasma salivarium</td>
<td>ATCC 23064</td>
</tr>
<tr>
<td>Neisseria mucosa</td>
<td>ATCC 25996</td>
</tr>
<tr>
<td>Parvimonas micra</td>
<td>ATCC 33270</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>ATCC 49031</td>
</tr>
<tr>
<td>Porphyromonas endodontalis</td>
<td>ATCC 35406</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>ATCC 25611</td>
</tr>
<tr>
<td>Prevotella melaninogenica</td>
<td>ATCC 25845</td>
</tr>
<tr>
<td>Prevotella nigrescens</td>
<td>ATCC 33563</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 27853</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>ATCC 12633</td>
</tr>
<tr>
<td>Solobacterium moorei</td>
<td>CCUG 39336</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>Staphylococcus pasteuri</td>
<td>ATCC 51129</td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>ATCC 27823</td>
</tr>
<tr>
<td>Streptococcus gordonii</td>
<td>ATCC 10558</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>ATCC 49456</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>ATCC 25175</td>
</tr>
<tr>
<td>Streptococcus oralis</td>
<td>ATCC 35037</td>
</tr>
<tr>
<td>Streptococcus parasanguinis</td>
<td>ATCC 15911</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>ATCC 25975</td>
</tr>
<tr>
<td>Streptococcus sanguinis</td>
<td>ATCC 10556</td>
</tr>
<tr>
<td>Streptococcus sobrinus</td>
<td>ATCC 27352</td>
</tr>
<tr>
<td>Tannerella forsythia</td>
<td>ATCC 43037</td>
</tr>
<tr>
<td>Treponema denticola</td>
<td>ATCC 35405</td>
</tr>
<tr>
<td>Veillonella parvula</td>
<td>ATCC 10790</td>
</tr>
</tbody>
</table>
Table 2: Target species per collection site ordered by decreasing mean proportion and their detected frequencies.

<table>
<thead>
<tr>
<th></th>
<th>ZA+DX</th>
<th>Teeth</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposed bone</td>
<td>Teeth</td>
<td>Teeth</td>
</tr>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 6</td>
</tr>
<tr>
<td>S.pasteuri</td>
<td>8</td>
<td>S.oralis</td>
<td>P.gingivalis</td>
</tr>
<tr>
<td>S.parasanguinis</td>
<td>8</td>
<td>S.mitis</td>
<td>M.salivarium</td>
</tr>
<tr>
<td>S.mitis</td>
<td>8</td>
<td>S.gordonii</td>
<td>S.mutans</td>
</tr>
<tr>
<td>S.gordonii</td>
<td>7</td>
<td>S.pasteuri</td>
<td>S.mitis</td>
</tr>
<tr>
<td>S.oralis</td>
<td>7</td>
<td>S.mutans</td>
<td>S.gordonii</td>
</tr>
<tr>
<td>S.salivarius</td>
<td>7</td>
<td>S.parasanguinis</td>
<td>8</td>
</tr>
<tr>
<td>S.mutans</td>
<td>7</td>
<td>S.moorei</td>
<td>E.faecalis</td>
</tr>
<tr>
<td>S.sanguinuis</td>
<td>7</td>
<td>S.sobrinus</td>
<td>T.denticola</td>
</tr>
<tr>
<td>P.putida</td>
<td>6</td>
<td>S.sanguinuis</td>
<td>S.constelatus</td>
</tr>
<tr>
<td>C.glabrata</td>
<td>7</td>
<td>M.salivarium</td>
<td></td>
</tr>
<tr>
<td>S.moorei</td>
<td>7</td>
<td>T.denticola</td>
<td></td>
</tr>
<tr>
<td>S.sobrinus</td>
<td>6</td>
<td>T.forsythia</td>
<td></td>
</tr>
<tr>
<td>C.tropicalis</td>
<td>7</td>
<td>V.parvula</td>
<td></td>
</tr>
<tr>
<td>P.micra</td>
<td>5</td>
<td>P.putida</td>
<td></td>
</tr>
<tr>
<td>S.aureus</td>
<td>6</td>
<td>C.albicans</td>
<td></td>
</tr>
<tr>
<td>C.oralis</td>
<td>6</td>
<td>C.tropicalis</td>
<td></td>
</tr>
<tr>
<td>P.melaninogenica</td>
<td>2</td>
<td>P.intermedia</td>
<td></td>
</tr>
<tr>
<td>F.periodonticum</td>
<td>5</td>
<td>S.sanguinuis</td>
<td></td>
</tr>
<tr>
<td>V.parvula</td>
<td>6</td>
<td>E.corrodens</td>
<td></td>
</tr>
<tr>
<td>P.nigrescens</td>
<td>5</td>
<td>E.faecalis</td>
<td></td>
</tr>
<tr>
<td>C.dubliniensis</td>
<td>7</td>
<td>S.sobrinus</td>
<td></td>
</tr>
<tr>
<td>E.corrodens</td>
<td>6</td>
<td>P.micra</td>
<td></td>
</tr>
<tr>
<td>C.krusei</td>
<td>7</td>
<td>C.dubliniensis</td>
<td>5</td>
</tr>
<tr>
<td>E.faecalis</td>
<td>4</td>
<td>L.casei</td>
<td></td>
</tr>
<tr>
<td>S.constelatus</td>
<td>5</td>
<td>S.constelatus</td>
<td></td>
</tr>
<tr>
<td>K.pneumoniae</td>
<td>6</td>
<td>E.corrodens</td>
<td></td>
</tr>
<tr>
<td>T.denticola</td>
<td>3</td>
<td>E.ceiae</td>
<td></td>
</tr>
<tr>
<td>M.salivarium</td>
<td>3</td>
<td>P.nigrescens</td>
<td></td>
</tr>
<tr>
<td>C.albicans</td>
<td>6</td>
<td>C.albicans</td>
<td></td>
</tr>
<tr>
<td>C.krusei</td>
<td>7</td>
<td>P.intermedia</td>
<td></td>
</tr>
<tr>
<td>F.periodonticum</td>
<td>0</td>
<td>E.ceiae</td>
<td></td>
</tr>
<tr>
<td>P.intermedia</td>
<td>4</td>
<td>P.gingivalis</td>
<td></td>
</tr>
<tr>
<td>T.forsythia</td>
<td>4</td>
<td>L.casei</td>
<td></td>
</tr>
<tr>
<td>P.gingivalis</td>
<td>3</td>
<td>P.intermedia</td>
<td></td>
</tr>
<tr>
<td>L.casei</td>
<td>3</td>
<td>P.gingivalis</td>
<td></td>
</tr>
<tr>
<td>C.gingivalis</td>
<td>3</td>
<td>P.gingivalis</td>
<td></td>
</tr>
<tr>
<td>N.mucosa</td>
<td>4</td>
<td>C.gingivalis</td>
<td></td>
</tr>
<tr>
<td>E.coli</td>
<td>4</td>
<td>N.mucosa</td>
<td></td>
</tr>
<tr>
<td>P.aeruginosa</td>
<td>4</td>
<td>E.coli</td>
<td></td>
</tr>
<tr>
<td>Aa_a</td>
<td>2</td>
<td>P.aeruginosa</td>
<td></td>
</tr>
<tr>
<td>P.melaninogenica</td>
<td>2</td>
<td>Aa_a</td>
<td></td>
</tr>
<tr>
<td>P.endodontalis</td>
<td>2</td>
<td>P.melaninogenica</td>
<td>0</td>
</tr>
<tr>
<td>F.nucleatum</td>
<td>1</td>
<td>P.endodontalis</td>
<td>0</td>
</tr>
<tr>
<td>P.anaerobios</td>
<td>0</td>
<td>F.nucleatum</td>
<td>P.anaerobios</td>
</tr>
<tr>
<td>Aa_b</td>
<td>0</td>
<td>P.anaerobios</td>
<td></td>
</tr>
<tr>
<td>B.fragilis</td>
<td>0</td>
<td>Aa_b</td>
<td></td>
</tr>
<tr>
<td>C.rectus</td>
<td>0</td>
<td>B.fragilis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C.rectus</td>
<td></td>
</tr>
</tbody>
</table>

124
Figure 1: Mean proportions (% ± SEM) of target species detected in rats.

†Statistically significant difference (p<0.05) between the teeth of the ZA+DX rats and control rats.
Figure 2: Scatter plots of the mean bacterial proportions (%) showing significant within-group non-parametric correlations between the bacteria colonizing the exposed bone and teeth ($r=0.818, p<0.0001$).
Figure 3: Images of the EXO sites in the maxilla and mandible of the experimental and control groups showing optimal healing (white arrows). Signs of inflammation and bone exposure were noted in the EXO sites of the ZA+DX group (black arrows).
Figure 4: (a) Seven measurements were taken and averaged to cover the site of the extracted first molar. (b) Box plots showing significantly larger alveolar bone height in the mandible with a similar trend in the maxilla of the ZA+DX group compared to the control group.
Figure 5: (a) Box plots show the results of micro-CT quantification of the non-resorbed bone fragments in the mandibular and maxillary extraction sites, which indicates statistically significant increases in the volume, surface area, and number of the separated fragments in the mandibular sites of the ZA+DX group. *p<0.05. Arrows show the atypical remodeling of the alveolar ridge associated with non-resorbed bone fragments in the extraction sites of the ZA+DX group compared to the round alveolar ridge indicating a normal healing process in the control group. (b) Histological sections (VonKossa x10) of mandible and maxilla showing a healing of the EXO site of the control group. Arrows point to the non-resorbed mineralized tissues embedded in the mucosa of the EXO sites in the mandible and maxilla of the ZA+DX group (*Root of the second molar).
Figure 6: (a) Plot box showing the percentage of bone volume to total tissue volume (%BV/TV) in the mandible and maxilla in the area of the non-extracted first molar. *p<0.01. (b) Cross-sectional micro-CT images of the mandible in the area of the non-extracted first molar that show a reduced bone marrow space in the body of the mandible and between the roots of the first molar consistent with the increased %BV/TV of the ZA+DX group.
Chapter five: General discussion
5.1 BRONJ patients at the Montreal General Hospital

In the present case series, 11 patients received intravenous bisphosphonates for cancer, and 3 patients received oral bisphosphonates for osteoporosis and osteopenia. The majority of these patients reported developing BRONJ following tooth extraction or wearing dentures. Our cohort of patients represented characteristics similar to those previously described in the literature (Wang, Goodger et al. 2003; Salesi, Pistilli et al. 2006; Migliorati, Woo et al. 2010; Manfredi, Merigo et al. 2011). Usually, the patients are exposed to combinations of drugs, such as the corticosteroid prednisone and different chemotherapeutic agents that significantly influence bone and its vascularization (Ruggiero, Dodson et al. 2014). In our case series, prednisone was the second most common drug prescribed after bisphosphonates, and it was taken by 4 patients. In addition, steroid use has been reported to produce avascular necrosis in the femoral head in humans and rats (Urbaniak and Harvey 1998; Amin Kerachian, Cournoyer et al. 2010). We used this observation in our rat model to generate BRONJ-like disease at the site of tooth extraction by administering intraperitoneal injections of the bisphosphonate zoledronic acid and the corticosteroid dexamethasone.

5.2 Management of BRONJ

5.2.1 Conservative and surgical management of BRONJ

In our cohort of patients, at the last follow-up, conservative treatment alone had resulted in BRONJ stage 0 in 7 out of 11 sites. Surgical treatment was delivered only to unresponsive cases, which resulted in BRONJ stage 0 in 5 out of 8 sites. It appears that surgical intervention yielded positive outcomes in cases that did not respond to
conventional therapy (Stanton and Balasanian 2009; Williamson 2010; Curi, Cossolin et al. 2011). In addition, it appears that surgical treatment provides a faster resolution of the symptoms associated with BRONJ stage 2 (Chapter 3.1, Figure 2). In the surgical cases, the faster response could be due to the removal of the necrotic bone sequestrum, which is colonized by bacteria and cannot be permeated by systemic antibiotics. Surgical treatment also was helpful for reducing the overall burden of BRONJ, and therefore, it was considered as providing an improvement in the quality of life of severely ill patients who were non-responsive to conservative treatment.

5.2.2 Bisphosphonate withdrawal

After discussion with the treating specialist, temporary bisphosphonate discontinuation was considered in 9 out of the 14 cases of BRONJ patients treated at the Montreal General Hospital (Chapter 3.1, Table 1). To reduce the risk of developing BRONJ, reduced bisphosphonate exposure or discontinuation before dental interventions has been proposed by the American Association of Oral and Maxillofacial Surgeons (2009) and the Canadian consensus for practice guidelines on BRONJ (Khan, Sandor et al. 2008; Ruggiero, Dodson et al. 2009). It has been reported that interrupting or reducing bisphosphonate exposure might have beneficial effects on the development of BRONJ (Corso, Varettoni et al. 2007). However, bisphosphonates stay in the bones for an extended period of time after drug interruption (Russell, Xia et al. 2007), so it is unlikely that bisphosphonate discontinuation for a short period of time will produce a significant reduction in the risk of developing BRONJ (Allen and Burr 2011). Currently, little data has shown the beneficial effects of reducing or interrupting bisphosphonate therapy (Corso, Varettoni et al. 2007; Campisi, Fedele et al. 2009). Since it is not clear yet how
long bisphosphonate interruption or reduction is required to achieve a significant decrease in the risk of developing BRONJ, a group of rats in our animal experiment had bisphosphonate withdrawal after tooth extraction to determine any associated beneficial effect. We noted that the resorption of sequestra appeared 2 weeks post-EXO on serial x-rays in rats with zoledronic acid withdrawal. This result suggested a resumption of bone remodeling following bisphosphonate withdrawal. Furthermore, at the end of the experiment, we did not find a statistically significant difference in the surface of the mandibular sequestra between the withdrawal and control groups (Chapter 4.1, Figure 7). This observation also could indicate a beneficial effect of bisphosphonate withdrawal with respect to the resumption of bone remodeling. However, the volume of sequestra remained significantly higher in the withdrawal group when compared to the control group (Chapter 4.1, Figure 7), which indicates that longer periods for ZA withdrawal should be investigated further. Therefore, bisphosphonate withdrawal may promote bone-remodeling reactivation following dental extraction.

5.3 Rat model to study jaw bone changes

Retired-breeder rats were used in the current study, since the risk of developing BRONJ increases with patients’ age (Khosla, Burr et al. 2007). Rats have been used extensively as a pre-clinical model to examine the impact of various interventions on bone metabolism. For the present experiment, the small animal size, easy manipulation, fast metabolism, and short lifespan make these rats an ideal model for obtaining reliable outcomes. All experimental procedures were performed according to the Principles of Laboratory Animal Care established by the Canadian Council on Animal Care (CCAC) and after approval by the Animal Ethic Committee at McGill University. The Animal Use
Protocol of the present investigation falls into the C category, which includes minor surgeries (teeth extractions) under anesthesia.

Alveolar bone is part of the maxillofacial complex that forms the primary support structure for the teeth. The process of alveolar wound healing in rats already has been studied and shown to be a suitable model for the study of the bone healing process in humans (Pietrokovski and Massler 1967; Guglielmotti and Cabrini 1985). After tooth extraction, the alveolar bone repair starts immediately and is influenced by various systemic factors, such as drug administration and nutrition (Gorustovich, Steimetz et al. 2008). In addition, several studies have used rats to investigate the bony changes following bisphosphonate administration and showed development of bone exposure in the site of tooth extraction similar to BRONJ, which lead to BRONJ-like disease in these rats (Sonis, Watkins et al. 2009; Perilli, Le et al. 2010).

In this rat model of BRONJ-like disease, we introduced a common traumatic injury in the form of single tooth extraction to test the association between different protocols for bisphosphonate administration and trauma on the onset and development of BRONJ. We used the contra-lateral side of the mouth as a within-subject control to monitor the development of BRONJ without the presence of trauma. In our investigation, we did not find any clinical or radiographic signs of exposed necrotic bone in the contra-lateral sides, which suggests that BRONJ is mostly triggered by traumatic injury to the jaw bones.
5.4 Assessment of bacteria associated with BRONJ

5.4.1 Study design in BRONJ patients

Only a few studies in the literature have explored the relationship between the presence of certain bacterial species and the development of BRONJ (Hansen, Kunkel et al. 2006; Sedghizadeh, Kumar et al. 2008; Ji, Pushalkar et al. 2012; Wei, Pushalkar et al. 2012). Therefore, our investigations have attempted to describe the bacterial population colonizing different sites of the oral cavity, including the area of exposed bone, teeth, and tongue of BRONJ patients.

Our results suggest that the area of exposed bone of BRONJ patients is predominantly contaminated by non-pathogenic opportunistic microbial species, such as S. gordonii and S. constellatus. Also prevalent were species linked to periodontal diseases and bone infections such as E. corrodens and S. aureus. However, pathogenic species frequently associated with periodontal diseases such as C. rectus, C. gingivalis, B. fragilis, and A. actinomycetemcomitans were rarely noted. The presence of non-pathogenic microbial species colonizing the exposed bone could be explained by some inhibitory effect of the antibiotics that the patients were taking (Chapter 3.2, Table 2). In our investigation, the bacterial profile of the exposed bone was unique and did not correlate with the bacterial profile of the teeth or soft tissues of the oral cavity of BRONJ patients.

A particular strength of the current experiment is the collection of microbial biofilm from multiple sites of the mouth within the same patient (i.e., the exposed bone, adjacent teeth, contra-lateral teeth, and tongue). This design provided insight into the microbial profile
of each site and enabled within-subject comparisons. However, the cross-sectional design of the current study does not provide information about the sequence of events that may have led to the development of BRONJ. It is still not clear whether BRONJ is the result of microbial infection or that the presence of exposed bone in the oral cavity promotes bacterial colonization. Longitudinal studies with a larger number of patients are necessary to identify the etiology of BRONJ and to better understand the change in the oral microbiota in cancer patients. In addition, it is important to determine if other factors such medications, gender, or ethnicity could play a role in the development of BRONJ.

5.4.2 Study design in rats

A prospective experimental design to study the bacteria colonizing exposed jaw bone in humans with BRONJ is extremely difficult. In addition, the small number of BRONJ patients available associated with the wide range and complex interactions among oral bacterial species is an additional challenge with respect to identifying specific pathogenic species that are present in BRONJ sites. Therefore, an animal model to study the oral bacteria associated with BRONJ is desirable. Although the oral microbiotas of humans and animals are not identical, the bacteria found in the oral cavity of animals susceptible to periodontal diseases similar to the periodontal diseases in humans are widely described in the literature, for example, mice, rats, cats, dogs, sheep, horses, and non-human primates (Colyer 1947). Given their close similarities to humans, primates may represent the best animal model to study oral microbiota (Holt, Ebersole et al. 1988; McArthur, Magnusson et al. 1989). However, the large expenses and ethical considerations to develop bone necrosis limit their use. Rats are the commonly used animals to study bacteria in experimental periodontitis models (Graves, Kang et al. 2012). Although the
oral cavity of humans and rats share several bacterial species and immunological aspects, it is recognized that the use of rats to study the oral bacteria of humans might not provide an accurate representation. By studying the oral bacteria in BRONJ patients and rats with BRONJ-like disease, we have attempted to find common species colonizing the area of bone necrosis. In addition, we aimed to identify any changes in the oral microbiota following bisphosphonate administration. The result of our experiment suggested that, similar to humans, BRONJ-like lesions in rats were colonized mainly by non-pathogenic microorganisms. In addition, we concluded that the administration of a high dose of bisphosphonates and corticosteroids changes the oral microbiota in rats.

5.4.3 The use of the DNA checkerboard hybridization method

The DNA–DNA checkerboard hybridization method is capable of identifying and quantifying a large number of species quicker and more accurately than staining or culture methods (Socransky, Smith et al. 1994; Socransky, Haffajee et al. 2004). Since the bacterial probes used in the current experiment were prepared and extracted from the human oral bacteria, we tested first to determine whether these probes could hybridize with the microbial species taken from the oral cavity of rats. While the amount of material collected from the oral cavity of rats was small, we detected quantifiable hybridization signals from 31 out of 33 tested probes in the control group. Although the *S. mutans* and *S. mitis* species were not detected in this experiment, it is possible that they were present in both groups, but in low quantities not detectable by the DNA checkerboard method. These two species are frequently associated with tooth cavities in humans, and our animals were free of caries and periodontal diseases.
5.5 Limitations of the studies and future research

5.5.1 Limitations of the studies

Given the relatively low incidence of BRONJ, the clinical investigations had a cross-sectional design with small sample size. The limited number of patients did not allow to achieve conclusive evidence about the treatment outcomes of BRONJ or conduct elaborated analyses to account for bacterial clusters. Longitudinal studies with a larger number of patients are desired to better assess the treatment outcomes of BRONJ and to clarify any changes in the oral microbiota following bisphosphonates administration.

In addition, the sensitivity of the DNA checkerboard method was adjusted in the current series of microbiological studies to allow the detection of $10^4$ bacterial cells. Therefore, bacterial genomes present in quantities below this threshold could not be detected. Furthermore, stains on the membranes, or the potential nonspecific binding of the chemoluminescent agent or the antibody conjugate to non-DNA debris may increase cross-reaction signals, which leads to difficulties in bacterial cell quantitation.

5.5.2 Future clinical research

The current case series aimed to evaluate the outcomes of the conservative and surgical treatment of BRONJ stage 2. Future clinical studies should focus on the role of controlled exposure to bisphosphonates and different co-medications in the development of BRONJ. Early diagnoses and treatment of BRONJ stage 1 also should be investigated to better understand the progression of the disease. Longitudinal studies should be conducted to identify the outcomes of early diagnosis and treatment of BRONJ and its relation to other
cancer co-medications. Future research should also focus on factors that could help to predict, prevent, and manage the manifestations of BRONJ; and establish therapeutic protocols to improve the quality of life of affected patients.

5.5.3 Future translational research

Data generated from this project will help in the design of further animal research to test the effect of chemotherapy drugs and other antiresorptive bone agents such as denosumab on the development of jaw bone necrosis. The bony changes of the maxilla, mandible, and appendicular skeleton also should be compared after exposure to the same regimen of osteoclast-mediated bone inhibitors and chemotherapy to better understand why jaw bone necrosis occurs only in the oral cavity.

In addition, future animal research should use bigger sample sizes to evaluate the early changes in bone tissues after bisphosphonate exposure. The optimal bisphosphonate dosages and discontinuation period also should be further investigated to maximize the effect of bisphosphonates and minimize the risk of BRONJ. Insightful preliminary information about changes in jaw bone could be obtained by using newly available technologies such as in vivo micro-CT. This instrument could be used to assess and monitor changes in jaw bone following bisphosphonate administration. In addition, it could help in investigating the course of the disease leading to the development of BRONJ in experimental animals. Serial histological analysis of sections cut at 5 micron for staining of endothelial vascular analysis CD34, fibrous tissue (Toluidine blue), osteoblasts (ALP), and osteoclasts (TRAP) activity and apoptosis also could add useful information.
Furthermore, future studies should investigate the effect of bisphosphonates on jaw bone vasculature. Ischemia or reduced jaw bone vascularization following bisphosphonate administration has been suggested to explain the etiology of BRONJ (Scavelli, Di Pietro et al. 2007). Avascular bone necrosis is a recognized etiological factor in other parts of the skeleton such as the hip (Talamo, Angtuaco et al. 2005). Current studies described in the literature used histological sections to quantify the number of vessels and their level of dilation (Sonis, Watkins et al. 2009; Lopez-Jornet, Camacho-Alonso et al. 2010) or used the alizarin complexone and BSA–fluorescein isothiocyanate conjugate (FITC-BSA) to histochemically mark the newly formed vessels (Bi, Gao et al. 2010). Although these techniques are widely used and considered accurate, they are limited to the tissue area in the histological section (Barou, Mekraldi et al. 2002). A novel three-dimensional analysis of the microarchitecture of the vascular network by injecting a radio-opaque contrast material and micro-CT has been recently described for long bones (Fei, Peyrin et al. 2010; Sider, Song et al. 2010). Other recent studies have used molecular techniques to determine the vascular etiology of BRONJ by measuring the level of angiogenesis-related genes such as CD31 and VEGF (Yamashita, Koi et al. 2011).

Finally, future translational research also should evaluate the possible delivery or local application of certain mediators in the development or prevention of BRONJ. One example is the systemic or local delivery of nitric oxide. Another example is the use of a scaffold to carry signaling molecules such as RANKL or VEGF.
5.5.4 Future microbial research

The current clinical and pre-clinical investigation showed that each region of the mouth, particularly the site of exposed bone, has its own microbial characteristics. Therefore, future research should study the role of each species on microbial infections and the ability of microorganisms to adhere to hard tissues such as bone. In addition, the role of the prophylactic use of antibiotics should be assessed regarding the prevention of BRONJ development following traumatic injury to the jaw bone.

In addition, the pathway of how bisphosphonates, corticosteroids, and chemotherapy drugs affect the oral biofilm should be further explored. This focus also will help to clarify whether BRONJ is a primary bone lesion caused by systemic factors or has resulted from a bacterial infection leading to non-healing bone exposure, or whether both events happen simultaneously. In a primary bone lesion, bone necrosis occurs after bisphosphonate administration followed by gingival retraction and bone exposure to the oral cavity (Otto, Hafner et al. 2010). In our investigation, the sites of exposed bone were mainly colonized by non-pathogenic species. Furthermore, pathogenic species such as C. rectus, C. gingivalis, B. fragilis, and A. actinomycetemcomitans were rarely noted. New molecular sequencing techniques such as 16S rRNA sequencing could be used to identify microbial species that were not tested or detected in the current investigations.

5.6 Scientific contribution to clinical practice

The current research project was designed to better understand the physiopathology of BRONJ and its relation to the bisphosphonate regimen and presence of trauma. BRONJ
management, including bisphosphonate withdrawal and surgical and conservative treatments, was investigated. The results of the chart review suggest that both conservative and surgical treatments could result in the resolution of BRONJ stage 2. Data generated by our serial experiments support the premises of the potential beneficial effect of bisphosphonate discontinuation, and that bisphosphonate withdrawal could help to resume bone remodeling after tooth extraction. In addition, the impact of high doses of bisphosphonates on the jaw bone microarchitecture and the effect of co-medications such as corticosteroids were evaluated. The current investigations suggest that exposure to a combination of bisphosphonate and corticosteroid increases the risk of developing BRONJ in comparison with exposure to bisphosphonates alone. The administration of corticosteroids alone, although immunosuppressant, did not result in bone necrosis or changes in bone microarchitecture. Furthermore, the bacterial profile of BRONJ patients and rats with BRONJ-like disease was explored throughout these experiments. The exposed bone had a different microbial profile than other sites of the oral cavity, which was mainly composed of non-pathogenic bacteria species and species linked to bone infections and periodontal diseases. These bacterial outcomes could provide additional information for clinicians when selecting antibiotics to target the related species. An extrapolation of the results of the rat investigations to humans suggests that the systemic administration of bisphosphonates and corticosteroids could change the oral microbiota colonizing the teeth.
Chapter six: Conclusions
Conclusions

The following specific conclusions could be highlighted:

• Both surgical and conservative treatments result in the resolution of BORNJ stage 2.
• Surgical treatment allows a faster resolution of BRONJ stage 2.
• Bisphosphonate administration results in a decrease of jaw bone remodeling.
• Bisphosphonate withdrawal may have a beneficial effect on the development of BRONJ and can help to resume bone remodeling.
• Bisphosphonate administration results in an increase in the number, volume, and surface area of sequestra at the site of tooth extraction.
• Probes prepared from the oral microbiota of humans hybridize with the whole genome extracted from the oral cavity of rats.
• The microbial profile of the exposed bone of BRONJ patients is different from other sites of the mouth.
• A systemic administration of bisphosphonates and corticosteroids results in changes in the oral microbiota.
References


Allen, M. R. and D. B. Burr (2010). "Bisphosphonate effects on bone turnover, microdamage, and mechanical properties: What we think we know and what we know that we don't know." Bone.


Kobayashi, Y., T. Hiraga, et al. (2010). "Zoledronic acid delays wound healing of the tooth extraction socket, inhibits oral epithelial cell migration, and promotes
proliferation and adhesion to hydroxyapatite of oral bacteria, without causing osteonecrosis of the jaw, in mice." Journal of Bone and Mineral Metabolism 28(2): 165-175.


dexamethasone-based regimens and high-dose chemotherapy." Journal of Clinical Oncology 23(22): 5217-5223.


Appendices
CONSENT FORM
For Research on Bacteria in the Oral Cavity

RESEARCH TITLE
Pilot evaluation of bacteria associated with bisphosphonate related osteonecrosis of the jaw (BRONJ).

PRINCIPAL INVESTIGATORS
Dr Rubens Albuquerque
Faculty of Dentistry, McGill University
Strathcona Anatomy and Dentistry
Room M-63, 3640 University Street
Montreal, Quebec H3A 2B2
Tel.: 514-398-7203 ext: 00090

Dr Janet E Henderson
Faculty of Medicine, McGill University
Montreal General Hospital
Room A5.169, 1650 Cedar Ave
Montreal, Quebec H3G 1A4
Tel: 514-934-1934 ext: 43358

Dr Michel El-Hakim
Faculty of Dentistry, McGill University
Montreal General Hospital
Room B3-119.1, 1650 Cedar Ave
Montreal, Quebec, H3G 1A4
Tel.: 514 934-1934 ext: 42468

Zaher Jabbour, PhD candidate
Faculty of Dentistry, McGill University
Strathcona Anatomy and Dentistry
Room M-64, 3640 University Street
Montreal, Quebec H3A 2B2
Tel.: 514-398-7203 ext: 09958
INTRODUCTION

You are being invited to participate in this study because you are 18 years and older, have been seen in the Dental Clinics at the Montreal General Hospital, for jaw bone necrosis related to the use of bisphosphonate medications (BRONJ), taking medications that might lead to BRONJ, or because you are a healthy individual not taking any medications.

Before you decide to participate, it is important that you understand the contents of this consent form, the risks and benefits to make an informed decision, and ask the study doctor or study nurse any questions if you do not understand. Please read this entire consent form and take your time to make a decision. If you decide to participate in this study, you will be asked to sign this informed consent form.

BACKGROUND

BRONJ is an important clinical problem associated with bisphosphonate medications. It is not clear yet how BRONJ develops. BRONJ was observed in patients undergoing interventions related to the teeth or gums. This leads to bone exposure to oral bacteria and an inflammation that does not heal.

PURPOSE OF THE STUDY

The purpose of this study is to identify the type of bacteria that could be associated with BRONJ.

STUDY DESCRIPTION AND PROCEDURES

If you agree to take part in this study, you will be asked to provide a sample of bacteria from your exposed oral bone, saliva and sites of plaque accumulation around the teeth at the time you are seen in the Dental Clinics at the Montreal General Hospital.

These collected samples will be tested in the laboratory for identification of the types of bacteria that they contain. The laboratory tests and analyses will results in the destruction of the samples.

Your participation in this study will not require any extra time, other than for the time to explain the study to you and to collect the samples (around 10 minutes).

About 105 subjects will be enrolled in this study at the Montreal General Hospital.

If changes are made to the study or new information becomes available about the type of bacteria you have, would you like to be informed?

Yes ☐ No ☐
Are you taking or have you taken any antibiotics other than what was prescribed to you by your dentist in the past year?

Yes ☐ No ☐ If yes, please specify:____________________________________

STORAGE AND SAFEKEEPING OF DNA SAMPLES

Your sample will permit us to perform basic research about bacteria. This research study was reviewed and approved by the researchers’ institutions.

Your sample will be assigned a code and stored at the Wong Laboratories for Mineralized Tissue Research located at the Genome building, McGill University, 740 Dr. Penfield Ave, Montreal, QC, H3A 1A4 while waiting for the laboratory analysis. The laboratory analysis will result in the destruction of your sample.

The use of your bacterial sample or medical information is not intended to provide you or your physician with test results. The study sponsor will not make any results available to you, any insurance company, your employer, your family, the study doctor, or any other physician who treats you now or in the future. Research information from this study will not become part of your medical records.

We will protect the confidentiality of the samples by assigning them a specific code. Your sample will not be specifically identified but only a code will link you or your name and the sample. Dr. Albuquerque, the principal researcher, will keep this code under lock and key in his office. Decoding can only be performed by Dr. Albuquerque, or an individual authorized by him.

BENEFITS

You should not expect any direct benefit from taking part in this study. However, the information gathered from this study may help improve future patients.

RISKS AND DISCOMFORTS

There are no foreseeable risks associated with your participation in this study. The samples collected are not expected to cause you any harm or discomfort.

ALTERNATIVE TREATMENT TO RESEARCH

You do not need to take part in this study to receive dental treatment. The investigators and treating dentist will explain all treatment options to you in full detail.

COSTS, REIMBURSEMENTS AND PAYMENTS

You will not be reimbursed for your participation in this study.
INDEMNIFICATION/ COMPENSATION IN CASE OF INJURY

We are not expecting any injuries related to this study.

By accepting to participate in this project, you are not waiving any of your legal rights nor discharging the researchers or the institution of their civil and professional responsibility.

CONFIDENTIALITY

The team of researchers of the Montreal General Hospital may consult your medical file to take note of the relevant data to this research project (including your medical and dental history, physical examinations, laboratory results and/or any information relevant to your medical health).

All information will be kept strictly confidential. The results from this study may be published, and other investigators participating in this research study may have access to your records related to this research study. However, your identity will not be revealed in the combined results. In order to verify the research study data, the Quality Assurance Officer from the MUHC Research Ethics Boards may review these records.

By signing this consent form, you give us permission to release information regarding your participation in this study to these individuals, and to inform your treating physician of your participation in the research study. Your confidentiality will be protected to the extent permitted by applicable laws and regulations.

VOLUNTARY PARTICIPATION AND/OR WITHDRAWAL

Your participation in this study is strictly voluntary. You may refuse to participate or you may discontinue your participation at any time without explanation, and without penalty or loss of benefits to which you are otherwise entitled. If you decide not to participate, or if you discontinue your participation, you will suffer no prejudice regarding your medical care or your participation in any other research studies. If you decide to withdraw, you can withdraw your sample if it was not destroyed by the laboratory tests. However, the information collected up until that time will remain in the general results.

COMMERCIALIZATION/RENUNCIATION

The analysis of your bacteria DNA sample may contribute to the creation of commercial products from which you will receive no financial benefit.

CONTROL OF THE ETHICAL ASPECTS OF THE RESEARCH PROJECT

The GEN Research Ethics Board of the MUHC approved this research project and is responsible for its follow-up. In addition, it will first approve any review and amendment made to the information/consent form and to the study protocol.
FUNDING OF THE RESEARCH PROJECT

The treating dentist and study investigators are not being paid for including you and looking after you during your participation in this study.

QUALITY ASSURANCE PROGRAM

The MUHC implemented a Quality Assurance Program that includes active continuing review of projects (on site visits) conducted within our establishment. Therefore, it must be noted that all human subject research conducted at the MUHC or elsewhere by its staff, is subject to MUHC Routine and Directed Quality Improvement Visits.

QUESTIONS AND/OR CONTACT INFORMATION

Should you have any questions concerning the study, you may contact Dr. Rubens Albuquerque at (514) 934-1934 ext. 00090.

If you wish to obtain additional information regarding your rights as participant in a research project or regarding any damage attributable to the research, harmful side effects to your health, you may contact The Montreal General Hospital Ombudsman at (514) 934-8306.

DECLARATION OF CONSENT

I have read this consent form, and I agree to participate in this research study. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I will be given a signed copy of this consent form. By signing this consent form, I have not given up any of my legal rights.

☐ I authorize the research team to contact me for future studies
☐ I do not authorize the research team to contact me for future studies

_________________________________________  ______________________________  ___________
Participant (Print Name) Date

I confirm that I have explained the nature and purpose of the study to the subject named above. I have answered all questions.

_________________________________________  ______________________________  ___________
Investigator and/or Delegate (Print Name) Date
FORMULAIRE DE CONSENTEMENT
pour la recherche sur les bactéries dans la cavité buccale

TITRE DE LA RECHERCHE
Évaluation pilote des bactéries associées à l'ostéonécrose des maxillaires reliée aux bisphosphonates (BRONJ).

CHERCHEURS PRINCIPAUX

Dr Rubens Albuquerque
Faculté de médecine dentaire, université McGill
Strathcona Anatomy and Dentistry
3640 rue Université, M-63
Montréal, Québec H3A 2B2
Tel.: 514-398-7203 ext: 00090

Dr Janet E Henderson
Faculté de médecine, université McGill
Hôpital général de Montréal
1650 Cedar Ave, A5.169
Montréal, Québec H3G 1A4
Tel: 514-934-1934 ext: 43358

Dr Michel El-Hakim
Faculté de médecine dentaire, université McGill
Hôpital général de Montréal
1650 Cedar Ave, B3-119.1
Montréal, Québec, H3G 1A4
Tel.: 514 934-1934 ext: 42468

Zaher Jabbour, étudiant au doctorat
Faculté de médecine dentaire, université McGill
Strathcona Anatomy and Dentistry
3640 rue Université, M-64
Montréal, Québec H3A 2B2
Tel.: 514-398-7203 ext: 09958
INTRODUCTION

Vous êtes invités à participer à cette étude parce que vous avez 18 ans et plus, vous avez été examinés aux cliniques dentaires de l'Hôpital général de Montréal pour ostéonécrose des maxillaires reliée aux bisphosphonates (BRONJ), vous prenez des médicaments qui pourraient être associés au BRONJ, ou parce que vous êtes un individu sain ne prenait aucun médicament.

Avant de décider de participer, il est important que vous compreniez le contenu de ce formulaire de consentement, les risques et les avantages de prendre une décision éclairée, et que vous demandez à l'infirmière ou au médecin de l'étude toute question que vous ne comprenez pas. S'il vous plaît, lisez ce formulaire de consentement entier et prenez votre temps pour prendre une décision. Si vous décidez de participer à cette étude, il vous sera demandé de signer ce formulaire de consentement éclairé.

CONTEXTE

BRONJ est un problème cliniquement important associé aux médicaments de bisphosphonates. Il n'est pas encore clair comment BRONJ se développe. BRONJ a été noté chez les patients subissant des interventions liées aux dents ou aux gencives. Cela peut mener à l'exposition de l'os des mâchoires aux bactéries orales et une inflammation qui ne guérit pas.

BUT DE L'ÉTUDE

Le but de cette étude est d'identifier le type de bactéries qui pourraient être liées au BRONJ.

DESCRIPTION DE L'ÉTUDE ET DES PROCÉDURES

Si vous acceptez de prendre part à cette étude, il vous sera demandé de fournir un échantillon de bactéries de votre os exposé à la cavité orale, de votre salive et des sites d'accumulation de la plaque autour des dents au moment où vous êtes vus aux cliniques dentaires à l'Hôpital général de Montréal.

Ces échantillons seront testés dans le laboratoire pour l'identification des types de bactéries qu'ils contiennent. Les tests et les analyses de laboratoire mènent à la destruction des échantillons.

Votre participation à cette étude ne nécessitera pas de temps supplémentaire, autre que le temps de vous expliquer l'étude et de recueillir les échantillons (environ 10 minutes).

Environ 105 sujets seront recrutés dans cette étude à l'Hôpital général de Montréal.

Si des modifications sont apportées à l'étude ou de nouvelles informations deviennent disponibles sur le type de bactéries que vous avez, aimeriez-vous être informé?

Oui ☐  Non ☐
Est-ce que vous prenez ou avez-vous pris des antibiotiques autres que ceux qui sont prescrites par votre dentiste pendant l'année passée?

- Oui ☐ Non ☐ Si oui, s'il vous plaît indiquez les: __________________________

DÉPÔT ET GARDE DES ÉCHANTILLONS D'ADN

Votre échantillon nous permettra d'effectuer des recherches fondamentales sur les bactéries. Cette étude a été examinée et approuvée par les institutions des chercheurs principaux.

Votre échantillon sera attribué un code et stockées dans les laboratoires de recherche Wong pour les tissus minéralisés situés dans le bâtiment du génome à l'université McGill, 740, Dr. Penfield Ave, Montréal, QC, H3A 1A4, en attendant les analyses de laboratoire. Les analyses de laboratoire mèneront à la destruction de votre échantillon.

L'utilisation de votre échantillon bactérien ou de vos informations médicaux n'est pas destinée à vous fournir ou fournir votre médecin les résultats des tests. La personne en charge de cette étude ne mettra pas les résultats à votre disposition, à la disposition d'aucun compagnie d'assurance, votre employeur, votre famille, le médecin de l'étude, ou tout autre médecin qui vous traite maintenant ou dans l'avenir. Les informations recherchés des de cette étude ne feront pas partie de votre dossier médical.

Nous allons protéger la confidentialité des échantillons en leur donnant des codes spécifiques. Votre échantillon ne sera pas spécifiquement identifié, à l'exception d’un code qui relie votre nom et l'échantillon. Dr Albuquerque, le chercheur principal, va conserver ce code sous clé dans son bureau. Le décodage ne peut pas être effectué que par Dr Albuquerque, ou une personne autorisée par lui.

AVANTAGES

Vous ne devriez pas attendre aucun bénéfice direct de participer à cette étude. Toutefois, les informations recueillies de cette étude peuvent bénéficier les futurs patients.

RISQUES ET LES MALAISES

Il n'y a aucun risque prévisible lié à votre participation à cette étude. Aucun dommage ou inconfort est attendu suite à la collection des échantillons.

TRAITEMENT ALTERNATIVE À LA RECHERCHE

Vous n'êtes pas obligé de prendre part à cette étude pour recevoir un traitement dentaire. Les chercheurs et le dentiste traitant vous expliqueront toutes les options de traitement en détail.

COÛTS, REMBOURSEMENTS ET PAIEMENTS

Vous ne serez pas remboursés pour votre participation à cette étude.
INDEMNISATION EN CAS DE BLESSURES

Aucune blessure liée à cette étude n'est pas attendue.

En acceptant de participer à ce projet, vous ne renoncez à aucun de vos droits légaux, ni déchargez les chercheurs ou l'institution de leur responsabilité civile et professionnelle.

CONFIDENTIALITÉ

L'équipe de chercheurs de l'Hôpital général de Montréal peuvent consulter votre dossier médical pour prendre des notes des données pertinentes à ce projet de recherche (y compris votre histoire médical et dentaire, les examens physiques, les résultats de laboratoire et / ou toute autre information pertinente pour votre santé médicale).

Toutes les informations seront gardées d’une façon strictement confidentielle. Les résultats de cette étude peuvent être publiés, et d'autres chercheurs participants à cette étude peuvent avoir accès à vos dossiers liés à cette étude. Cependant, votre identité ne sera pas révélée dans les résultats. Afin de vérifier les données des études de recherche, l'agent de l’assurance de la qualité du bureau de l'éthique de la recherche du CUSM peut examiner ces dossiers.

En signant ce formulaire de consentement, vous nous donnez la permission de divulguer l'information concernant votre participation à cette étude à ces individus, et d'en informer votre médecin traitant de votre participation à l'étude. Votre confidentialité sera protégée dans la mesure permise par les lois et règlements applicables.

PARTICIPATION VOLONTAIRE ET / OU RETRAIT

Votre participation à cette étude est strictement volontaire. Vous pouvez refuser de participer ou vous pouvez cesser votre participation à tout moment sans explication, sans pénalité ou sans perte des avantages auxquelles vous avez droit par ailleurs. Si vous décidez de ne pas participer, ou si vous cessez votre participation, vous ne subirez aucun préjudice concernant vos soins médicaux ou votre participation à toute autre étude. Si vous décidez de vous retirer, vous pouvez retirer votre échantillon s'il n'a pas été détruit par les tests de laboratoire. Toutefois, les informations recueillies jusqu'à ce moment resteront dans les résultats généraux.

COMMERCIALISATION / RENONCIAISON

L'analyse de votre échantillon de bactéries peut contribuer à la création de produits commerciaux à partir de laquelle vous ne recevrez aucun avantage financier.

CONTRÔLE DES ASPECTS ETHIQUES DU PROJET DE LA RECHERCHE

Le bureau GEN de l’éthique de la recherche du CUSM a approuvé ce projet de recherche et il est responsable de son suivi. De plus, il approuvera d'abord toute révision ou modification apportées aux informations / formulaire de consentement et au protocole de l’étude.
FINANCEMENT DU PROJET DE RECHERCHE

Les chercheurs de l'étude et le dentiste traitant ne sont pas payés pour vous inclure et s'occuper de vous pendant votre participation à cette étude.

PROGRAMME D'ASSURANCE DE LA QUALITÉ

Le CUSM a implémenté un programme d'assurance de la qualité qui contient des examens continus et actifs (visites sur place) des projets conduits au sein de notre établissement. Par conséquent, il faut noter que toutes les recherches avec les sujet humains au CUSM ou par ses personelles, sont soumises à des visites routines et dirigées par le CUSM afin de l’amélioration de la qualité.

QUESTIONS ET / OU RENSEIGNEMENTS

Si vous avez des questions concernant l'étude, vous pouvez contacter le Dr Rubens Albuquerque au (514) 934-1934 ext. 00090.

Si vous souhaitez obtenir des informations supplémentaires concernant vos droits en tant que participant à un projet de recherche ou concernant tout dommage relié à la recherche, les effets secondaires nocifs sur votre santé, vous pouvez communiquer avec un médiateur à l'Hôpital général de Montréal (514) 934-8306.

DÉCLARATION DE CONSENTEMENT

J'ai lu ce formulaire de consentement, et je m'engage à participer à cette étude. J'ai eu l'occasion de poser des questions et toutes mes questions ont été répondues à ma satisfaction. J'ai eu suffisamment de temps pour examiner les informations ci-dessus et à demander un conseil si je choisis de le faire. Je vais recevoir une copie signée de ce formulaire de consentement. En signant ce formulaire de consentement, je n'ai pas renoncé à aucun de mes droits légaux.

☐ J'autorise l'équipe de recherche de me contacter pour des futures études
☐ Je n'autorise pas l'équipe de recherche à me contacter pour des futures études

_______________________________ (Nom imprimé) ________________ Date
Participant(e)

Je confirme que j'ai expliqué la nature et le but de l'étude sur le sujet susmentionné. J'ai répondu à toutes ses questions.

_______________________________ (Nom imprimé) ________________ Date
Chercheur(e) et / ou Délégué(e)
PATIENT EVALUATION FORM

Patient Name

Identification Code

Date

Site of bone exposure

Area of plaque sample collection

Score 0 = The tooth surface is clean.
Score 1 = Dental plaque can be removed from the gingival third with a sharp explorer.
Score 2 = Plaque is visible along the gingival margin.
Score 3 = The tooth surface is covered with abundant plaque.

Presence of bone exposure

Presence of purulent discharge

Presence of pain

Is the patient taking any antibiotics

If yes, please specify______________________________________

For how long? _______________________________________

Is the patient taking any other medication

If yes, please specify______________________________________

For how long? _______________________________________

168