THE ROLE OF THE INNATE IMMUNE SYSTEM IN BISPHOSPHONATE-INDUCED OSTEONECROSIS OF THE JAW

By

Carol Forster

A thesis submitted in conformity with the requirement for the Degree of Master of Science
Graduate Department of Dentistry
University of Toronto

© Copyright by Carol Forster 2011
THE ROLE OF THE INNATE IMMUNE SYSTEM IN BISPHOSPHONATE-INDUCED OSTEONECROSIS OF THE JAW

Carol Forster 2011, Degree of Master of Science, Graduate Department of Dentistry, University of Toronto

ABSTRACT

Bisphosphonate-induced osteonecrosis of the jaw (BPONJ) has been identified as a severe complication of dental treatment in 1-10% of patients previously treated with intravenous bisphosphonates. The mechanism by which bisphosphonates induce BPONJ is uncertain. It has been noted that necrotic bone from BPONJ sites display signs of bacterial infection that suggests that an immune defect may play a role in the pathophysiology of BPONJ. The purpose of this thesis examined the effect of a potent bisphosphonate, zoledronate, on the innate immune system, specifically, neutrophil function, differentiation and survival with in vitro and in vivo murine models. Zoledronate exposure leads to decreased neutrophil migration, neutrophil NADPH oxidase activity, circulating neutrophil counts, as well as neutrophil survival, however does not appear to affect neutrophil differentiation. We present evidence that bisphosphonates have the potential to depress the immune system in mice and a subset of patients, possibly contributing to the pathogenesis of BPONJ.
Acknowledgements

Special thanks to Dr. Glogauer, who kindly accepted me as a summer research student many years ago and then as a Masters student when I had little knowledge and experience in molecular biology research. This study would not have been possible without his guidance, support and encouragement.

I add my sincere thanks to my supervisory committee members, Drs. Sean Peel and Morris Manoloson for their time in regular attendance to the committee meetings and for their advice and critical discussion of my work.

I would also like to thank everyone in the CIHR Group in Matrix Dynamics. Special thanks to Chunxiang Sun, and Jan Kuiper for their help with the GST-pull down assay and apoptosis assays and other technical support in the Glogauer lab.

Finally, I would not have come this far without the love and support from my parents, my sibling and my friends.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................ii

ACKNOWLEDGEMENTS .................................................................................. iii

TABLE OF CONTENTS .................................................................................. iv

LIST OF TABLES & FIGURES ......................................................................... vii

ABBREVIATIONS ............................................................................................ viii

Chapter 1: INTRODUCTION .......................................................................... 1

I. INTRODUCTION .......................................................................................... 2

II. BISPHOSPHONATES
   A. Chemical Structure .................................................................................. 4
   B. Mechanism of Action ............................................................................ 5
   C. Targeting of Bisphosphonates to Bone and Cellular Uptake ............... 6
   D. Distribution of bisphosphonates in bone .............................................. 7
   E. Uptake and retention of bisphosphonates in bone .............................. 8
   F. Speed of onset of anti-fracture effects ................................................ 9
   G. Offset of Action ..................................................................................... 10
   H. Relating properties of individual bisphosphonates to their clinical effects.. 11
   I. Clinical uses of bisphosphonates .......................................................... 12
      i. Osteoporosis ....................................................................................... 12
      ii. Paget’s disease ................................................................................ 12
      iii. Bone oncology ................................................................................ 13
   J. Side-effects of bisphosphonates ............................................................ 14

III. BISPHOSPHONATE-INDUCED OSTEONECROSIS OF THE JAW (BPONJ)
   A. Definition and Diagnosis ...................................................................... 15
   B. Staging Criteria ..................................................................................... 17
   C. Incidence ................................................................................................ 17
      i. Randomized control trials ................................................................. 18
      ii. Retrospective chart reviews ............................................................. 18
   D. Prevention ............................................................................................ 20
   E. Management ........................................................................................ 22

IV. NEUTROPHILS
   A. Bone Marrow Origin of the Neutrophil ............................................. 24
   B. The Fate of Circulating Neutrophils .................................................... 25
      i. Leukocyte Rolling ............................................................................. 25
Hypothesis and Objectives of Thesis ................................................................. 36

Chapter 2: MATERIALS AND METHODS....................................................... 38

2.1. Antibodies and reagents ........................................................................... 39
2.2. Animals ...................................................................................................... 39
2.3. In vivo Zoledronate treatment ................................................................. 39
2.4. Isolation of murine bone marrow neutrophils ......................................... 40
2.5. Measurement of NADPH oxidase activity ............................................... 40
2.6. Zigmond chamber chemotaxis ................................................................. 41
2.7. Sodium periodate peritonitis ..................................................................... 41
2.8. Circulating neutrophil levels ..................................................................... 41
2.9. GST-pulldown assay ................................................................................ 42
2.10. Apoptosis assay ........................................................................................ 42
2.11. In vitro generation of neutrophils ............................................................ 42
2.12. BPONJ patient selection .......................................................................... 43
2.13. Patient blood neutrophil chemotaxis and isolation .................................. 43
2.14. Patient oral rinse samples ...................................................................... 44
2.15. Statistics .................................................................................................. 44

Chapter 3: RESULTS ..................................................................................... 45

3.1. Effect of Zoledronate on in vitro Neutrophil Function ............................... 46
3.2. Effect of Zoledronate on in vivo Neutrophil Function ............................... 46
3.3. RhoGTPase Assay .................................................................................... 47
3.4. Effect of Zoledronate on Circulating Neutrophil counts ........................... 47
3.5. Effect of Zoledronate on Neutrophil Differentiation and survival ............ 47
3.6. Blood neutrophil chemotaxis in BPONJ patients ....................................... 49
3.7. Oral neutrophil recruitment in BPONJ patients .......................................... 49
List of Tables & Figures

Table 1. The chemical structure, MOA, potency and trade names of common BPs.............11
Table 2. Proposed clinical descriptions of BPONJ ...............................................................16
Table 3. Staging criteria for BPONJ ....................................................................................17

Figure 1-A. Effect of Zoledronate on in vitro neutrophil chemotaxis..............................50
Figure 1-B. Effect of Zoledronate on in vitro NADPH oxidase activity.............................50

Figure 2-A. Effect of in vivo Zoledronate treatment on in vitro chemotaxis.......................50
Figure 2-B. Effect of in vivo Zoledronate treatment on in vivo peritoneal recruitment...... 50

Figure 3. Possible mechanisms through which ZOL affects neutrophil functions............. 51

Figure 4. Effect of Zoledronate on circulating neutrophil levels.................................... 51

Figure 5. Effect of Zoledronate on differentiation and apoptosis of mature neutrophils..... 52
Figure 5-A. Effect of Zoledronate on in vitro neutrophil differentiation from bone marrow stem cells .................................................................52
Figure 5-B. Quantification of neutrophil cell death following ZOL exposure....................52
Figure 5-C. ZOL affects mature neutrophil survival during the process of differentiation...52
Figure 5-D. Effect of ZOL on neutrophil apoptosis in the absence of G-CSF....................52
Figure 5-E. Effect of ZOL on neutrophil apoptosis in the presence of G-CSF..................52

Figure 6-A. Neutrophil Chemotaxis is perturbed in BPONJ patients...............................54
Figure 6-B. Oral neutrophil counts in BPONJ patients vs. healthy control patients...........54
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AAOMS</td>
<td>American Academy of Oral and Maxillofacial Surgery</td>
</tr>
<tr>
<td>ASBMR</td>
<td>American Society for Bone Mineral Research</td>
</tr>
<tr>
<td>BP</td>
<td>Bisphosphonate</td>
</tr>
<tr>
<td>BPONJ</td>
<td>Bisphosphonate-(associated/induced/related) osteonecrosis of the jaw</td>
</tr>
<tr>
<td>CTX</td>
<td>Cross-linked C-telopeptide</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl diphosphate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-Formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>G-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl diphosphate</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope-Coded Affinity Tags</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MMP8</td>
<td>Matrix metalloprotein-8</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenosine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>N-BP</td>
<td>Nitrogenous bisphosphonate</td>
</tr>
<tr>
<td>NonN-BP</td>
<td>Non-nitrogenous bisphosphonate</td>
</tr>
<tr>
<td>NTX</td>
<td>Cross-linked N-telopeptide</td>
</tr>
<tr>
<td>ONJ</td>
<td>Osteonecrosis of the jaw</td>
</tr>
<tr>
<td>ORN</td>
<td>Osteoradionecrosis</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>ZOL</td>
<td>Zoledronate</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
I. INTRODUCTION

Bisphosphonates have become the primary treatment for the prevention of osteoporosis and are used to treat hypercalcemia of malignancy and to reduce skeletal-related symptoms and events in patients with multiple myeloma and metastatic bone lesions of solid tumor cancers such as breast, prostate and lung cancer [1][2]. Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption. An important characteristic of these drugs is their highly selective localization and retention in bone compared to other tissues [3]. This quality makes them ideal candidates for treatment of bone diseases. Bisphosphonates work by reducing bone turnover, increasing bone mass, and decreasing the risk of fracture.

In recent years it has been noted that a small subset of patients (1-10%) treated with intravenous bisphosphonates are at an increased risk of developing osteonecrosis of the jaw (BPONJ) [4, 5]. The publicity surrounding this complication and the lack of information about its underlying pathogenesis has led to apprehension amongst patients and dental clinicians when treating patients with a history of bisphosphonate therapy [6]. Critical information that will help alleviate these concerns and improve oral treatment approaches in this patient population is required.

The mechanism by which BPs induce BPONJ is uncertain. Several possible theories have been cited in literature including: reduced bone turnover, ischemia, bone toxicity, soft tissue toxicity and infection.
In light of the more consistent observation that bacterial colonization is a common, if not universal finding in BPONJ, we sought to explore the possibility that BPs may impart an irreversible effect on the immune system of the host. There have been reports that in some patients, bisphosphonates can cause neutropenia[7], inhibit neutrophil enzymes that impact on wound healing such as MMP8 [8] and decreased generation of reactive oxygen species formation [9, 10]. This is not surprising in view of the mechanism of action of BPs which target small Rho GTPases that are signaling proteins integral to neutrophil differentiation and function. It is important to note that the BP-related neutrophil defects indicate that this side effect does not have any severe clinical effects such as increased susceptibility to infection [11]. Clearly other aspects of the immune system are able to compensate for the neutrophil effects systemically. However it is possible that in a small subset of these patients, prolonged BP use may effect neutrophil differentiation and function which could manifest in poor oral innate immunity, poor oral wound healing and susceptibility to BPONJ.

Being able to identify and understand the possible mechanisms through which bisphosphonates affect normal bone/wound healing in the jaw will help identify patients at risk of developing BPONJ and improve patient management. The goal of this thesis is to look into the possible role that the immune system plays in the pathogenesis of this rare condition.
II. Bisphosphonates

A. Chemical Structure

Bisphosphonates (BPs) are pyrophosphate analogues, meaning that the central oxygen atom in the molecule is replaced by a carbon atom, resulting in a P-C-P bond. The existence of this bond not only enables the binding of the bisphosphonate to hydroxyapatite, the naturally occurring mineral in bone, but also renders the molecule highly resistant to hydrolysis under acidic conditions or by pyrophosphatases[12]. As a result, BPs are not converted into metabolites in the body and are excreted unaltered. It has been suggested that the two phosphate groups are required for binding to the bone mineral, where varying one or both phosphonate groups can dramatically reduce the affinity of the BP for bone mineral[13]. In the bisphosphonate molecule, two side chains are attached to the central carbon atom (R1-the short side chain and R2-the long side chain) which determine the potency of the particular bisphosphonate[12]. R1 substituents such as hydroxyl or amino enhance chemisorption to mineral[14], while varying the R2 substituent’s results in differences in anti-resorptive potency of several orders of magnitude. Risedronate and zoledronate have been shown to be two of the most potent anti-resorptive BPs in several animal models[15], due to the nitrogen atom within the heterocyclic ring side group at R2. The increased anti-resorptive potency observed with the different R2 groups is linked to its ability to affect the biochemical activity e.g., inhibition of the farnesyl diphosphate (FPP) enzyme[16]. The presence of nitrogen in the R2 chain, as is the case in the second-generation bisphosphonates, increases the potency of the drug by allowing the formation of a tridentate conformation that is able to bind Ca\(^{2+}\) more effectively[17, 18].
B. Mechanisms of Action

(i) Non-nitrogenous Bisphosphonates

Two classes of bisphosphonates exist depending on their chemical structure and mechanism of function. As the name suggests, the non-nitrogen bisphosphonates (e.g. clodronate, tiludronate and etidronate) do not have a nitrogen moiety within their side chains, leading to a relatively lower potency drug. The non-nitrogenous BPs are metabolized in the cell to generate cytotoxic analogs of ATP[19, 20]. These ATP analogs form a non-functional molecule that competes with ATP in the cellular energy metabolism and as a result, interferes with mitochondrial function[21]. In essence, the induction of osteoclast apoptosis following the accumulation of such metabolites, appears to be the major mode of action of these BPs in inhibiting bone resorption[3].

(ii) Nitrogenous Bisphosphonates

In contrast, the highly potent nitrogenous bisphosphonates (N-BPs) (e.g., pamidronate, alendronate, ibandronate, risedronate and zoledronate) are not metabolized by osteoclasts but instead bind to and inhibit farnesyl diphosphate (FPP) and geranylgeranyl disphosphate (GGPP) [22] in the HMG-CoA reductase pathway (also known as the mevalonate pathway). Inhibition of the HMG-CoA-reductase pathway at the level of FPP and GGPP prevents the formation of two metabolites; farnesol and geranylgeraniol. This ultimately disrupts the prenylation and membrane targeting of members of the Rho family of small GTPases such as Ras, Rho, Rac, Rab and Cdc42 [23, 24]. These small GTPases are essential for numerous immune and osteoclast cell functions [25]. As a result, N-BPs may interfere with various intracellular processes such as cytoskeleton
reorganization, vesicle transport, exocytosis and ultimately, apoptosis in the targeted cells [26, 27].

C. Targeting of Bisphosphonates to Bone and Cellular Uptake

*Nitrogenous BPs*

The pronounced selectivity of bisphosphonates for bone rather than other tissues is the basis for their value in clinical practice. The bone targeting property of the bisphosphonates comes from the fact that they have a high avidity for Ca\(^{2+}\) ions[18]. Once in the circulation, the bisphosphonates selectively absorb onto exposed bone surfaces in active areas of bone remodeling and concentrate there[28]. During the bone resorptive process, the space beneath the osteoclast, known as the ruffled border is acidified by the action of vacuolar-type proton pumps. The acidic pH of this environment causes dissolution of the hydroxyapatite bone mineral and as a result leads to the release of free, non-mineral bound bisphosphonate. Since the osteoclasts are here in the highest proportion, they are therefore exposed to the highest concentration of drug[28, 29]. Because the osteoclasts are highly endocytic during the bone resorption process, bisphosphonates located in the resorption lacuna in bone are internalized in the osteoclast. The N-BPs carry a highly negative charge and are therefore considered membrane impermeable, and the manner in which bisphosphonates cross the vacuolar membrane to enter the cytoplasm is not well established[30, 31]. In a previous study, Felix et al. used radiolabeled bisphosphonates to study uptake by calvarial cells *in vitro* and confirmed that the bisphosphonates could enter the cytoplasm as well as mitochondria and other organelles[32]. Studies with slime mold amoebae, where the growth is inhibited by bisphosphonates[33], have also demonstrated that cellular uptake
occurs by fluid-phase endocytosis. The endocytosis of the BPs by the osteoclasts leads to a disruption of the cytoskeleton, loss of ruffled border, inhibition of lysosomal enzymes, and protein tyrosine phosphatases[34]. In addition to their primary action on the osteoclast activity and life span, bisphosphonates also reduce the number of active osteoclasts by inhibiting their recruitment and finally they inhibit the osteoclast-stimulating activity of osteoblasts[34].

D. Distribution of Bisphosphonates in bone

At the microscopic level, BPs initially localize to areas of active bone turnover[28]. This pattern of localization has the advantage of targeting BPs specifically to disease sites in Paget’s disease and metastatic cancer in bone, and possibly even in osteoporosis. A majority of the BPs that attach to the bone surface become quickly buried in the bone where they can be retained for long periods of time. Small amount of BPs can be measured over several weeks or months in the urine following cessation of the drug due to the release from the skeleton[35, 36]. This suggests that BPs must be present in the circulation and ready for re-absorption into bone for prolonged periods, and may be responsible for their ongoing pharmacological actions.

In theory, the release of BPs from bone can occur through at least two mechanisms; either chemical desorption and/or ostoclastic resorption. The binding of BPs to hydroxyapatite surfaces is a reversible physiochemical process[16, 37], such that BP absorption will occur when extracellular concentrations are high and desorption will occur when extracellular levels are low[16, 37].
Bisphosphonates will also be released from bone during osteoclastic resorption. It is unknown the relative contribution of desorption versus cell-mediated resorption. It is assumed however that physico-chemical desorption to be more important immediately after dosing, before the drug becomes buried under bone. However in the longer term, after the cessation of dosing, release of BPs may occur readily as a result of liberation during bone resorption[38].

**E. Uptake and retention of BPs in bone**

The retention of BPs in the skeleton has been studied in both animal and human models. The use of radiolabeled BPs has been the main method used to measure the uptake and retention of BPs in different parts of the skeleton. From these studies, approximately one to two thirds of the administered BP dose becomes incorporated into the skeletons, whereas the remainder is predominantly excreted into the urine within the first few hours following administration. One of the difficulties in these retention studies has been the fact that retention is affected by the disease state of the individual, renal function and retention is also dependent on the specific BP used[39].

The concept of half-lives of retention can be quite misleading based on the fact that the half-lives of the drugs are not equivalent to the half-lives of their effects. Much of the BPs kept for long periods of time within the skeleton is likely to be rapidly “buried” within the mineral phase rendering it pharmacologically inactive[40]. Thus the release of BPs from bone is highly dependent on the remodeling and resorption. It is reasonable to assume that the figures quoted in the literature that have previously referred to half-lives of 10 years or greater, compared with days or months, do not offer valid comparisons.
among BPs. These figures also fail to give a true indication of the persistence of their pharmacological effects[17].

F. Speed of onset of anti-fracture effects

From a clinical standpoint, it is advantageous to know the speed with which various BPs achieve fracture reduction. There is evidence from individual randomized control trials (RCTs), post-hoc analysis and observational studies to suggest that these effects occur to varying degrees with the different BPs. However these results are not directly comparable between the different BPs since there have been few head to head studies. One of these studies performed was a 15 month, randomized study that compared intravenous pamidronate to zoledronate in 90 subjects with active Paget’s disease of bone [41]. Paget's disease is a disorder that involves abnormal bone destruction and regrowth, which is typically monitored with blood levels of alkaline phosphatase (ALP), where elevated levels suggest increased bone destruction. The primary efficacy endpoint used in this study was therapeutic response at 6 months, which was defined as normalization of alkaline phosphatase (ALP) or a reduction of at least 75% in total ALP excess. At 6 months, 97% of patients receiving zoledronate had a therapeutic response compared with 45% of patients receiving pamidronate. In the zoledronate group, normalization of ALP was achieved in 93% of patients and in 35% of patients in the pamidronate group. ALP normalization was maintained in 79% and 65% of zoledronate-treated patients after 12 and 15 months, respectively; loss of therapeutic response was observed in 2 of 30 (6%) at 12 and 15 months[41].
When looking at radiographic vertebral fractures, several RCTs with aledronate[42] and ibandronate[43] show a significant reduction following 24 months. While risedronate and zoledronate show a significant reduction following only 12 months[11, 44].

G. Offset of Action

Information about the speed of reversal of effect following discontinuation of the BP is limiting as no head-to-head studies of the different BPs have been undertaken. Bone turnover markers, such as type-1 collagen crosslinked N-telopeptide (NTX) and type-1 collagen cross-linked C-telopeptide (CTX), can be used as an indirect measure of bone turnover. Following 5 years of treatment with aledronate, bone turnover markers appeared to be at least partially suppressed for up to 5 years after treatment was discontinued[45, 46]. Similarly, bone turnover markers remained reduced for 1 year after a single IV dose of zoledronate at 4 or 5-mg and remained significantly reduced at the 2 year follow up [11, 47]. While a variety of BPs have been shown to affect the duration of suppression of bone turnover, data from animal and human studies suggest that the dose of the BP also plays a role. In general, the higher the dose or the higher the bone affinity of the drug, the longer bone turnover remains reduced[47, 48].
**H. Relating properties of individual BPs to their clinical effects**

Due to the diverse structures of the BPs, each one can display unique biochemical potency on FPP and potential differences in mineral targeting and release (Table 1). The rank order of the most critical properties is as follows[17]:

Mineral affinity: clodronate < etidronate < risedronate < ibandronate < alendronate < pamidronate < zoledronate

FPP enzyme inhibition: etidronate < clondronate (extremely weak inhibitors) <<<< pamidronate < alendronate < ibandronate < risedronate < zoledronate

<table>
<thead>
<tr>
<th>Agent</th>
<th>$R_1$ side chain</th>
<th>$R_2$ side chain</th>
<th>MOA</th>
<th>Potency (relative to that of etidronate)</th>
<th>Trade Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>-OH</td>
<td>-CH$_3$</td>
<td>Non-N</td>
<td>1</td>
<td>Didronel</td>
</tr>
<tr>
<td>Clodronate</td>
<td>-Cl</td>
<td>-Cl</td>
<td>Non-N</td>
<td>10</td>
<td>Bonefos</td>
</tr>
<tr>
<td>Tiludronate</td>
<td>-H</td>
<td>-S-Cl</td>
<td>Non-N</td>
<td>10</td>
<td>Skelid</td>
</tr>
<tr>
<td>Pamidronate</td>
<td>-OH</td>
<td>-CH$_2$-CH$_2$-NH$_2$</td>
<td>Nitrogenous</td>
<td>100</td>
<td>Aredia</td>
</tr>
<tr>
<td>Neridronate</td>
<td>-OH</td>
<td>-(CH$_2$)$_5$-NH$_2$</td>
<td>Nitrogenous</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Olpadronate</td>
<td>-OH</td>
<td>-(CH$_2$)$_2$N(CH$_3$)$_2$</td>
<td>Nitrogenous</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Alendronate</td>
<td>-OH</td>
<td>-(CH$_2$)$_3$-NH$_2$</td>
<td>Nitrogenous</td>
<td>500</td>
<td>Fosamax</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>-OH</td>
<td>-CH$_2$-CH$<em>2$N($\text{CH}</em>{3}$)</td>
<td>Nitrogenous</td>
<td>1000</td>
<td>Boniva</td>
</tr>
<tr>
<td>Risedronate</td>
<td>-OH</td>
<td></td>
<td>Nitrogenous</td>
<td>2000</td>
<td>Actonel</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>-OH</td>
<td></td>
<td>Nitrogenous</td>
<td>10000</td>
<td>Aclasta</td>
</tr>
</tbody>
</table>

Table 1. The chemical structure, mechanism of action, potency and trade names of common bisphosphonates
I. Clinical Uses of Bisphosphonates

Since it was first recognized that BPs are selectively absorbed by the skeleton, oral and intravenous forms of BPs have become widely used for the treatment of osteoporosis, Paget’s disease, and in oncology.

(i) Osteoporosis

Oral and in a fewer cases, intravenous bisphosphonates, are the preferred pharmacological agents in the treatment of osteoporosis. Bisphosphonates are effective in increasing bone mineral density and lowering the risk of fractures at the spine by 30-70%, and also to varying degrees at other skeletal sites in postmenopausal women[42, 49, 50]. Etidronate was the first BP approved for osteoporosis[51, 52], followed by alendronate, risedronate[44], and ibandronate[43], and most recently zoledronate[11].

(ii) Paget’s Disease

Many different BPs have been prescribed to treat Paget’s disease. The order of potency of the different BPs used to suppress the rapid bone turnover observed in Paget’s disease appears to be similar to their order of anti-resorptive potency observed in an animal model: etidronate < clodronate < pamidronate < alendronate < risedronate < zoledronate[53, 54]. Recently, zoledronate and risendronate, newer and more potent BPs, have been observed to produce an even greater suppression in disease activity. In the most recent study[48], a single 5mg infusion of zoledronate was found to produce a greater and longer lasting suppression of excess bone turnover than oral risedronate given at 30 mg daily over 2 months.
(iii) Bone Oncology

Bisphosphonates are quite effective in the treatment of bone complications associated with malignancy, including hypercalcemia and/or increased bone destruction and now are considered the standard of care when treating these patients. The concept behind using these osteoclastic inhibitors in the therapeutic armamentarium for bone metastases is explained by the pathophysiology of tumor-induced osteolysis. Once cancer cells colonize the bone marrow, they are attracted to the surface of the bone by products from bone resorption. The cancer cells have the ability to increase osteoclast differentiation of hematopoietic stem cells and over time destroy the bone via direct osteoclast stimulation[55, 56]. As well, in the case of cancers of the breast, the propensity to metastasize and proliferate is best explained by the “seed and soil” theory [57]. The “seed” (the breast cancer cell), secrete osteolytic factors, such as parathyroid hormone-related peptide (PTHrP), which tend to increase the development of metastases in the skeleton, which is thought of as the “soil” since it is rich in cytokines and growth factors that are known to stimulate cancer cell growth. These cytokines are thought to induce osteoclast differentiation from hematopoietic stem cell as well as activate mature osteoclasts in the bone. The overall increase in osteoclast number and activity leads to local foci of osteolysis, and enhanced released of growth factors that further stimulate cancer cell proliferation. Thus a viscous cycle is created where the bone resorption-induced release of growth factors from the bone matrix tends to encourage growth of breast cancer cells and the production of osteolytic factors.

Zoledronate, clodronate, ibandronate, and pamidronate are all approved for clinical use based on placebo-controlled trails, though not all are available in all countries.
J. Side Effects of Bisphosphonates

Adverse effects are similar with oral bisphosphonates and include upper gastrointestinal irritation, which may be manifested with heartburn, esophagitis, abdominal pain, or diarrhea. Rarer adverse effects include bone, joint, and muscle pain that subsides after the drug is discontinued. The i.v. preparation is associated with osteonecrosis of the jaw, ocular inflammation manifesting as scleritis, uveitis, or conjunctivitis, fever, leukopenia and nephritic syndrome[58, 59].
III. Bisphosphonate-induced osteonecrosis of the Jaw (BPONJ)

A. Definition and Diagnosis

Bisphosphonate-(induced/associated/related) osteonecrosis of the jaw (BPONJ) was first described in the dental literature in 2003 [60]. There is no one agreed upon definition of BPONJ however several professional groups have attempted to develop a more uniform definition of BPONJ (Table 2). The diagnosis of BPONJ is primarily based on clinical findings. The clinical findings that appears to be central to each associations definition of BPONJ is a patient who has had current or previous treatment with a bisphosphonate, and has exposed, necrotic bone in the maxillofacial region that has persisted for >8weeks; and has no history of radiation therapy in the jaws[61].
<table>
<thead>
<tr>
<th>Task Force/Association</th>
<th>Proposed Clinical Description of ONJ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>American Society for Bone and Mineral Research (ASBMR) Task Force (2007)</strong></td>
<td>BPONJ is defined as an area of exposed bone in the maxillofacial region that does not heal within 8 wk after identification by a healthcare provider, in a patient who is receiving or has been exposed to a bisphosphonate and has not had radiation therapy to the craniofacial region. Additional signs and symptoms may include pain, swelling, paresthesia, suppuration, soft tissue, ulceration, intra- or extraoral sinus tracts, loosening of teeth, and radiographic variability. However, these are neither individually nor collectively sufficient for a diagnosis in the absence of exposed bone as described above.</td>
</tr>
<tr>
<td><strong>American Academy of Oral and Maxillofacial Surgeons (AAOMS) (2006)</strong></td>
<td>Patients may be considered to have bisphosphonate-related ONJ if all of the following 3 characteristics are present: 1. Current or previous treatment with a bisphosphonate 2. Exposed bone in the maxillofacial region that has persisted for &gt;8wk 3. No history of radiation therapy to the jaws</td>
</tr>
<tr>
<td><strong>American College of Rheumatology (ACR) (2006)</strong></td>
<td>ONJ typically appears as an intraoral lesion with areas of exposed yellow-white hard bone with smooth or ragged borders, sometimes associated with extraoral or intraoral sinus tracts. Painful ulcers may be present in the soft tissues adjacent to the ragged bony margins of the lesion. Dental x-rays may be unremarkable in the early cases, but advanced cases demonstrate poorly defined areas of ‘moth-eaten’ radiolucencies, with or without radio-opaque bone sequestra. Pathologic jaw fractures have occurred in some cases. In its most severe form, ONJ may cause loss of a significant part of the mandible or maxilla.</td>
</tr>
<tr>
<td><strong>American Association of Endodontics (AAE) (2006)</strong></td>
<td>Patients presenting with bisphosphonate-associated ONJ typically present with at least some of the following signs and symptoms: 1. An irregular mucosal ulceration with exposed bone in the mandible or maxilla 2. Pain or swelling in the affected jaw 3. Infection, possibly with purulence 4. Altered sensation (e.g., numbness or heavy sensation)</td>
</tr>
<tr>
<td><strong>American Dental Association (ADA) (2006)</strong></td>
<td>The typical clinical presentation of BON includes pain, soft-tissue swelling and infection, loosening of teeth, drainage, and exposed bone. Symptoms may occur spontaneously in the bone or, more commonly, at the site of a previous tooth extraction. In some cases, patients seek dental care complaining of pain that may mimic a dental problem. Infection may or may not be present. The “dental” problem does not respond to routine dental therapy, and there is no clinically visible osteonecrosis. However, BON also may remain asymptomatic for weeks or months and may become evident only after the finding of exposed bone in the jaw during a routine examination. In some cases, the symptoms of BON can mimic dental or periodontal disease. Routine dental and periodontal treatment will not resolve the symptoms. In this case, if the patient is receiving bisphosphonate therapy, BON must be considered as a possible diagnosis, even in the absence of exposed bone. If BON is suspected, dentists are encouraged to contact the FDA’s MedWatch program.</td>
</tr>
<tr>
<td><strong>Multidisciplinary expert panel recommendations (2006)</strong></td>
<td>ONJ may remain asymptomatic for many weeks or months and is usually identified by its unique clinical presentation of exposed bone in the oral cavity. These lesions typically become symptomatic when sites become secondarily infected or if there is trauma to adjacent and/or opposing healthy soft tissue from irregular surfaces of the exposed bone. Signs and symptoms of ONJ include localized pain, soft-tissue swelling and inflammation, loosening of previously stable teeth, drainage, and exposed bone. These symptoms most commonly occur at the site of previous tooth extraction or other dental surgical interventions but may occur spontaneously. Some patients may present with atypical complaints such as “numbness”, the feeling of a “heavy jaw”, and various dysesthesias. Objective signs</td>
</tr>
</tbody>
</table>

Adapted from American Association of Endodontics[62], J Am Dent Assoc[63], American Academy of Oral and Maxillofacial Surgeons[64], J Bone Miner Res[65], Aust Fam Phys[66], American College of Rheumatology[67], and J Oncol Pract[68]
B. Staging Criteria

The model for cancer tumor staging has been classically used as the staging criteria for BPONJ (Table 3.). The American Academy of Oral and Maxillofacial Surgery (AAOMS) has been the dominant figure to date in the development of a more comprehensive staging and grading system[69]. In order to better define the severity and assess the treatment responses, this new system is not solely based on physical examinations and symptoms but also incorporates the imaging results[61].

Table 3. Staging Criteria of BPONJ

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>At risk</td>
<td>Patients that have been treated with oral or intravenous BPs that show no apparent exposed/necrotic bone</td>
</tr>
<tr>
<td>Stage 1</td>
<td>Exposed/necrotic bone in patients who are asymptomatic and have no evidence of infection</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Exposed/necrotic bone associated with infection with pain and erythema in the region of exposed bone with or without purulence</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Exposed/necrotic bone in patients with infection, pain, and ≥ 1 of the following: pathologic fracture, extraoral fistula, or osteolysis extending to the inferior border</td>
</tr>
</tbody>
</table>

Adapted from American Academy of Oral Maxillofacial Surgeons[64]

C. Incidence

Many of the estimates on the prevalence and incidence of BPONJ are weak, arising primarily from anecdotal reports and single institutions’ case reports and series. These data are hindered by inconsistent definitions, as well as incomplete reporting. The risk of BPONJ in patients receiving high potency BPs has been estimated to be about 1-10 per 100 patients depending on the duration of treatment.
(i) Randomized Clinical Trials

In the United States, RCTs of clinical trials of bisphosphonates marketed for the treatment of osteoporosis reported no cases of BPONJ, out of >17,000 patient exposed to alendronate, >44,000 patient-years of exposure to risedronate, and >12,000 patients exposed to ibandronate[61].

(ii) Retrospective Chart Reviews

A large systemic chart review of 4,019 cancer patients receiving IV bisphosphonates revealed 29 cases of ONJ, giving an overall prevalence of 0.72%. The criteria to be considered ONJ used in this study was exposed non-healing bone with or without pain ≥3 months duration. Of the 29 cases, 13 patients had multiple myeloma (13 of 548; 2.4%) and 16 had breast cancer (1.2%)[70].

A similar retrospective study was performed by Murad and colleagues[71] with 1,951 patients who had received oral or IV bisphosphonates or had a listed diagnosis suggestive of ONJ. Two patients, both with multiple myeloma were identified with ONJ, who had initially been treated with pamidronate for several years before being treated with zoledronate. They identified dental extractions as the precipitating event in both cases.

A case control study performed by Bamias and colleagues[72] found that the incidence of ONJ increased with time exposed to intravenous BPs from 1.5% among cancer patients treated for 4-12 months to 7.7% for treatment for 37-48 months. BPONJ was most prevalent in the mandible (65.3%) than the maxilla (29.2%) and combination of maxilla and mandible (4.8%).

King et al[73] reviewed 44 published case reports and case series describing 481 patients with BPONJ. They concluded that BPONJ occurs more frequently in patients receiving
intravenous bisphosphonates (94.2%) than in patients taking oral bisphosphonates (5.8%). With the intravenous patients with BPONJ, 43% were taking Zoledronic acid, 29.1% were taking Pamidronate, with 26.7% taking a combination of Zoledronic acid and Pamidronate with the remainder taking other combinations. In those patients who developed BPONJ following oral BP exposure, 85.7% took Aledronate, while 10.7% took Risedronate.

Cancer patients had a higher incidence of BPONJ (93.8%) than non-cancer patients. Among cancer patients, multiple myeloma was the most common (52.3%), followed by breast cancer (33%), other malignancies (14.6%). Among the non-cancer patients, osteoporosis was the most common (4.8%), followed by Paget’s disease (0.8%) then osteopenia (0.6%) [73]. The most common inciting event of ONJ was investigated and tooth extraction or other surgical or invasive dental procedure was reported as the most common (68.8%), followed by spontaneous occurrences (20.7%), and (7.6%) had periodontal disease, (0.4%) had oral ulcers, with (0.2%) having prior ONJ, maxillofacial trauma, tooth abscess and new oral prosthesis [73].

Abu-Id et al. [74] recently reviewed 7500 patients from seven case series and found ONJ to occur in 2-11% of multiple myeloma patients, 1-7% of breast cancer patients and 6-15% of those with prostate cancer and suggest an overall prevalence of 5%. These results suggest that these three malignancies do not differ in the risk of ONJ.

Hoff et al. [75] also reviewed charts of 4000 cancer patients treated with bisphosphonates at MD Anderson Cancer Center and found that half of the patients had either breast cancer or multiple myeloma, of which 1.2% and 2.8%, respectively developed BPONJ.
Of the other half suffering from other malignancies, only two cases of BPONJ were observed, giving a prevalence of 0.09%.

Recently a systematic review by Khan et al.[76] showed that prospective data evaluating the incidence of BPONJ are very limited. In cancer patients receiving a high-dose intravenous BP, BPONJ is dependent on the dose and duration of therapy, where 36 months of exposure has an estimated incidence of 1-12%. BPONJ was found to be quite rare in osteoporosis patients, with an estimated incidence of <1 case per 100,000 person-years of exposure. They found that there was insufficient evidence to confirm a causal link between low-dose BP therapy in the osteoporosis patient population and BPONJ, where high-dose BP therapy, primarily in the oncology patient population, is associated with BPONJ.

**Prevention**

Due to the absence of any randomized controlled trials in the area of prevention and treatment of BPONJ, the recommendations regarding the prevention of BPONJ are based on the available literature. Currently a number of institutions have developed recommendations for the prevention of bisphosphonate-induced osteonecrosis of the jaw. Current guidelines recommend that prior to starting BP therapy, a comprehensive dental exam take place that allows optimization of dental health including tooth cleaning, tooth extractions and any invasive surgical procedures. If the patient requires invasive surgical procedures to be completed, it is recommended they wait until the wounds have healed, generally 2-3 weeks after tooth extraction in the same manner as for patients who receive radiotherapy to the head and neck region[6, 68, 77, 78].
Once bisphosphonate therapy has begun (oral or intravenous), patients should avoid any invasive dental procedures if possible. If the patient requires oral surgical intervention while on oral bisphosphonates, it is recommended that they use chlorhexidine 0.12% mouth rinse for 2 months after surgery[6, 77]. The American academy of Oral and Maxillofacial Surgeons suggests that discontinuing oral bisphosphonates for 3 months before and 3 months after invasive procedures (a “drug holiday”) may decrease the risk of developing ONJ[64]. The idea of a drug holiday is supported by a study which uses a breakdown product of bone resportion known as C-terminal telopeptide (CTX), as a surrogate marker of bone turnover[79]. This telopeptide fragment is cleaved from the main crosslink chains of collagen by the osteoclast during bone resorption. Its level in the blood is therefore proportional to the amount of osteoclastic resorption occurring at the time the blood is drawn. The authors suggest that CTX values less than 100 pg/mL represent a high risk of BPONJ, CTX values between 100 pg/mL and 150 represent a moderate risk, and CTX values above 150 pg/mL represent a minimal risk. Of clinical importance, this study showed evidence that a 6-month drug holiday from oral bisphosphonates had direct and significant improvements in the CTX values in every patient. This improvement correlated to either spontaneous resolution of the exposed bone, a significant improvement in the amount of exposed bone, or an uncomplicated healing response after surgery[79]. The results of this study are controversial to many who criticize that the lack of a control group render the results questionable, ie. patients taking BP that had not developed BPONJ were not included.
It is important to keep in mind that many of these recommendations are based on anecdotal evidence, not data from clinical trials, and physicians must consider the potential risks of discontinuing bisphosphonate therapy. During intravenous bisphosphonate therapy, the oral cavity must be frequently monitored for any signs of change. The optimal frequency for dental examinations for a patient treated with intravenous BPs without signs of osteonecrosis is between two to four times per year taking into account the dental hygiene of the patient[6, 77]. Migliorati et al suggest that a maxillofacial CT-scan in the follow-up of the malignant disease may be beneficial to provide additional information with regard to early development of subclinical ONJ lesions. However, the number of exams are not well defined[80].

E. Management

In the absence of a clear case definition of BPONJ, the development of guidelines to manage the condition is a considerable challenge. Several groups have developed guideline for recommendations. For example, the AAOMS Position Paper states that the management of BPONJ primarily depends on the staging categories of the disease[77], where the ASBMR task force recommendations provide general guidelines regarding management of infection, surgical management, and discontinuation of bisphosphonate therapy[65]. In a majority of the available guidelines[77],[65, 68, 81], pain control, treatment of infection and prevention of disease progression are the main goals of therapy where no one therapeutic modality has proven efficacy.
The treatment of BPONJ should initially include the treatment of infection. Once the infection has been managed, the clinician should then focus on the treatment of necrotic bone.

Treatment of an isolated infection consists first of antimicrobial testing to identify the microbes specific to the environment then the appropriate antibiotics (possibly clindamycin or amoxicillin in combination with metronidazole)[82]. It is also recommended to combine systemic antibiotics with a local antiseptic treatment such as chlorhexidine in combination with analgesics if necessary. If the infection has spread to the adjacent soft tissues, then drainage of the abscess must be completed[83].

As a majority of the patients who have BPONJ were initially being treated for an underlying malignant disease, one must take into account the life expectancy of patients when considering how to properly manage the condition. In patients with a short-life expectancy, supportive cares are recommended where is it suitable to leave the spontaneously evolved disease. In individuals with longer-life expectancies, a curative attitude may be more effective with the removal of exposed and surrounding bone[77, 83]. Biological factors such as fibrin or autologous platlet-rich plasma to cover the osseous surface may provide some benefit in wound healing and bone regeneration[84, 85]. Primary closure of the surgical site with tension-free sutures is also advisable to isolate the surgical site from the diseased environment[84].

In the posterior maxilla, most cases of BPONJ also present with an underlying sinusitis due to a communication between the oral environment and the maxillary sinus. Before considering closure of this communication, it is first advisable to resolve the sinusitis with antibiotics[83].
IV. NEUTROPHILS

1. THE LIFE OF THE NEUTROPHIL

A. Bone Marrow Origin of the Neutrophil

The bone marrow is the site of origin for neutrophils. Neutrophils originate from committed progenitor cells, which arise from pluripotential hematopoietic stem cells differentiating in response to specific growth factors. The hematopoietic stem cell upon replication, generates a new stem cell along with a committed progenitor[86]. Neutrophils delivered to the circulation are mature, and are around 12-15μm in diameter. They have a characteristic segmented nucleus with abundant cytoplasmic granules (commonly referred to as a polymorphonuclear neutrophilic granulocyte), while its immature myelocytic progenitor, known as the ‘band neutrophil’, possess a band-shaped nucleus with few cytoplasmic granules. Much of the machinery and molecules needed for neutrophil responses are prepackaged into the cells’ cytoplasmic granules or plasma membrane, and thereby is available for cellular functions when needed when the cells are initially isolated from blood[86].

For the purposes of having only functionally capable cells present at the front line of an infection, only functional mature neutrophils are released from the bone marrow into the blood stream. In healthy individuals, the levels of blood neutrophils remains fairly constant at approximately 10^9 cells/L blood[87]. The lifespan of the neutrophil in the circulation is brief, displaying a half-life of approximately 6 hours. During the onset of infection, the levels of blood PMNs rise quickly, in response to endotoxins released by
gram-negative bacteria, and the neutrophils adhere to the endothelial cells and migrate into infected tissues where the neutrophils survive for only 1-2 days.

**B. The Fate of Circulating Neutrophils**

At the sites of inflammation in the systemic circulation, post-capillary and collecting venules are the principal sites for neutrophil adhesion and emigration. Once neutrophil have left the blood circulation, they will never return. Neutrophils leave the circulating blood stream by first tethering to and then rolling on the inflamed endothelium lining the blood vessel lumen. Adhesion of neutrophils to the venule endothelium occurs normally in the presence of fluid shear rates ranging from 150 to 1600 per second[86].

**i) Leukocyte rolling**

_The Role of Selectins in neutrophil rolling_

All the known members of the selectin family mediate neutrophil rolling on venule endothelium[88-96]. The selectin family has three members, L-selectin (CD62L), P-selectin (CD62P), and E-selectin (CD62E). L-selectins are expressed on the surface of neutrophils, lymphocytes, monocytes and eosinophils[97]. Their localization to the surface projections (ruffles), allows for close positioning to facilitate interactions with the inflamed endothelial surface[98, 99]. Shedding of L-selectins occurs rapidly (minutes) following neutrophil activation. P-selectin are expressed on the surface of activated platelets and endothelial cells and translocate to the cell surface following stimulation with thrombin, LTC₄, histamine calcium ionophore A23187, complement proteins C5b-9 or phorbol esters. E-selectins on the other hand, are only expressed on the surface of activated endothelial cells and a wide range of inflammatory mediators induce E-selectin expression, including IL-1β, TNFα, bacterial endotoxin and substance P [100, 101]. With
the development of mice deficient in one or more selectins, it is now apparent that each 
of the selectins may mediate leukocyte rolling at different velocities which is also 
dependent on the dose, type and timing of inflammatory stimuli [86, 102-104]. 
Selectins are constitutively active when expressed and bind with rapid rates of association 
and dissociation that allow rolling in response to high forces of flowing blood.

ii) Leukocyte Arrest and Activation

The Role of Integrins

The function of integrins is to arrest rolling cells on the inflamed endothelial surface. 
Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-
substrate interactions[105]. They are intimately involved in the regulation of a variety of 
cellular functions including embryonic development, metastasis, programmed cell death, 
hemostasis and leukocyte homing and activation. Each integrin is composed of an α and 
β subunit that associate to form a non-covalently bound heterodimer. The β2 family of 
integrins is exclusively expressed on leukocytes. The involvement of the leukocyte 
integrin family β2 (CD18), in contrast to selectins, exhibit low binding avidity unless 
activated[86, 106-109]. Endothelial ICAM-1 (CD54) is the principal ligand for the β2 
integrins, LFA-1 (CD11a/CD18) and Mac-1 (cd11b/CD18). While many vessels 
constitutively express ICAM-1, its expression is greatly increased following stimulation 
with inflammatory mediators (e.g. IL-1β, TNFα, INFγ and endotoxin). β2 integrin 
adhesion function can also be strengthened with cross-linking L-selectin [110-112] and 
the leukocyte receptor(s) for E-selectin[113].
Chemokines on the surface of endothelial cells are thought to trigger the rapid transition of neutrophil rolling to stationary adhesion[108, 114]. The binding of integrins to immunoglobulin superfamily members, such as ICAM1 and VCAM1, expressed by endothelial cells during the rolling process, results in rapid increases in chemokines and other chemoattractants. Modulation of the affinity of β2 integrin for its ligand is well documented as a crucial step in chemokine-induced arrest under static conditions[115], however the evidence documenting the role of surface bound chemokines in the transition of rolling to stationary adhesion to endothelium under flow conditions in neutrophils is less convincing[108].

Neutrophils have numerous ligands for selectins on their surface[116, 117] which may not only be important in the tethering and rolling function but may also allow transduction of signals for neutrophil activation[118]. For example, transient increases in Ca^{2+} flux, the production of reactive oxygen species, IL-8 and TNFα have been observed following L-selectin crosslinking [119, 120].

**iii) Transendothelial Migration**

A short time following arrest on the endothelial cells, most neutrophils begin to crawl, searching for a gateway to cross the endothelium[121]. Transendothelial migration, also referred to as diapedesis, is the step of leukocyte recruitment in which leukocytes move across the endothelial cell layer toward a site of inflammation. While the preceding steps (e.g. tethering, rolling, and firm adhesion) are reversible, diapedesis is the “point of no return” for a leukocyte. While the events leading up to transendothelial migration are well characterized in neutrophils, for instance, the selectin-mediated rolling and integrin-mediated firm adhesion, controversy exists in the mode by which neutrophils migrate
across the endothelium[122, 123]. Currently, two models for neutrophil transendothelial migration have been proposed: (1) Paracellular migration and (2) Transcellular migration

*Paracellular Migration:* It is widely believed for years and popularized in review articles that neutrophil migration across the endothelium is primarily paracellular with leukocytes squeezing through and between adjacent endothelial cells[124, 125]. This involves penetration (distruption) of the intercellular junctions (zonula occludens or tight junctions; zona adherens or adherens junctions). Within the cleft, the neutrophil is thought to interact with endothelial gap junctions and resident adhesion molecules (PECAM-1 (CD31), CD99, JAMs) [126]. This model is supported by previous electron microscopic observations of neutrophil transmigration in *in vivo* models of inflammation.

*Transcellular Migration:* By this pathway, neutrophils migrate through an individual endothelial cell. More recently, Feng and colleagues [127] showed that neutrophils migrate through the endothelium of inflamed venules via a transcytotic pathway *in vivo*. In particular, the activation state of endothelial cells, and the ICAM-1 expression level appears to influence whether migrating leukocytes take the paracellular or the transcellular pathway. Yang et al.[128] reported that human neutrophils preferentially utilize the paracellular pathway to cross a monolayers of cultured human endothelial cells, when endothelial cells are stimulated *in vitro* for 4 h with tumor necrosis factor-α[128]. However, 24 h stimulus with this cytokine, which strongly up-regulates ICAM-1 expression, shifted the ratio of transmigrating neutrophils towards the transcellular pathway, with up to 20% of the cells taking the non-junctional route[128].
C. The Role of the Cytoskeleton

The driving machinery of neutrophils is the classical and ubiquitous contractile protein actin. In the resting spherical cell, the actin exists in two forms, globular monomeric (g-actin) and polymerized, filamentous actin (F-actin), with the polymerized form primarily located at the cell periphery[129]. Upon stimulation, nearly all of the g-actin rapidly polymerizes, and as the cell migrates forward, a seemingly constant amount of polymerized actin (F-actin) remains towards the front leading edge[129]. The filamentous actin is then crosslinked into bundles and networks by actin binding proteins. This allows the cell to tailor the dimensions and mechanical properties of the bundles to suit specific biological functions[130, 131]. Examples of actins binding proteins include filamins, α-actinin, fibrins, and spectrins.

D. Cell Morphology changes

Neutrophils are spherical in shape when in suspension, but when they are allowed to adhere to a surface, the cytosol spreads from the contact site in all directions with no obvious polarity. The presence of a chemical stimuli results in the neutrophil rapidly organizing itself into a clearly defined polarized structure which is essential for cell motility. The polarized structure consists of a leading edge “lamellipodium”, and a trailing end also known as the uropod. The lamellipodium present at the leading edge of the mobile cell is made up of an actin mesh which is believed to be the actual motor that pulls the cell forward during migration[132]. Within the lamellipodium are small, finger-like projections of actin filaments which, when spread beyond the lamellipodium frontier are called filopodia[132]. Filopodia are thought to be responsible for probing the extracellular milieu and detecting the chemical gradient[133]. On the other end of a
polarized neutrophil, myosin-based contraction and retraction of the uropod facilitates substrate de-adhesion, resulting in forward propulsion.

E. Chemotaxis

Neutrophil chemotaxis involves a complex array of individual components, leading to an organized development and dissolution of actin filaments and focal adhesions along a highly structured cytoskeletal network, as well as contractile processes that operate in a coordinated manner along the length of the cell.

i) The Rho Family of Small GTPases:

The Rho family of small GTPases have been shown to play pivotal roles in regulating the biochemical pathways involved in cell migration [134]. They constitute a family of intracellular messengers that are regulated both by their location and state of activation[135].

Rho-related small GTPases, including Rho, Rac, and Cdc42, play key roles in regulating cell shape, motility, and adhesion through the reorganization of the actin cytoskeleton[136]. Small GTPases are monomeric guanine nucleotide-binding proteins (molecular masses=20-25 kDa) that serve as molecular switches to regulate growth, morphogenesis, and cell motility. The Rho family of small GTPases is of special interest because they transduce extracellular stimuli into intracellular signals. Small GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states. Following stimulation, GTPases release GDP and bind to GTP, a reaction mediated by guanine nucleotide exchange factors (GEFs). In their active GTP-bound state, Rho GTPases interact with a variety of effector proteins to promote cellular responses. The active state of GTPases is transient because of their intrinsic GTPase activity, which is stimulated
further by GTPase activating proteins (GAPs). Guanine nucleotide dissociation inhibitors (GDIs) are thought to block the GTPase cycle by sequestering and solubilizing the GDP-bound form [137][138]. Upon activation, the RhoGTPases become prenylated allowing them to translocate and anchor to the plasma membrane where they can then interact with cellular target proteins (effectors) to generate a downstream response[139]. The three main RhoGTPases are Rac (Rac1 and Rac2), Cdc42, and RhoA. In general, Rac localizes to the leading edge of a migrating cell and its primary role is to generate a protrusive force through the localized polymerization of actin to generate lamellipodia. Specifically, it has been shown that the two Rac isoforms, Rac1 and Rac2, play distinct roles in neutrophils during immune cell function, including cell migration and bacterial killing[140, 141]. While Rac1 has been shown to be essential for neutrophil chemoattractant gradient detection and orientation toward the chemoattractant source, Rac2 is the primary regulator of actin assembly providing the molecular motor for neutrophil translocation during chemotaxis[141].

Cdc42 also induces actin polymerization to generate filopodia observed at the leading edge, but also plays a crucial role at the front of cells in controlling the direction of migration. RhoA on the other hand, localizes to the rear of the migrating cell and is associated with regulating the assembly of contractile, actin:myosin filaments necessary for cell body contraction and rear end retraction [26, 133, 136, 142]. Similar to other motile cells, dynamic reorganization of the neutrophils actin cytoskeleton allows for changes in cell shape and also generates the forces necessary for cell locomotion.
**F. Neutrophil chemoattractants**

Neutrophils respond to a variety of soluble stimuli including those that act on seven transmembrane-spanning domain receptors (f-met-leu-phe, C5a, IL-8, Substance P, PAF) and those that are receptor independent (the phobol esters, PMA)[143]. These agents increase random neutrophil motility when in the absence of a chemoattractant gradient [144](chemokinesis) and also direct the migration of the neutrophil towards the source in the presence of a chemoattractant gradient (chemotaxis). The majority of neutrophil chemotaxis studies use fMLP as the stimulus. Unlike mammalian protein synthesis in which methionine is the “starter” amino acid, in bacteria the methionine is formylated[145]. This difference allows neutrophils to detect bacteria at a distance[146]. This mechanism may be the most important in the body, since it does not rely on the cooperation of other cells or systems. All of the agonists stimulate, as an early event, the hydrolysis of PIP2 with the consequent generation of IP3 leading to Ca$^{2+}$ mobilization[147]. Another response to a chemotactic agonist is activation of tyrosine kinases, resulting in the tyrosine phosphorylation of multiple intracellular substrates[148-150].

**G. Phagocytosis**

Following extravasation from the circulation and chemotaxis to the site of infection, the neutrophil arrives at the location and encounters the invading microbe. The process of phagocytosis may be divided into recognition and attachment of a particle to the surface of the phagocyte and internalization of the bound particle[151-153]. In contrast to attachment, engulfment is a process that requires energy from glycolysis. Physiologically, the particle to be internalized will be opsonized, meaning that it will be coated with a
molecule for which the neutrophil has receptors[151, 152]. There are essentially two types of opsonization, either with the complement fragment C3bi or with antibodies specific for antigens on the particle surface, thus displaying the Fc region of the antibody to the neutrophil. Interaction between the opsonic ligands and their receptors on the neutrophil surface induces a circumferential flow of phagocyte membrane which gradually surrounds the microorganism to be ingested. The elaborated pseudopod meet in a single point, and fusion of opposing membranes at this point pinches off the resulting phagosome into the cytoplasm [153, 154].

**H. Neutrophil Respiratory Burst Oxidase**

The normal function of neutrophils is to protect the individual from infection by seeking out and destroying invading microbes. These cells possess a membrane-bound enzyme system which can be activated to produce toxic oxygen radicals in response to a wide variety of stimuli[153]. The enzyme responsible for the respiratory burst in neutrophils is the membrane bound multi-component NADPH-oxidase, which consists of a membrane-spanning electron transport chain, with cytosolic NADPH as the electron donor and oxygen as the electron acceptor[155]. In unstimulated resting neutrophils, this NADPH-oxidase system is normally dormant. The activation of this system can be triggered by phagocytosis or by a number of other soluble physiological or experimental agents such as the complement fragment, C5a, fMLP, platelet activating factor and PMA[156]. Once this system is triggered, the enzyme system is rapidly activated in the walls of the phagocytic vacuole, where electrons are shuttled from cytosolic NADPH to oxygen present in the
extracellular fluid. Under normal circumstances, one single electron is donated to molecule of oxygen. This results in the generation of one molecule of superoxide (O$_2^-$). The overall reaction catalyzed by the oxidase is:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

The 2 molecules of superoxide (O$_2^-$) then interact either spontaneously (dismutase reaction) or enzymatically to form one molecule of hydrogen peroxide (H$_2$O$_2$):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Further reactions of O$_2^-$ and H$_2$O$_2$ are possible and in the presence of transition metals such as iron and copper salts a complex series of redox reactions is seen to occur with the generation of other more powerful reactive oxidant species such as the hydroxyl radical (OH$^\cdot$)[157, 158].

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2$$

The bulk of the O$_2^-$ generated by triggering the NADPH-oxidase system dismutates to H$_2$O$_2$.

The heme protein myeloperoxidase is found in the azurophilic granules of neutrophils. This enzyme can use Cl$^-$, Br$^-$, or I$^-$(as a reducing substrate) to greatly potentiate H$_2$O$_2$ toxicity by converting it into hypocholorous acid (HOCl)[159]. HOCl is the major hypohalous acid produced during neutrophil oxidase activation because the plasma concentration of Cl$^-$ is more than a thousand times that of other halides[143]. HOCl is an extremely potent cytotoxin for bacteria, viruses, fungi, mycoplasmas and cultured mammalian cells[152].
I. Oral Neutrophils as a Diagnostic Test

Recent research from our lab and others suggest that the mouth provides an opportunity to monitor the innate immune system non-invasively through the use of an oral rinse that measures oral neutrophil levels[160-162]. The underlying principle of this test is that for normal innate immunity, neutrophils must be able to enter the bacteria-rich oral cavity in sufficient numbers to maintain health. Any defects in this normal recruitment can be monitored using a standardized rinse test to quantify oral neutrophil levels much like a standard complete blood count. During infections or following surgery neutrophil levels spike indicating an active immune and healing response. In addition to using an oral rinse to monitor periodontal disease [163] we have also used an oral rinse assay to monitor susceptibility to infection and acute neutropenia (low blood neutrophil counts) during hematopoietic stem cell transplantation. We have confirmed that oral neutrophil levels can be used to monitor and report on the state of the innate immune system in pediatric and adult patients with successful bone marrow transplants [161, 162, 164].
Hypothesis and Objectives of Thesis

Bisphosphonate-associated osteonecrosis of the jaw (BPONJ) has been identified as a severe complication of dental treatment and oral trauma in 1-10% of patients previously treated with intravenous bisphosphonates[165]. It is believed that in the subset of susceptible patients, bisphosphonates perturb normal osseous wound healing through inhibition of osteoclast formation and function. However, it has been noted that necrotic bone from ONJ sites also display signs of bacterial infection that suggest to us that an immune defect may play a role in BPONJ pathophysiology in affected patients [166]. This thesis focuses on the possible role of neutrophils which are critical elements of normal wound healing, particularly in the oral cavity where bacterial biofilms have such a strong impact on oral health and disease. This thesis determined in a novel fashion whether bisphosphonates alter neutrophil function and if this defect could explain the development of BPONJ, and the physiological mechanisms through which bisphosphonates mediate BPONJ in susceptible patients. This thesis tested the novel hypothesis that bisphosphonates dampen neutrophil functions in an animal and human model and may result in poor innate immunity and an increased risk of BPONJ following oral surgery or trauma. The specific objectives of this thesis were:

Objective 1). Determine if bisphosphonates cause functional defects in neutrophils

i) Analyze neutrophil functions in mice treated with the bisphosphonate zoledronate. Neutrophils will be isolated from mice treated one month previously with a single dose of zoledronate. Using standard neutrophil function assays for chemotaxis
and NADPH oxidase function, we will characterize the effect of previous BP therapy on neutrophil functions in vitro. The neutrophil function results from the in vitro assays will be confirmed in vivo using standard murine acute models that measure neutrophil influx into inflamed/infected sites and neutrophil mediated bacterial killing efficiency in those sites.

ii) Analysis of neutrophil functions from patients being treated with bisphosphonates. In order to determine if neutrophil functions are impaired by BP therapy, blood neutrophils will be isolated from patients diagnosed with BPONJ. In vitro functional tests (as in i, above) will be used to monitor and compare neutrophil functionality during the high dose BP therapy.

iii) Analysis of neutrophil delivery to the oral cavity in patients diagnosed with BPONJ

In order to determine if neutrophil delivery in the mouth is impaired in patients diagnosed with BPONJ, oral rinses will be collected from these patients and compared to healthy controls.
CHAPTER 2

MATERIALS AND METHODS
2.1 Antibodies and reagents

A mouse monoclonal antibody against Rho A (sc-418) and a mouse monoclonal antibody against Cdc42 (B-8) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody against Rac was purchased from Upstate Cell Signaling Solutions (Charlottesville, VA). The QIAamp DNA Blood Extraction mini Kit (Cat.no.51104) was purchased from QIAGEN (Santa Clarita, CA). N-formyl-methyonyl-leucyl-phenylalanine (fMLP) and Percoll were purchased from Sigma (St Louis, MO). Glutathione-Sepharose beads, horseradish peroxidase-coupled anti-mouse antibody and horseradish peroxidase-coupled anti-Rabbit antibody were purchased from Amersham Pharmacia Biotech UK (Buckinghamshire, England). 50X protease inhibitor cocktail was purchased from BD PharMingen.

2.2 Animals

All experiments were performed with 3-month old mice bred at the University of Toronto Animal Care Facility. All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and were approved by the University of Toronto Animal Care Committee.

2.3 in vivo zoledronate treatment

Using a modification of a previously described rat protocol [168, 169], 3 month old mice were treated with 2 or 20µg of Zoledronate or vehicle alone. The dosing was based on the recommended human therapeutic dose of 4mg every 4 weeks. Thus the dose on a µg ZOL per gram BW basis (assuming an average weight of 60Kg) is 4,000µg ZOL divided
by 60,000 g BW = 0.06667 µg/g. For a 30g mouse then the equivalent dose would be 2 µg/mouse. The higher dose was used to model longer durations of treatment.

2.4 Isolation of murine bone marrow neutrophils
As described previously [170], mouse neutrophils were isolated from the bone marrow of mice which is the primary neutrophil storage site in the mouse. Briefly, Zoledronate treated or the vehicle control treated mice were euthanized by cervical dislocation. The femur and tibia were dissected, cut proximally and distally, and flushed with ice-cold 1x HBSS (supplemented with 10 mM HEPES, pH 7.5), and the eluant was centrifuged at 2,600 rpm for 30 min at 4 °C, and layered over a three-layer Percoll gradient (52, 65, and 85%). The neutrophil-rich fraction was then collected at the interface of the 65 and 85% layers, resuspended in 1x HBSS, and centrifuged at 1,500 rpm for 5 min at 4 °C. Pelleted cells were resuspended in 1x HBSS containing 2.5% heat-inactivated fetal bovine serum and used immediately. The murine neutrophil isolation protocol routinely yields cell suspensions that are >90% neutrophils with >98% viability as judged by Wright stain and trypan blue exclusion, respectively. All of the neutrophil studies are carried out at 37 °C.

2.5 Measurement of NADPH oxidase activity
Neutrophil associated oxidant content was measured using dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR). Isolated bone marrow neutrophils were incubated in the presence of 1 mM DHR for 15 min, followed by incubation with $10^{-5}$ M PMA and $10^{-6}$ M fMLP. Cell-associated fluorescence was then measured 10 min later using a flowcytometer [171].
2.6 Zigmond chamber chemotaxis

To assess activation and determine whether there was a chemotactic defect, isolated bone marrow neutrophils were suspended in HBSS and 1% gelatin. A neutrophil suspension (1 x 10⁶/mL) was allowed to attach to bovine serum albumin (BSA)–coated glass coverslips (22 x 40 mm) at 37°C for 20 minutes. The coverslip was inverted onto a Zigmond chamber, and 100 µL HBSS media was added to the left chamber with 100 µL HBSS media containing fMLP[10⁻⁶] added to the right chamber. Time-lapse video microscopy was used to examine neutrophil movements in the Zigmond chamber. The images were captured at 20-second intervals with Nikon Eclipse E1000 Microscope. Cell-tracking software (Retrac version 2.1.01 Freeware) was used to characterize cellular chemotaxis from the captured images.

2.7 Sodium periodate peritonitis

To induce an experimental peritonitis, 1 mL of 5 mM sodium periodate (Sigma, Oakville, ON, Canada) in phosphate-buffered saline (PBS) was injected intraperitoneally. The mice are then euthanized 3 hours later and the peritoneal exudate is collected by lavage with chilled PBS (5 mL/mouse). Neutrophils were counted by a hemocytometer and an electronic cell counter (Becton Dickinson). The neutrophil numbers between the BP treated mice and the vehicle controls are compared to their untreated (no peritonitis) controls.

2.8 Circulating neutrophil levels

Prior to euthanasia, 200 µl of blood was taken from the great saphenous leg vein of the mouse. A complete blood count was measured using a hemavet (Hemavet®950, Drew Scientific, Oxford, CT).
2.9 GST-Pull down Assay

The Pak-binding domain (PBD) and Rhotekin-binding domain (RBD) assays were carried out as described previously[172]. Briefly, isolated bone marrow neutrophils were exposed to Zoledronate (20 μM) for 15 minutes, followed by a one minute exposure to fMLP(10^{-6}M) at 37°C. They were immediately lysed with and glutathione-S-transferase (GST)–PBD or GST-RBD beads (Amersham Pharmacia Biotech Buckinghamshire, England) were added to the lysates. The GST-PBD or GST-RBD were recovered with Sepharose beads and immunoblotting was completed using either a Cdc42 antibody (B-8; Santa Cruz Biotechnology), Rac antibody (Upstate Cell Signaling Solutions (Charlottesville, VA), or a RhoA antibody (sc-418; Santa Cruz Biotechnology). Image J software version 1.31v (National Institutes of Health) was used for blot densitometry. Data were normalized to resting control neutrophils and are expressed as the mean value from 3 separate experiments.

2.10 Apoptosis Assay

The percentage of apoptotic neutrophils was determined by Annexin-V staining using the Annexin V-FITC Apoptosis Detection Kit from BioVision according to the manufacturer's protocol. Stained cells were analyzed by FACS and cells positive for both annexin-V and propidium iodide (PI) or annexin-V alone were considered apoptotic.

2.11 In vitro generation of neutrophils

Hematopoietic progenitors were isolated from bone marrow using the EasySep™ Hematopoietic Progenitor Enrichment kit from Stemcell technologies (Vancouver,
Stem cells were subsequently differentiated into neutrophils as described previously [173]. In brief, progenitors were cultured for 3 days in IMDM (Gibco), 10% fetal calf serum (FCS), 50 uM beta-mercaptoethanol, 10 u/ml penicillin/streptomycin, 2 mM glutamine, SCF, 50 ng/ml), FLT-3 (50ng/ml), GM-CSF (0.1 nM), IL-3 (0.1 nM) and G-CSF (30 ng/ml). After this initial period the medium was replaced every 3 days with medium containing only G-CSF (30ng/ml). Cells were harvested between day 9 and 12 (as mentioned in the results section).

2.12 BPONJ patient selection

Five patients diagnosed with bisphosphonate-induced osteonecrosis of the jaw and three patients diagnosed with osteoradionecrosis were recruited from the dental clinic at Sunnybrook Health Sciences Centre in Toronto, Canada by dental staff practitioners. The patients diagnosed with BPONJ met the criteria for diagnosis set by the AAOMS[77]. This study was run in accordance with the institutions Research Ethics Board-approved protocols and after informed consent had been obtained.

2.13 Patient Blood Neutrophil Isolation and chemotaxis

A 10-ml blood sample was drawn into an EDTA-containing vacutainer. Neutrophils were isolated using a one-step neutrophil isolation solution. Briefly, 4 ml of the blood was layered carefully over 4 ml of Optiprep (Sigma, Oakville CA), and the tube was centrifuged at 1,600 revolutions per minute (rpm) for 30 minutes at 4C. The neutrophils were harvested from the lower of two bands and washed by centrifugation with Hanks balanced salt solution at 2,500 rpm for 5 minutes. Any remaining red blood cells were lysed with 1 ml distilled water, and a neutrophil-rich pellet is obtained by centrifugation at 2,500 rpm for 5
minutes. The cells were resuspended in 1 ml Hanks balanced salt solution. A cell count was obtained with a hemocytometer. This method yields neutrophils with >95% cell viability as determined by trypan blue staining.

To assess activation and determine whether there was a chemotactic defect, isolated neutrophils were placed into the upper well of a transwell chamber which is separated from the bottom chamber, containing vehicle alone or $10^{-6}$ M fMLP (Sigma) and a glass cover slip, by a membrane with 3 micron pores (COSTAR). The transwell plates were placed at 37°C for 1 hour and cells migrating through the membrane and onto the bottom cover slip were washed once and quantified. Chemotaxis was assayed by counting the mean number of cell per field over 6 fields [174].

### 2.14 Patient Oral Rinse Samples

The neutrophils from the oral cavity were collected following a one-minute oral rinse with 5-ml of a sterile bicarbonate solution. One rinse was collected from each patient in a sealable falcon tube and immediately transported to the Glogauer laboratory at the University of Toronto for analysis. Neutrophils in each oral rinse sample was quantified using the acridine orange staining technique [162]. This technique gives a very characteristic fluorescent bi-lobed nuclear staining pattern in neutrophils facilitating easy identification and counting on a hemocytometer chamber.

### 2.15 Statistics

Normally distributed data were analyzed by unpaired Student’s $t$ tests. Analysis of variance (ANOVA) with Bonferroni correction was used for multiple comparisons. A $P$ value of $<0.05$ was considered to be significant. Data are expressed as mean ± SD unless indicated otherwise.
CHAPTER 3

RESULTS
RESULTS

Although bacterial colonization is a common finding in patients with BPONJ, it remains unclear whether infection plays a primary causative or is secondary to the appearance of the lesion. We chose to test the hypothesis that zoledronate exposure leads to impaired neutrophil function in an in vitro and in vivo murine model.

3.1 Effect of Zoledronate on in vitro Neutrophil Function

Isolated murine neutrophils exposed to increasing concentrations of zoledronate for 15 minutes showed a dose dependent decrease in neutrophil function (p<0.05). Both in vitro chemotaxis speed (Figure 1A) as well as in vitro NADPH oxidase activity (Figure 1B) were diminished with increasing concentrations of zoledronate (p<0.01), suggesting that a brief direct exposure to the drug may not only detrimentally effect the neutrophils ability to migrate to the site of infection but also diminish its ability to destroy the invader at the site.

3.2 Effect of Zoledronate on in vivo Neutrophil Function

To determine if in vivo zoledronate treatment has any residual effects of neutrophil function, mice were treated 4 weeks prior to experimentation with 0, 2 or 20µg of Zoledronate. While a dose dependent relation was not observed with systemic treatment of zoledronate, an obvious decrease is chemotaxis speed was observed in mice treated previously with zoledronate at both 2 and 20µg (p<0.05). Both sodium periodate-induced in vivo neutrophil recruitment (Figure 2A) as well as fMLP-mediated in vitro chemotaxis (Figure 2B) show decreases in neutrophil chemotaxis speed (p<0.05).
3.3 RhoGTPase Assay

As bisphosphonates have been well documented to inhibit a crucial step in RhoGTPases prenylation, we hypothesized that zoledronate treatment may affect the function of these key signaling proteins involved in neutrophil function. As indicated in Figure 3, while fMLP activation of Rac1, and Cdc42 was normal in cells pretreated with 20 micromolar zoledronate for 15 minutes in vitro, RhoA activity downstream of fMLP was depressed by almost 50% at the same 20 micromolar zoledronate dose (P<0.05). This data confirms that this bisphosphonate can affect a key regulator of neutrophil functions.

3.4 Effect of Zoledronate on Circulating Neutrophil Counts

While abnormalities in bacterial colonization and impaired wound healing observed in patients with BPONJ may be related to neutrophil functional defects, one must also consider a possible defect in circulating neutrophil numbers. Neutropenia was observed in mice 2-weeks following a 2 micromolar pretreatment with zoledronate (P<0.05) (Figure 4). This finding led us to consider the possibility that zoledronate may decrease neutrophil survival and/or neutrophil differentiation in the bone marrow.

3.5 Effect of Zoledronate on Neutrophil Differentiation and Survival

In order to verify that zoledronate could impact neutrophil differentiation in the bone marrow we used a standard in vitro neutrophil differentiation assay to monitor neutrophil differentiation in the presence of this commonly used bisphosphonate. Control stem cells were differentiated into neutrophils for 12-14 days in the presence of G-CSF.
During this differentiation period, cells were treated with varying concentrations of zoledronate. After 12 and 14 days of differentiation, cells were analyzed by Diff-Quick staining. Figures 5A illustrates a dose dependent decrease in viable mature neutrophils (dark blue nucleus) with increasing zoledronate concentration, suggesting that zoledronate exposure leads to increased cell death during neutrophil differentiation in vitro (p<0.05).

We were also interested in determining if ZOL’s effect on neutrophil survival during differentiation is irreversible in nature. Stem cells from mice pre-treated 4 weeks prior with ZOL were isolated in vitro and differentiated into neutrophils for 10-12 days in the absence of ZOL. Figure 5C illustrates that stem cells from zoledronate-pretreated mice (‘Z-M’) are able to differentiate normally in vitro with similar survival rates as control cells (“C-M”) if zoledronate is absent during differentiation. However, the addition of zoledronate to control stem cells during in vitro differentiation increases the number of apoptotic events (Figure 5C; “C-M+ZOL”) (p<0.05). These results suggest that ZOL must be present during neutrophil differentiation to affect mature neutrophil survival.

We also determined that a key growth factor, G-CSF must be present in the immediate environment in order for us to observe the negative affects ZOL has on mature neutrophil survival. When mature neutrophils were isolated from ZOL pre-treated mice and left to undergo physiologic apoptosis, there was no difference in levels of apoptosis compared with control cells (Figure 5D). However, when G-CSF was added to the media, increased levels of neutrophils apoptosis was observed (Figure 5E).
3.6 Blood neutrophil chemotaxis in BPONJ patients

In order to determine if a similar neutrophil chemotaxis defect is observed in a human disease model, blood samples were taken from five patients diagnosed with BPONJ, three patients with diagnosed ORN and three control patients. Neutrophils were isolated and their ability to migrate through a porous membrane was measured using fMLP as a chemoattractant in a Boyden chamber set-up. Significantly fewer numbers of neutrophil from BPONJ patients were observed to migrate toward the fMLP stimulus compared with control neutrophils and ORN neutrophils (p<0.05) (Figure 6A).

3.7 Oral neutrophil recruitment in BPONJ patients

To determine if the in vitro defects manifest as reduced neutrophil function in the oral cavity in these patients, we used an oral rinse protocol to quantify neutrophil delivery to the oral cavity. Figure 6B illustrates a trend toward increased neutrophil delivery to the oral cavity in patients with BPONJ as well as ORN, however significance was not achieved for either group (p>0.05).
In order to determine if Zoledronate had a direct effect on neutrophil functions, mature neutrophils were isolated from mouse bone marrow as described previously [141]. Neutrophils were treated for 15 minutes at the indicated concentration of Zoledronate followed by the assessment of either A) fMLP mediated chemotaxis in a Zigmond chamber or B) fMLP mediated NADPH oxidase activity. As indicated in A and B, zoledronate inhibits both fMLP mediated chemotaxis in a dose dependent manner and NADPH oxidase activity. [All experiments were repeated 3 times with each experiment using 3 mice per group. Errors are standard deviation and all points are significantly different from control p<0.01].

A) In order to determine if in vivo Zoledronate treatment had a residual effect on in vitro neutrophil functions, mature neutrophils were isolated from bone marrow of 4 month old mice treated 30 days previously with 0, 2 or 20 micrograms of Zoledronate [168, 169]. fMLP mediated neutrophil chemotaxis was assessed in a Zigmond chamber [167]. As indicated in A) zoledronate given 30 days prior has a residual inhibitory effect on neutrophil chemotaxis. B) In order to verify these results, the same mouse treatment and Zoledronate dosage protocol was used to test in vivo neutrophil recruitment to sites of inflammation. Briefly a peritonitis model was used in which an irritant (sodium periodate) is injected into the peritoneum and 3 hours later the cellular infiltrate is collected and quantified [141, 175]. In this model greater than 95% of cells collected are neutrophils. As indicated in B, mice previously treated with Zoledronate display a clear reduction in neutrophil recruitment to the inflamed peritoneum. [All experiments were repeated 3 times with each experiment using 3 mice per group. Errors are standard deviation and all points are significantly different from control p<0.01].
Figure 3: Possible mechanisms through which ZOL affects neutrophil functions: In order to verify that Zoledronate could affect key signaling proteins involved in neutrophil functions we measured chemoattractant mediated Rho small GTPase activity in neutrophils treated with Zoledronate in vitro. As indicated in this figure, while fMLP activation of Rac1, and Cdc42 was normal in cells pretreated with 20 micromolar Zoledronate for 15 minutes, Rho activity downstream of fMLP was depressed by almost 50% at the same 20 micromolar Zoledronate dose. This data confirms that this bisphosphonate can affect a key regulator of neutrophil functions. Errors are standard deviation and * is different from control p<0.01.

Figure 4: Effect of zoledronate on circulating neutrophil levels: In order to determine if Zoledronate had a direct effect on neutrophil numbers, circulating blood was collected from 4 months old mice that had been pre-treated with a 2mg dose of zoledronate 2 weeks prior and compared with controls. A significant decrease in circulating neutrophils is observed following zoledronate treatment, suggesting that zoledronate may be either causing early apoptosis of cells or is negatively affecting the production of neutrophils. [Experiments were repeated 2 times with each experiment using 5 mice per group. Errors are standard deviation and ( *) is significance at p<0.05].
Figure 5: Effect of Zoledronate on differentiation and apoptosis of mature neutrophils - In order to determine if zoledronate treatment has an effect on neutrophil differentiation and apoptosis, we quantified the numbers of mature cells that had been derived from either (A-C) in vitro neutrophil differentiated precursors cells or (D, E) mature neutrophils extracted from bone marrow. (A) In order to determine if Zoledronate could impact neutrophil differentiation in the bone marrow we used a standard in vitro neutrophil differentiation assay to monitor neutrophil differentiation in the presence of this commonly used bisphosphonate. Bone marrow cells were harvested from 3 control mice and hematopoietic progenitors were isolated using a negative selection kit from StemCell Technologies. Progenitors were cultured for 3 days in IMDM medium containing 10% FSC, 50 μM β-mercaptoethanol, SCF (50 ng/ml), FLT-3 (50 ng/ml), IL-3 (10 ng/ml), GM-CSF (1 ng/ml) G-CSF and (30 ng/ml). The medium was replaced every three days with IMDM medium containing 10% FSC, 50 μM β-mercaptoethanol, G-CSF (30 ng/ml) and Zoledronate (1, 10, 50 and 200 μM). After 12 and 14 days the differentiation of neutrophils was assessed by Diff-quick staining. The panels in A, shows representative images of Diff-quick stained cells after 12 days in vitro. Live cells stain with a dark blue nucleus, while dead cells stain with a pale blue nucleus. Cells were counted in 4 random fields and the percentage of neutrophils was calculated for 12 days. As demonstrated in this figure zoledronate reduces mature neutrophil survival in a dose dependent manner, but does not appear to affect neutrophil differentiation since similar numbers of total cells are present (live and dead) compared with control. (B) Quantification of the percentage of cells that have undergone mature
neutrophil apoptosis in response to increasing concentrations of zoledronate during the process of differentiation. A significant increase in apoptosis compared with control is observed when neutrophils are differentiated in the presence of zoledronate concentrations greater that 10μM. (C) Progenitors from 3 mice pretreated with 2μg of zoledronate 2 months prior (‘Z-M’) and 3 control mice (‘C-M’) were differentiated similar to that in (A) but without the addition of zoledronate to the media. No significant difference is noted in neutrophil survival between (Z-M and C-M). As a positive control, 3 control mice were differentiated in an exact manner as was performed in (A) in the presence of 20 μM zoledronate (C-M + Zol). The presence of ZOL to the media during differentiation leads to increase neutrophil apoptosis. (D) Mature neutrophils were extracted from the bone marrow of mice that had been pre-treated 2 weeks prior with 2μg of zoledronate and subjected to a spontaneous 20H apoptosis assay in the absence of G-CSF. The levels of apoptosis were not significantly different (‘NS’) between mature neutrophils from control and zoledronate pre-treated mice. (E) Mature neutrophils were extracted from the bone marrow of mice that had been pre-treated 2 weeks prior with 2μg of zoledronate and subjected to a spontaneous 20H apoptosis assay in the presence of G-CSF (30 ng/ml). Zoledronate pre-treated mice in the presence of G-CSF show significantly higher levels of apoptosis of mature neutrophils compared with control mice. These levels of apoptosis are similar those observed during the in vitro differentiation of precursors cells in (A). Annexin-positive but PI-negative cells are defined as early apoptotic cells, whereas annexin-positive and PI-positive cells are defined as late apoptotic cells. [Experiment was preformed with n=4 per group. Errors are standard deviation and * is significance of p<0.05].
Figure 6 (A) Neutrophil Chemotaxis is perturbed in BPONJ patients. The final data that confirms that there is value in testing the novel hypothesis proposed comes from comparing neutrophil chemotaxis in control neutrophils, patients with osteoradionecrosis (ORN) and from patient with bisphosphonate induced osteonecrosis of the jaw (BPONJ). We measured the neutrophils ability to migrate through a porous membrane using fMLP (10^{-6} M) as a chemoattractant in a Boyden chamber set-up. Neutrophils from 5 patients with BPONJ were analyzed and compared with neutrophils from 3 patients with ORN and 3 healthy control patients for migratory impairments. Cells that migrated through the 3μm pores were DAPI-stained, and 5 random fields of view (FOV) were counted using a fluorescent microscope under 40X magnification. The level of migration was expressed as the average number of neutrophils per FOV. Control, ORN and BPONJ neutrophils show significantly increased migration toward fMLP. However, significantly fewer numbers of BPONJ neutrophils were observed to migrate toward the fMLP stimulus compared with control neutrophils as well as ORN neutrophils, suggesting a neutrophil migratory defect in the BPONJ patients. *p<0.05, error bars are S.D. (B) Oral neutrophil counts in BPONJ patients vs. healthy control patients-Oral Neutrophils in the rinse samples were easily identified using the acridine orange staining technique described previously[162]. The neutrophils were visualized with fluorescence microscopy and counted using a hemocytometer. Although there was a trend toward increased neutrophil recruitment in the BPONJ and ORN patients, the levels did not reach statistical significance with p>0.05, error bars are S.E.M.
CHAPTER 4

DISCUSSION
In recent years it has been noted that a small subset of patients (1-10%) treated with intravenous bisphosphonates are at an increased risk of developing osteonecrosis of the jaw (BPONJ) [4, 5]. The publicity surrounding this complication and the lack of information about its underlying pathogenesis has led to apprehension amongst patients and dental clinicians when treating patients with a history of bisphosphonate therapy [6].

4.1 Pathogenesis

Several mechanisms by which bisphosphonates (BP) are thought to cause bisphosphonate-induced osteonecrosis of the jaw (BPONJ) have been discussed in the literature. Apart from the earliest idea that BPs lead to a decrease in bone remodeling due to an increase in oseoclastic cell death, several other theories have emerged.

4.1-1 Anti-angiogenesis and Matrix Necrosis

One of such theories is the idea that the anti-angiogenic properties of the bisphosphonates which compromise the blood supply in the bone, is the initiating event of BPONJ[176]. Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. There has been evidence that bisphosphonates can interfere with the proliferation, adhesion, migration, and vessel sprouting of endothelial cells thereby reducing the formation of new blood vessels[177][178]. However the effects observed in the aforementioned studies were found at greater-than-human equivalent dose, and have not been demonstrated in humans. The most recent human study to support this theory looked at 31 patients with BPONJ who underwent jaw bone biopsy and tissue sampling. Light microscopy and confocal scanning laser microscopy examination demonstrated prominent and dense woven bone formation with
fewer and distant blood vessels along with reduced osteoclastic activity. This study also supported the notion that anti-angiogenic effects by BPONJ may be caused from an ischemic necrosis due to the osteogenic and nonosteogenic expansion of the bone without an increase in blood supply. The authors propose that this new dense bone with less blood supply would eventually lead to necrosis and secondary infection [179].

With this being said, it is believed by some that these drugs have the potential to cause avascular necrosis. This etiology has been reported to be unsubstantiated by many who argue that if the bone was in fact avascular, the ONJ tissue should not bleed at the time of surgery which has been reported [180]. As well, Hansen et al[181] noted patent vessels in seven out of eight BPONJ cases studied histologically. The vascular supply of the mandible is inherently restricted, and interferences with this blood supply. Moreover, other more potent anti-angiogenic medications such as thalidomide, used for chemotherapy, are not associated with BPONJ in humans[176].

In a beagle dog model of BPONJ, Burr and Allen [182] showed that bone matrix necrosis occurs in bones with higher remodeling rates such as the jaw bone that has been treated with with clinical doses of aledronate and zoledronate, while none of the control animals in the study acquired osteonecrosis.

4.1-2. Bone Turnover and Microcracks

Another theory arises from the well documented mechanisms of action of the bisphosphonates in reducing bone turnover. It is believed by some that an excessive reduction in bone turnover exists in some individuals taking bisphosphonates leading to an accumulation of microcracks. In essence, the microcracks may make the bone more susceptible to necrosis when there is increased demand for osseous repair such as in cases
of trauma to the jaw[176, 183, 184]. This theory is supported by a recent human study using scanning electron microscopy[185]. Microcracks were present in 54% of patients with BPONJ compared with 29% in patients without BPONJ but taking BPs, and 17% in osteoporosis patients not taking BP, whereas no microcracks were observed in patients with osteoradionecrosis of the jaw or osteomyelitis of the jaw without BP therapy. The results of this study have been questioned by some who criticize the use of scanning electron microscopy to evaluate microcrack damage. Allen [186] proposes that the gold standard method for evaluating microdamage in bone is basic tuchsin staining, which stains the bone prior to processing. Allen argues that the importance of staining specimens prior to processing is that it allows separation of microcracks that existed prior to processing and those that are due to specimen preparation. Without such staining, it is impossible to know whether any damage is due to processing or not. In his own study, Allen et al. [187] showed that three years of daily oral alendronate treatment in beagle dogs increases microdamage in vertebral bone but does not significantly increase it beyond levels of microdamage found after 1 yr of treatment. He suggests microdamage accumulation peaks during the early period of bisphosphonate treatment and does not continue to accumulate with longer periods of treatment. Refuting the notion that BPs lead to increase in microcracks in bone is Chapurlat et al[188] who examined transiliac bone biopsies from postmenopausal women and found no significant differences in microcracks between these women and control groups following at least 3 years’ treatment with a bisphosphonate (3 subjects on I.V. pamidronate, 37 on oral alendronate, and 10 on oral risedronate) despite a marked reduction in bone turnover. A similar study was conducted by Stepan et al[189] where
they compared transiliac bone biopsies from 38 postmenopausal women that were being treated with daily or weekly doses of alendronate for at least 5 years. They found no differences in crack density and crack surface density between the control and study groups. A major drawback of these studies however, is that the majority of the women in the study population were on oral doses of bisphosphate rather than intravenous administration of the drug. It is well documented that BPONJ is associated with intravenous administration rather than oral administration [65, 184]. As well, the bone examined in these studies were not taken from the jaw bone where BPONJ occurs. One point to keep in mind is the idea that if reduced bone turnover was in fact the etiology of BPONJ, it seems odd that other conditions associated with chronically reduced bone turnover, such as hypoparathyroidism, do not show BPONJ-like lesions [61].

Other compelling evidence suggests that bone turnover is not even reduced within BPONJ lesions. Several groups [181, 190, 191] have observed deep osteoclastic pits in BPONJ lesions, as well, Hansen et al. [181] showed up to a four-fold increase in osteoclast number in BPONJ patients compared with those from osteoradionecrosis and control patients suggesting that bone turnover is not suppressed at sites of BPONJ. Enhanced bone turnover has also been demonstrated with bone scintigraphy, where Abu-Id et al. found that areas of necrotic bone had increased tracer uptake in 15 of the 16 BPONJ subjects examined [74].

4.1-3. Soft Tissue Toxicity

Soft tissue toxicity to the oral mucosa may lead to compromised healing of traumatic lesions that arise in the soft tissue, with extension of the lesion to the
underlying bone[192]. Much of the focus of BPONJ has centered upon bisphosphonates and bone, an ‘inside-out’ theory. However the toxic effects of bisphosphonates on traumatized oral epithelium may play a critical role in the development of BPONJ, a so-called “outside-in” hypothesis. Several studies have confirmed that idea that BPs can negatively affect the function of several cell types including macrophages, periodontal ligament fibroblasts [193], endothelial cell[177, 194-196], a variety of tumor cells, osteoblasts and epithelial cells[197-199]. Bisphosphonate toxicity to epithelial cells is a well documented side effect with the use of oral nitrogen-bisphosphonates with the formation of gastric erosions and ulcers. Coxon et al[200] explored the idea that bisphosphonates on bone surfaces are toxic to adjacent cells. Interestingly, they observed that when the bisphosphonates were not bound to bone but present free in solution, they appeared to have toxic affects on cells. However, when the bisphosphonates were in the presence of bone, the toxicity was greatly reduced, suggesting that the when the bisphosphonates are bound to bone, they are unable to concentrate and toxify the surrounding cells. On the other hand, when osteoclasts were added to the cultures in an attempt to simulate a normal physiologic environment, the bisphosphonates were mobilized from the bone and began to concentrate in the surrounding solution leading to toxicity in the adjacent cells[201].

With respect to bone, the amount of drug that is deposited in the maxillofacial bones is unknown. Likewise, the amount of active bisphosphonate present in the gingival crevicular fluid is unknown, however the presence of active periodontal disease would be expected to increase the level of bisphosphonates in the area[61]. This is supported by
Marx [176], who suggests that approximately 85% of affected patients have had peridontitis during the diagnosis of BPONJ.

Similarly, areas of high bone turnover observed in the alveolar bone following a traumatic extraction would be expected to present with higher concentrations of bisphosphonates. Studies using $^{99m}$Tc-labeled bisphosphonates have confirmed the hypothesis that there is preferential uptake of tracer at these sites[202], however the exact concentration of the bisphosphonates is unknown. Given this potential mechanism, it is not surprising how one may believe a scenario in which trauma to the oral mucosa can represent an initiating event of BPONJ and release of bisphosphonates from the underlying bone can inhibit mucosal wound healing with spread to the adjacent bone[61].

4.1-4. Infection

One of the final common theories discussed in literature as a possible initiating factor in the pathogenesis of BPONJ is presence of an infection. This idea came about from the consistent, if not universal histological finding of bacterial colonization in biopsies taken from BPONJ patients[74, 181, 203, 204].

Recently, a study looking at biopsies of four BPONJ patients using scanning electron microscopy, made the observation that all four patients had the presence of microbial biofilms[190]. A biofilm is a complex aggregation of microorganisms growing on a solid substrate. Biofilms are characterized by structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances[205]. They have a characteristic feature of having resistance to both host defenses (antibodies and phagocytes) and to antibacterial agents, making them difficult to
penetrate. Physical removal of the biofilm is usually required to effect a cure. The presence of infection may be significant in producing one of the unpredicted but constant characteristics of this condition; increased bone resorption despite the presence of bisphosphonate in bone[201]. Several bacteria that reside in the biofilm, such as gram negative bacteria, have the ability to stimulate bone resorption, and some even have the capability to inhibit bone formation. Many species of Gram-negative bacteria are pathogenic, meaning that they can cause disease in a host organism. This pathogenic capability is usually associated with certain components of Gram-negative cell walls, in particular the lipopolysaccharide (also known as LPS or endotoxin) layer. In humans, the LPS mediates osteolysis through stimulation of local cytokine production and immune system activation[206]. However, there are several other factors produced by bacteria that have similar negative effects on the host that act through different mechanisms. For example, studies have shown that several proteins from Porphyromonas gingivalis can directly regulate the production of RANKL and osteoprotegerin in human periodontal ligament cells and gingival fibroblasts, increasing the stimulation of osteoclastogenesis[207].

The theory that infection may play a role in the pathogenesis of BPONJ may explain the exclusive predisposition of BPONJ to the oral cavity. The oral cavity, unlike other parts of the body, represents an open environment where bone has the potential to be easily colonized by an abundant flora of bacteria. Some authors have even suggested that BPONJ can be subclinical, where no mucosal breakdown and bone exposure occurs in the oral cavity. In cases such as these, it not unreasonable to suggest that an infection can
ensue from bacterial colonization through odontogenic or periodontal infection extending to the bone through fistulas in the absence of mucosal breakdown. The jaws are typically resistant to microbial colonization, in part, by the constant influx of neutrophils which migrate into the mouth from the periodontal tissues that surround and support the teeth [192, 208]. However in a situation where the immune system in compromised, the presence of oral infection is not uncommon[209, 210].

4.2 Purpose of Thesis

The goal of this thesis tested the novel hypothesis that bisphosphonates dampen neutrophil functions and differentiation resulting in poor oral innate immunity. The compromised oral immunity increases the risk of infection following surgery or trauma which can subsequently develop into BPONJ. This theory is based on the fact that there have been reports that some bisphosphonates can cause neutropenia[7], inhibit neutrophil enzymes that impact on wound healing such as MMP8 [8] and decreased generation of reactive oxygen species formation [9, 10]. This is not surprising in view of the mechanism of action of bisphosphonates which target small RhoGTPases that are signaling proteins integral to neutrophil differentiation and function. Despite this fact, the idea that BPs may negatively affect the innate immune system has not received much focus in the literature.

4.3 Plausible Mechanism through which ZOL Affects the Innate Immune System

The plausible mechanism for this hypothesis is based on the biology of bisphosphonates and small GTPases where 1) it is known that BP target the Rho family of small GTPases which are signaling proteins [211] not only in osteoclasts but also in neutrophils and 2)
neutrophils with defects in small GTPases display perturbed differentiation and defective antibacterial functions [170, 212].

The mechanism of action of the nitrogenous bisphosphonates involves the perturbation of several metabolic reactions, most notably the mevalonate biosynthetic pathway. This affects cell activity and cell survival by interfering with protein prenylation and therefore, the signaling function of key regulatory proteins. The mevalonate pathway is a biosynthetic route responsible for the synthesis of cholesterol, other sterols, and isoprenoid lipids such as isopentyl diphosphate, farnesyl diphosphate (FPP) and geranylgeranyldiphosphate (GGPP)[213, 214]. FPP and GGPP are involved in post-translational modification (prenylation) of small GTPases such as Ras, Rab, Rho, and Rac[215]. Prenylation is necessary for the correct function of these proteins because the lipid prenyl group serves as an anchor for the proteins in the cell membranes[23]. The small GTPases are important signaling proteins that regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and some plants. Three members of the family have been studied a great deal: Cdc42, Rac1, and RhoA. Rho proteins have been described as "molecular switches" and play a role in cell proliferation, apoptosis, gene expression, and multiple other common cellular functions[216]. In osteoclasts, the small RhoGTPases are essential for cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles, and apoptosis[40]. Neutrophil function is also dependent on the rhoGTPases, where they control among other things, filodopida and lamellopodia formation and neutrophil chemotaxis[216]. While many of the theories regarding the pathogenesis of BPONJ have focused on how bisphosphonates negatively affect osteoclast function, very little has been written on the
effect that the bisphosphonates may impart on other cell types in the myeloid lineage such as the neutrophil.

4.4 Oral Innate Immunity

The mouth contains a constant supply of bacteria in the form of surface coating biofilms on teeth, tongue and other shedding surfaces [217]. This bacterial presence is kept under control, in part, by the constant influx of neutrophils which migrate into the mouth from the periodontal tissues that surround and support the teeth. In order to maintain a state of oral health, an individual’s immune system must be kept in tight control. A shift to either side of this equilibrium may result in a state of immune under-activity, observed in cases of AIDS, where a reduced number of neutrophils in the oral cavity can result in an overgrowth of unwanted fungi termed “thrush or candidiasis” or lead to a rapid destruction of the soft and hard tissue of the periodontium known as ‘necrotizing ulcerative periodontitis’ (NUP) [218]. Interestingly, Marx has shown that approximately 85% of affected patients have had periodontitis during the diagnosis of BPONJ [176]. Conversely, excessive neutrophil levels or cell hyperactivity may result in a state of immune over-activity and can lead to diseases of the periodontium known as ‘refractory periodontitis’[219, 220].

The observation that a majority of cases of BPONJ, present with an active infection lead us to suspect that the immune system may be perturbed in these individuals.

4.5 The Effect of Zoledronate on Neutrophil Function and Survival

A state of immune under-activity can result from either (1) a decrease in the functional capability of the immune cells, or (2) a decrease in the number of immune cells that are present to fight off the infection, or a combination of the two.
We chose to test this hypothesis by first performing functional test on neutrophils following both *in vitro* and *in vivo* zoledronate exposure.

4.5-1. **Zoledronate negatively affects Neutrophil Function**

Our results show that both *in vitro* and *in vivo* zoledronate exposure leads to diminished neutrophil chemotaxis *in vitro*, diminished neutrophil recruitment to the peritoneum *in vivo*, as well as diminished NAPDH oxidase activity *in vitro*.

Although both *in vivo* and *in vitro* models confirm that zoledronate exposure leads to diminished neutrophil chemotaxis, only the *in vitro* model resulted in a dose dependent response; where incremental increases in concentration of zoledronate, resulted in near proportional decreases in neutrophil speed. The differences in response with increasing BP dose observed between the *in vitro* and *in vivo* models may be the fact that the level of exposure *in vivo* may have been different to what the neutrophils were exposed to *in vitro* where the *in vivo* dose may have already been maxed out. For the zoledronate pre-treated mice, we chose to use a dose of 2ug/mouse. This dose has been previously calculated for mice to correspond to the dose that is typical for a human patient (detail explanation found in ‘Materials and Methods’).

Since there are no studies that have determined the concentration of zoledronate in the bone marrow, we chose to use data from a previous study in rats to determine the appropriate concentration of zoledronate to use for our *in vitro* mouse model. Using radiolabeled zoledronate in rats, Green et al. [221] quantified the concentration of zoledronate in bone, soft tissue and blood from 1 to 280 days after a single 2ug infusion. Since the bone marrow is apposed to the bone, we estimated that the concentration of ZOL in the bone marrow is somewhere in between the bone value and the soft tissue
value. Taking this into account, we used values from 20-100 uM \textit{in vitro} to try and reproduce the 2ug dose \textit{in vivo}. The differences in dose response between the \textit{in vitro} and \textit{in vivo} models, is possibly due to the fact that although we attempted to expose the neutrophils to similar ZOL concentrations in both models, we were unable to do so precisely. It is possible that a 2ug dose of zoledronate elicits the maximum effect on neutrophils that can be achieved, where any further increase in dose does not have any further negative effect. Whereas \textit{in vitro}, the dose from 20uM-100uM may be still low enough that incremental increases lead to further decreases in cell speed.

All of our initial experiments were performed after 30 days post-zoledronate injection to recapitulate the dosing cycle in humans. Typically human patients are given a regime of 4mg/30days of a bisphosphonate (usually zoledroante) for a variable number of months depending on the severity of their condition. Interestingly the results that were obtained following 30 days post-zoledronate infusion were the same as those obtained after only 14 days of exposure. The lack of difference between 14 and 30 days post injection is consistent with previous work in a rat model, where the levels of zoledronate in bone and soft tissue remained relatively constant from the day of infusion to approximately 60 days post-infusion. These long term effects observed with ZOL can be attributed to the idea that there is continual recycling of BP off and on back onto the bone mineral surface [28, 29]. This notion is supported by the observation the BPs can be found in plasma and urine many months after dosing [54]. The observation that neutrophil function is also perturbed at 14 days post-ZOL exposure implies that; because of the intrinsically short life of the neutrophils, one will see the deficits in the neutrophils within a few days and
this will continue until the levels of circulating BP drop below some critical concentration, possibly months or years after the last injection.

4.5-2. **Zoledronate Affects Neutrophil Function through the small RhoGTPase RhoA**

Neutrophil chemotaxis and NADPH oxidase activity is dependent upon the appropriate localization and activation of the small RhoGTPases; Rac, Rho and Cdc42, within the neutrophil. Unlike Cdc42 and Rac which localize to and are responsible for the formation and protrusion of the leading edge of the neutrophil, RhoA localizes to and is responsible for regulating the myosin II-based contraction and retraction of the trailing uropod during neutrophil chemotaxis[26, 133, 136, 222-224]. Our results confirm that zoledronate perturbs the activation of RhoA but does not appear to disrupt the activation of either Cdc42 or Rac1 following fMLP stimulation. The diminished RhoA activation may explain the reduced migratory ability of the zoledronate-exposed neutrophils compared with controls, where these neutrophils may have an inhibitory to fully retracting their tails during migration. These results are in accordance with several other groups who have also observed neutrophil migration defects when RhoA activation is impaired [225, 226].

To address the second possible scenario that a decrease in the number of immune cells leads to diminished immune function, we quantified the number of circulating neutrophils in zoledronate-treated mice at 14 and 30 days following a 2ug dose of ZOL. We demonstrate that both time points result in a similar significant reduction in circulating neutrophil counts, leading us to propose that ZOL may decrease either mature neutrophil survival and/or neutrophil differentiation in the bone marrow.
4.5-3. **Zoledronate Decreases Mature Neutrophil Survival**

Neutrophil progenitors isolated from control mice and differentiated in the presence of ZOL *in vitro* demonstrated an increase in apoptosis in a concentration dependent manner (Figure 5A). Since the total number of mature neutrophils derived from the progenitors (live + dead) were unaffected by ZOL treatment we conclude that local zoledronate exposure does not affect the process of neutrophil differentiation, but instead increases mature neutrophil apoptosis. While our results show that; (i) ZOL elicits its effect during neutrophil differentiation (Figure 5A), and (ii) the effects that ZOL imparts on neutrophils are long lasting, where mature neutrophils from ZOL pre-treated mice exhibit increased apoptosis even when in a ZOL-free environment (Figure 5E); we are still unable to determine at what phase of neutrophil differentiation that ZOL is eliciting its effects on neutrophils.

4.5-4. **Zoledronate and G-CSF**

Interestingly, when we attempted to recapitulate the above findings by subjecting mature neutrophils isolated from ZOL-treated mice to a 20 hour spontaneous apoptosis assay, no increase in mature neutrophil apoptosis was observed (Figure 5D). In an attempt to understand why ZOL exposure affects the survival of mature neutrophils when differentiated *in vitro* (Figure 5A), while no difference in survival was observed when the neutrophils were directly isolated from ZOL treated mice (Figure 5D), we considered that the major difference between these two experiments was the presence of a growth factor G-CSF. Granulocyte colony stimulating factor (G-CSF) is produced by a number of different tissues to stimulate the bone marrow to produce granulocytes and stem cells and
to promote survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils[227]. These complex functions necessitate G-CSF’s presence during in vivo and in vitro neutrophil differentiation from precursor cells. For this reason, G-CSF was only added to the initial experiment (Figure 5A) to encourage neutrophil differentiation from the progenitors and not to the later assay (Figure 5D) in which only mature neutrophils were tested. To adjust for this, G-CSF was added to the media containing mature neutrophils and apoptosis was re-analyzed. Figure 5E shows that the addition of G-CSF leads to a similar increase in mature neutrophil apoptosis, as was observed in Figure 5A. G-CSF is known to dramatically prolong the half-life of mature neutrophils, having a so-called ‘anti-apoptotic effect’, which supports the observation that even control cells show lower levels of apoptosis when in the presence of G-CSF (Figure 5D and E). The observation that G-CSF must be present for zoledronate to have a negative effect on neutrophil survival suggests that zoledronate inhibits the anti-apoptotic effects of G-CSF. Previous groups have shown that G-CSF signals via the G-CSF receptor requiring various GTPases, specifically Ras and Rap1[228, 229]. Our results suggest that ZOL may alter G-CSF receptor signaling, impeding the anti-apoptotic effect of G-CSF on neutrophils and ultimately resulting in higher levels of neutrophil apoptosis.

Our theory that zoledronate negatively effects the function and lifespan of neutrophils is consistent with previous work by Coxon et al. who has shown that bisphosphonates have the potential to leave the bone surface in the presence of osteoclasts and concentrate in solution to disrupt the function of several other cell types [200].
4.6 Neutrophil Function in BPONJ patients

We present convincing evidence that suggests that neutrophil function and differentiation in the presence of bisphosphonates are severely impaired. However some may argue that the effects that are observed in mice may not be recapitulated in human BPONJ patients. To address this issue, a study was set up to observe neutrophil function in diagnosed BPONJ patients. As expected, the preliminary data from these trials suggest that neutrophil chemotaxis is also perturbed in BPONJ patients, where neutrophils isolated from the blood of BPONJ patients did not migrate as well as control blood neutrophils (Figure 6A). As a positive control, we selected three patients that had been diagnosed with osteoradionecrosis (ORN), a condition which has a similar clinical presentation to BPONJ, however the pathogenesis is known to be the result from radiation therapy that negatively affects the vasculature of jaws. The use of these patients as a control is beneficial since we may observe what true neutrophil recruitment to a site with increased inflammation is in the absence of bisphosphonate therapy. No difference in chemotaxis was observed between control and ORN patients, however BPONJ patients had poorer chemotaxis in comparison to ORN neutrophils (p<0.05), confirming that the bisphosphonates do in fact perturb the migration of neutrophils, which may result in an overall reduced immune response. Although we initially expected to see a decrease in oral neutrophil recruitment in BPONJ patients, this was not the case. Both ORN and BPONJ patients exhibited a trend toward an increase in oral neutrophil delivery (p>0.05) however both did not reach statistical significance. The trend toward an increase in neutrophil delivery in this patient population is not surprising since an open area with active inflammation is present in the oral cavity in both cases. It may have been more
informative to test the specific bacterial killing and NADPH oxidase activity of neutrophils in the BPONJ group and compare it to the neutrophils present in the ORN group to identify if the \textit{in vitro} neutrophil defects can be recapitulated in the human oral cavity, which may contribute to the ongoing infection in BPONJ patients.

\section*{4.7 Questions Arising from our Study}

The results presented in this thesis may lead one to then ask the question, if zoledronate does cause affects on neutrophils, then why are there limited reports of increased susceptibility to infection in BP treated patients? As well, if bisphosphonates do lead to neutrophil defects, then why is this condition only observed in 1-10\% of BP-treated patients?

These questions can be addressed by the idea that not all patients with neutropenia for example, display equal susceptibility to infection, ie. While the BP may be imparting a defect in neutrophils, possibly only a subset of these patients (1-10\%) actually show a phenotype for increased infection susceptibility.

We believe that the neutrophil defect observed in the 1-10\% of BP users is quite mild and in normal circumstances is mild enough that it does not predispose the patient to increased risk of systemic infections.

However, in a case where the jaw of a BP-treated patient is now subjected to trauma, the immune cells which are typically recruited are now being brought to a site with impaired vascularity and accordingly, a reduced number of these cells are able to penetrate the site. Consequently, the neutrophils that are able to penetrate the wound are not functioning to 100\% capacity, and their ability to combat the abundant oral bacteria which normally resides in the mouth is impaired and an infection ensues. It is also worth mentioning that
along with ZOL imparting mild defects in neutrophils, a majority of patients who develop BPONJ are cancer patients that are also treated concurrently with corticosteroids. Corticosteroids act on the immune system by blocking the production of substances that trigger allergic and inflammatory actions, such as prostaglandins. As well, they also impede the function of white blood cells impeding the immune system from functioning properly. Hence it may be combination of both medications (ZOL and corticosteroids) that is imparting a deleterious effect on the innate immune response which predisposes certain individuals to BPONJ more than others.

Furthermore, it is likely that the individual defects that have been noted in BP-treated patients such as, impaired bone and soft tissue remodeling, impaired angiogenesis, and the impaired immune response, are mild enough that on their own do not cause BPONJ, but when all of these mild defects are coupled together, a vicious cycle is established where the bone and soft tissue lesion caused by a tooth extraction or even as small as oral ulceration is unable to heal because of persisting infection, resulting in sustained bone resorption and the release of cytotoxic bisphosphonates. The proposed multifactorial etiology of BPONJ may explain the rarity of this condition in BP-treated patients.

Despite infection being present in most BPONJ patients, difficulty arises when trying to determine if infection is a primary or secondary event in the pathophysiology of this condition. Although our data does not definitively confirm causality, we believe that infection is a primary etiology to BPONJ for two reasons. Firstly, our data clearly demonstrates that ZOL disrupts neutrophil functions as well as limiting neutrophil survival. Extrapolating these results suggest that if a BP-treated pt is now subjected to
trauma to the oral cavity, such as a tooth extraction, then the neutrophils which are not functioning or surviving to full capacity, will have difficulty initiating the wound healing response which potentiates the development of a non-healing wound. Secondly, it is also important to compare and contrast features of BPONJ with ORN. The pathogenesis of ORN is defined by a sequence of radiation, hypovascular-hypocellular-hypoxic tissue formation and trauma induced or spontaneous mucosa breakdown leading to a non-healing wound [230]. While both BPONJ and ORN show rather identical clinical presentations, one of the major differences histologically is the incidence of infection and bacterial colonization in biopsies taken from BPONJ patients. While BPONJ patients almost exclusively present with an active infection of the necrotic bone, ORN does not necessarily present in such a way. This is not to say that infection in ORN patients does not occur, however most recent studies show that the incidence of Actinomyces bacteria for example, is almost double in BPONJ patients than in ORN patients[181]. A second group showed in a 5-year retrospective study of 50 cases of ORN of the jaw, that only six cases (12%) of Actinomyces infection were found[231]. This is in contrast to several studies which have shown that almost 100% of the BPONJ specimens are colonized with multispecies microbial biofilms [190, 232]

This suggests that an additional mechanism, such as an alteration in the immune response, may be predisposing certain patients to infection which may subsequently develop into BPONJ.

4.8 Study Limitations

It is important to recognize that a specific limitation of this and many studies looking into the pathogenesis of BPONJ is the fact that a majority of patients diagnosed with BPONJ
are concurrently being treated for malignancy. Thus it may be the fact that not only is this patient population subjected to BPs with a higher dose and potency, but cancer therapy usually necessitates the use of several other medications, such as corticosteroids and cytotoxic drugs, that may either exacerbate the effects of the bisphosphonates or they themselves may be contributing to the pathogenesis of BPONJ.

A cancer treatment that may also affect this condition is the use of G-CSF. G-CSF is the new standard of care in certain cancer patients being treated with chemotherapy drugs. In oncology and hematology, a recombinant form of G-CSF is used to accelerate recovery from neutropenia and prevent infection following chemotherapy[233, 234]. Although we are unable to come to any firm conclusion about this, it is still worthwhile pointing out that it is quite possible that a majority of the patients that have developed BPONJ are also treated with G-CSF. The results of our study suggest that the assumed benefits from the exogenous G-CSF treatments in these patients may be diminished in those concurrently treated with zoledronate.

4.9 Future Work

It would be beneficial in the future to analyze neutrophil delivery to and function in the oral cavity of patients being treated with bisphosphonates. Oral neutrophil levels and oral neutrophil functions will be monitored prospectively in these patients where oral rinse samples will be collected from patients prior to initiation, and during high dose BP therapy to determine if BPs affect neutrophil oral delivery and function. As well, we suspect that the bisphosphonates may be disrupting the function of various other proteins involved in cellular activity apart from RhoA that was identified in this study. We hope
the use of Isotope-Coded Affinity Tags (ICAT) technology may provide additional information on possible protein targets of bisphosphonates by using quantitative comparison of the two proteomes.

Our study is the first to present clear evidence that the bisphosphonates negatively affect neutrophil function and survival, possibly compromising immune function.

Several rational theories have been proposed in the literature and it likely that this condition is multifactorial in origin where bisphosphonates are released at high concentrations into the immediate environment, and are taken up by various cells types including epithelial cells, vascular, mesenchymal and immune cells which lead to a state of impaired wound healing.
Thesis Summary

Bisphosphonates are anti-bone resorptive drugs that are primarily used for the prevention of osteoporosis and more recently as the standard of care to prevent skeletal-related symptoms and events in patients with multiple myeloma and metastatic bone lesions of solid tumor cancers such as breast, prostate and lung cancer [1] [2]. A small subset of patients (1-10%) treated with intravenous bisphosphonates are at an increased risk of developing a severe bone disease that affects the jaws, known as bisphosphonate-induced osteonecrosis of the jaw (BPONJ) [4, 5].

In recent years, an increased incidence of BPONJ has been associated with the use of high intravenous dosages of bisphosphonates, required by some cancer treatment regimens, especially when the patient undergoes subsequent dental procedures.

The publicity surrounding this complication and the lack of information about its underlying pathogenesis has led to apprehension amongst patients and dental clinicians when treating patients with a history of bisphosphonate therapy [6]. Critical information that will help alleviate these concerns and improve oral treatment approaches in this patient population is required. Being able to identify and understand the possible mechanisms through which bisphosphonates affect normal bone and soft tissue wound healing in the jaw will help identify patients at risk of developing BPONJ and improve patient management.

In this thesis, we show evidence that bisphosphonate therapy negatively affects the function and survival of cells that are key players in the immune system (neutrophils), and that patients with BPONJ, appear to have diminished neutrophil function in comparison to their healthy counterparts. These results suggest that a once overlooked area, the immune
system, may play a role as a contributing risk factor in the development of BPONJ. Future work will concentrate on determining if worsening neutrophil levels or function corresponds with a worsening of the disease or increased risk of the disease.

If oral neutrophil function is confirmed to be a contributing risk factor for the development of BPONJ, we may be able to identify those patients at risk of BPONJ prior to invasive dental treatment. This would be the first risk assessment screening tool for this condition. Additionally, in patients on BP therapy who require oral surgery or in those who develop BPONJ, neutrophil function assays may guide therapy in terms of discontinuing or modifying BP therapy during the peri-operative and healing or resolution periods.
REFERENCES


81. Rheumatology, A.C.o., Bisphosphonate-induced osteonecrosis of the jaw. 2006.


