The Effect of Bisphosphonate Therapy on Neutrophil Function: A Potential Biomarker Preliminary Findings

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Dentistry
University of Toronto
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THE EFFECT OF BISPHOSPHONATE THERAPY ON NEUTROPHIL FUNCTION: A POTENTIAL BIOMARKER

Christa Favot 2013, Degree of Master of Science, Graduate Department of Dentistry, University of Toronto

ABSTRACT

Bisphosphonate-related osteonecrosis of the jaws (BRONJ) occurs subsequent to intravenous and oral bisphosphonate exposure in a small subset of patients. Evidence of concurrent bacterial colonization at sites of bone necrosis, previous reports of neutrophil-related complications in some patients taking bisphosphonates along with perturbed neutrophil function in bisphosphonate-treated mice suggests an innate immune role in the development of bisphosphonate-related osteonecrosis of the jaws. This study investigates neutrophil function in BRONJ patients to determine if neutrophil functional defects may serve as a potential biomarker for BRONJ susceptibility. Patients with BRONJ and patients beginning intravenous pamidronate were studied. Eighteen patients with BRONJ and five patients beginning pamidronate therapy provided oral and blood neutrophil samples. Neutrophils from the population of patients with bisphosphonate-related osteonecrosis of the jaws and from those post-pamidronate treatment showed lower reactive-oxygen species production. These data suggest that a compromise in neutrophil function may be a potential biomarker for BRONJ susceptibility.
Acknowledgements

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Last but not least, I am grateful for my family, friends and co-residents who were generous with their support throughout my graduate work.
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MATERIALS LEGEND

¶ Sodium Citrate Vacutainer, Becton Dickinson, Rutherford, NJ.

§ 1-Step Polymorphs, Accurate Chemical and Science Corp, NY.

|| University of Toronto Media Preparation Services, Toronto, ON.

§& University of Toronto Media Preparation Services, Toronto, ON.

& Millipore Canada.

* University of Toronto Media Preparation Services, Toronto, ON.

¶¶ University of Toronto Media Preparation Services, Toronto, ON.

 §§ Sigma-Aldrich Chemical Co.

|||| Bovine Erythrocyte, Sigma-Aldrich Chemical Co.

** Sigma-Aldrich Chemical Co.

§§§ Sigma-Aldrich Chemical Co.

@ Costar® 3 micron pore membrane, Corning Inc., Lowell, MA.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AAOMS</td>
<td>American Academy of Oral and Maxillofacial Surgery</td>
</tr>
<tr>
<td>APD</td>
<td>3-amino-1-hydroxypropylidene-1, 1-bisphosphonate</td>
</tr>
<tr>
<td>ASBMR</td>
<td>American Society for Bone Mineral Research</td>
</tr>
<tr>
<td>BMU</td>
<td>Bone multicellular unit</td>
</tr>
<tr>
<td>BSU</td>
<td>Basic structural unit</td>
</tr>
<tr>
<td>BP</td>
<td>Bisphosphonate</td>
</tr>
<tr>
<td>BRONJ</td>
<td>Bisphosphonate-(associated/induced/related) osteonecrosis of the jaw</td>
</tr>
<tr>
<td>CTIBL</td>
<td>Cancer therapy-induced bone lesion</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F</td>
<td>Female</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-Formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl diphosphate</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl pyrophosphate synthase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphate-hydrolyzing enzyme</td>
</tr>
<tr>
<td>HAP</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Male</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial surface components which recognize adhesive matrix molecules</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>NHOK</td>
<td>Normal human oral keratinocyte</td>
</tr>
<tr>
<td>NHOF</td>
<td>Normal human oral fibroblast</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NonN-BP</td>
<td>Non-nitrogenous bisphosphonate</td>
</tr>
<tr>
<td>ONJ</td>
<td>Osteonecrosis of the jaw</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>ORN</td>
<td>Osteoradionecrosis</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PO</td>
<td>By mouth</td>
</tr>
<tr>
<td>PPi</td>
<td>Inorganic pyrophosphate</td>
</tr>
<tr>
<td>q</td>
<td>Every</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>yrs</td>
<td>Years</td>
</tr>
<tr>
<td>ZOL</td>
<td>Zoledronic acid</td>
</tr>
</tbody>
</table>
PREFACE

THESIS FORMAT

This MSc thesis is presented in the “Publishable Style”. The abstract and the chapters describing experimental data and the conclusions has been submitted for publication and are, in part, presented here in their published form. An Introduction and Thesis Summary are included to frame experimental content. The contributions made by collaborators are indicated before the chapter. Those who provided technical support are mentioned in the acknowledgements.

PUBLICATION FROM THIS THESIS

CHAPTER 1

THESIS INTRODUCTION
I. INTRODUCTION

Over the past ten years, the number of prescriptions for bisphosphonates have soared; consequently, recipient patients with malignancy have seen decreased skeletal-events and those with osteoporosis have experienced decreased fracture rates. However, this class of medication is linked to bisphosphonate-related osteonecrosis of the jaw (BRONJ) in a subset of patients.

BRONJ is a condition that affects patients with varying degrees of morbidity. It is characterized by “current or previous treatment with a bisphosphonate; [necrotic and often] exposed bone in the maxillofacial region … and no history of radiation therapy to the jaws” 2. Manifestations of BRONJ vary among patients, where early clinical findings may include dental mobility, spontaneous or trauma-induced areas of exposed non-vital bone with erythematous and edematous margins. These sites may be tender to palpation, often secondarily infected and are usually refractory to debridement. Symptoms including paresthesia, cutaneous or mucosal fistulae and pathologic fractures may manifest later in the clinical course 3.

Bisphosphate drugs are used for the treatment of numerous osseous conditions including hypercalcemia and osteolytic lesions associated with malignancy (such as Multiple Myeloma), Paget’s disease and prevention of bone metastases (such as in breast and prostate cancer). Those at higher risk include the intravenous bisphosphonate-treated
group. As analogs of pyrophosphate, they are synthetic inhibitors of osteoclastic activity and therefore function to increase bone density and prevent further loss.

Case reports of BRONJ are abundant in the literature. The positive correlation between bisphosphonate administration and BRONJ is well-defined in the literature\(^3\), \(^5\)-\(^10\), causality, however, remains to be established\(^11\). Likewise, there is no clear treatment regimen that has been outlined in the literature and BRONJ may resolve spontaneously or present with recalcitrant lesions that require long-term antibiotic therapy and multiple invasive surgical resections. While some lesions appear spontaneously in the course of bisphosphonate treatment\(^11\), the majority are sequelae of invasive dental care or trauma. Given this clinical scenario, many treatment providers and patients are hesitant to commit to dental therapy that may jeopardize their jawbone health. Elaboration of both BRONJ pathogenesis as well as predictors of BRONJ risk is required.

**II. BISPHOSPHONATES**

**A. Chemical Structure**

The chemical structure of bisphosphonate medications is based on the naturally occurring inorganic pyrophosphate (PPI) shown in Figure 1.

Two phosphonate groups (hence “bisphosphonate”) share a covalent bond to a central carbon, which replaces the central oxygen in the pyrophosphate molecule\(^12\),\(^13\). Two side chains are bound to the central carbon; R1, the short side-chain, and R2, the long side
chain. This central carbon with two phosphonate groups is the P-C-P motif that characterizes bisphosphonate drugs\textsuperscript{13}. The more recent bisphosphonates available for prescription, the nitrogen-containing bisphosphonates, are different from the earlier bisphosphonates\textsuperscript{14}. The R1 side chain in these medications is a hydroxyl group, which lends the molecule the property of irreversible binding to hydroxyapatite. These BP molecules, in which the R1 hydroxyl group is held common and the R2 group varies, have been studied \textit{in vitro}, where van Beek et al. and Leu et al. have shown that changing the R2 group will produce molecules with relatively constant bone affinity but differing osteoclast inhibition or macrophage function\textsuperscript{14-16}. A nitrogen atom at the R2 site yields higher potency, a property that is exaggerated when the nitrogen is contained in a ring form\textsuperscript{1}. The cyclic side-chain from zolendronate, for example, allows from a comparative potency of 10,000 times that of etidronate\textsuperscript{10}. Alendronate, which has the advantage of a nitrogen atom at the R2 site is 100 times more potent than etidronate but this amine form is significantly less potent than the cyclic form\textsuperscript{17}.

![Pyrophosphate and Bisphosphonate Structures](image)

\textbf{Figure 1.} Inorganic pyrophosphate and the basic bisphosphonate structure\textsuperscript{18}. 
Table 1. Common bisphosphonates: molecular structure and potency\textsuperscript{19, 10, 20}

<table>
<thead>
<tr>
<th>NonN-BP</th>
<th>R1</th>
<th>R2</th>
<th>Potency vs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>-OH</td>
<td>-CH\textsubscript{3}</td>
<td>1</td>
</tr>
<tr>
<td>Clodronate</td>
<td>-Cl</td>
<td>-Cl</td>
<td>10</td>
</tr>
<tr>
<td>Tiludronate</td>
<td>-H</td>
<td>-S\textsubscript{2}Cl</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-BP</th>
<th>R1</th>
<th>R2</th>
<th>Potency vs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pamidronate (APD, Aredia)</td>
<td>-OH</td>
<td>-CH\textsubscript{2}-CH\textsubscript{2}-NH\textsubscript{2}</td>
<td>100</td>
</tr>
<tr>
<td>Alendronate (Fosamax)</td>
<td>-OH</td>
<td>-(CH\textsubscript{2})\textsubscript{3}-NH\textsubscript{2}</td>
<td>500</td>
</tr>
<tr>
<td>Ibandronate (Boniva)</td>
<td>-OH</td>
<td>-CH\textsubscript{2}-CH\textsubscript{2}N - CH\textsubscript{3} (\text{N} - \text{CH}_2\textsubscript{3} )</td>
<td>1,000</td>
</tr>
<tr>
<td>Risedronate (Actonel)</td>
<td>-OH</td>
<td>\text{N} \text{N}</td>
<td>2,000</td>
</tr>
<tr>
<td>Zolendronate (Zometa, Aclasta)</td>
<td>-OH</td>
<td>\text{N} \text{N}</td>
<td>10,000</td>
</tr>
</tbody>
</table>

**B. Mechanism of Action**

The two different classes of bisphosphonate drugs, nitrogenous and non-nitrogenous, elicit their effects through two different mechanisms.
Nitrogen-containing bisphosphonates (N-BPs) act in the mevalonate pathway. It is here that they are able to interfere with the osteoclast regulatory enzyme farnesyl pyrophosphate synthase (FPPS), inhibiting the prenylation of small GTPases such as Rho, Rac and Rabs\textsuperscript{1,21-24}.

The mevalonate pathway (Figure 2) is present in all mammalian cells, yielding essential lipid molecules such as cholesterol and isoprenoids. The synthesis of the latter begins with acetyl coenzyme A and is biosynthesized from the intermediate molecule mevalonic acid. Isoprenoids are required for posttranslational prenylation (lipid modification) of small GTPases\textsuperscript{25}. Without proper lipid modification of these proteins, they cannot function properly\textsuperscript{1} which affects osteoclast function as these GTPases participate in osteoclast cell morphology control, signaling, membrane ruffling, vesicle transport, and apoptosis\textsuperscript{1,26-28}.

N-BPs act in this pathway by targeting FPPS and inhibiting protein prenylation. Because BPs have a high affinity for the hydroxyapatite (HA), they concentrate in bone. Subsequently, the primary effects are seen here as the loss of small GTPase proteins occurs in osteoclasts, inhibiting function and bone loss\textsuperscript{22,25,26}.

These antiresorptive agents afford the basic structural units (BSUs) an extended lifetime by reducing the formation of basic multicellular units (BMUs). This leads to an increase in the degree of mineralization of bone (DMB) by extending the secondary mineralization
process of the bone matrix. Otherwise, resorption of recently formed BSUs prior to complete mineralization would occur.\textsuperscript{29}

Non-nitrogenous bisphosphonates are a less potent medication group. Once taken up and metabolized by osteoclasts, a cytotoxic compound forms in vivo. This compound mimics ATP by forming bisphosphonate ATP-analogs, resulting in its uptake by osteoclasts\textsuperscript{13, 30}. The ATP analog is non-functional and cytotoxic to the cell trying to incorporate it into the energy cascade through disruption of mitochondrial function and result in apoptosis. In vivo, the result is decreased bone resorption\textsuperscript{13, 23}.

\textbf{C. Bisphosphonate tissue specificity, uptake and retention in bone}

In order to impact skeletal resorption, it is intuitive that bisphosphonates have an affinity for the calcium ions and therefore their large stock as a part of the calcium phosphate
mineral in osseous tissues\textsuperscript{13, 31}. This mineral-binding affinity is crucial to BP drug function, as it determines not only drug binding but also drug distribution within bone, it’s cellular effects and it’s release and recycling propensity\textsuperscript{23, 31}. In areas of bone formation and resorption, where the osteoclast population is dense and their activity is high, bisphosphonates become more concentrated\textsuperscript{14}. This occurs as a result of the osteoclasts at active resorption sites, which resorb bone partially through acidification and cause release of HAP-bound BPs. The unbound (active) BP is then endocytosed by local osteoclasts, leading to the osteoclasts’ cell death\textsuperscript{1, 13, 32}. This binding property has dramatic consequences as the effects of the drug can be prolonged long after discontinuation\textsuperscript{33, 34}, which explains extended dosing intervals with some N-BP medications.

In the nascent stages of bisphosphonate drug development, bisphosphonate drugs were employed as bone scintigraphy makers due to their dramatic ability to bind to the skeleton. These different drug-structure to mineral-binding-affinity relationships were further investigated and more bisphosphonates were designed, each with slightly different clinical impacts on the compromised skeleton. Some were directed to vertebral vs. non-vertebral sites, such as risendronate which has a more even distribution and an overall lower binding affinity\textsuperscript{35}. While the native structure of the drug affects affinity, the local pH has the capacity to alter these bonds to HAP, resulting in the release of the drug. This is mediated by a change in ionization of the side chain or phosphonate group, which alters the affinity for it’s site of attachment\textsuperscript{31}. 
Through it’s structural arrangement and interactions with the dynamic skeletal environment, bisphosphonate drugs exhibit a cycle of binding, release and reuptake in osseous tissue\textsuperscript{14}.

\textbf{D. Offset and onset of action}

The termination of drug effects (offset of action) for bisphosphonate medications is not clearly defined and long-term data that describes the clinical or molecular effects with time is not available. The early half-life of a dose of NBP is approximately ten days, whereas the terminal half life is approximately ten years (10.9 years for alendronate\textsuperscript{36,14,37}). The duration of effect of BP medications is demonstrated via several clinical studies. For instance, the FLEX study\textsuperscript{33} shows only a very small decline in BMD and no increase in non-vertebral fractures after an alendronate treatment time of 5 years followed by a drug holiday of 5 years vs. continuation of the drug. However, alendronate seemed to have a protective effect on vertebral fractures for those continuing with the drug. This is, however, opposed by the FIT trial\textsuperscript{34} where there was no decreased fracture incidence in the population of patients continuing on alendronate vs. drug holiday. On a molecular level, suppression of bone turnover markers has also been shown to continue during a five-year drug holiday, a residual effect supported by other work\textsuperscript{34,38,39}. Skeletal capacitance for bisphosphonate binding is unlimited and the function of bisphosphonates is self-protective in that it decreases bone turnover which is a factor for it’s release from osseous stores\textsuperscript{40}. It has been estimated that after an equivalent dose of alendronate 70 mg per week for 10 years, the amount of drug released over the ensuing months would be approximately 70 mg per month\textsuperscript{41}. Shorter-term data exists for a three-year treatment
regimen of risendronate 5mg daily and one-year drug holiday. Markers of bone turnover increased after this holiday and were equivalent to the control group who did not receive risendronate. The BMD decreased after the year off, however it remained higher than baseline and higher than in the patients who did not receive BP treatment. Similarly, there was a residual effect with vertebral fractures where the control group was at 46% higher risk of fracture. The markedly potent zoledronate, was reported by Eastell et al.\textsuperscript{40} to have a residual effect three-years after a one-time 5mg dose, almost halving bone turnover markers and increasing BMD by 4-7%. Longer remission times were noted during drug holiday in patients treated with pamidronate for Paget’s disease vs. patients not treated with the drug\textsuperscript{43}. These authors suggest that a treatment time of 3 months may yield a remission lasting almost a year as the biochemical effect, as measured by serum alkaline phosphatase and urine hydroxyproline levels, was reported to be quite dramatic after only two months of treatment, with the decrease tapering after 4 months. Overall, clinical and molecular investigations show a long duration of effects of BP medications, although precise data describing the termination of BP effects is not available.

E. Clinical indications for bisphosphonate treatment

i. Osteoporosis

The World Health Organization (WHO) defines osteoporosis as a bone mineral density (BMD) value greater than 2.5 SD below the young adult peak (T score > -2.5) \textsuperscript{44}. In addition, independent of BMD, those at-risk women with a low-force fracture can also be considered to have a clinical diagnosis of osteoporosis and an increased risk of future
fracture. The National Osteoporosis Foundation guidelines suggest women of advanced age with osteoporosis and an increased risk of fracture should undergo antiresorptive therapy\textsuperscript{45}.

Oral bisphosphonate medications, such as alendronate, are commonly prescribed for the treatment of osteoporosis which has resulted in the reduction of fracture incidence in patients with osteoporosis and a significant increase in spine and hip bone density\textsuperscript{46,47}. The effect of alendronate was prospectively evaluated by Black et al. in women with osteoporosis at a daily dose of 5 mg for 2 years, then 10 mg daily after the second year. Those with existing vertebral fractures received treatment for 3 years and those without were treated for 4 years. Those with a calcium intake below recommended daily values were prescribed a supplement with vitamin D (500mg and 150 IU, respectively). The results showed a statistically significant decrease in fracture risk of all clinical fractures including nonvertebral, clinical vertebral, hip and wrist sites\textsuperscript{48}. Two or 3 years of a daily alendronate dose of 10 mg in post-menopausal osteoporotic women experienced a 7-10% increase in the mean DMB from decreased bone remodeling\textsuperscript{29}.

\textit{ii. Multiple myeloma and malignancy-related osteolysis}

Nitrogenous bisphosphonates (such as pamidronate and zolendronic acid) are administered intravenously as part of the treatment regimen for patients with multiple myeloma and metastatic cancers in bone, interrupting the pathology of osteolytic lesions and the ensuring hypercalcemia, opposing cancer therapy-induced bone loss (CTIBL),
and improving the risk of pathologic fractures\textsuperscript{49, 50}. This approach has lead to an improved quality of live and longer life expectancy in cancer patients\textsuperscript{51}. Patients with bone metastases from breast cancer or multiple myeloma may receive zoledronate (ZOL) or pamidronate as part of their treatment regimen\textsuperscript{52}. Bone metastases from other solid tumor types, such as lung and prostate cancer, may be treated in part by ZOL\textsuperscript{53}. Zoledronate in particular has gained considerable attention in the cancer-research community as this newer, third-generation NBP seems to show promise for treatment of a broader spectrum of cancer types than originally suspected. Clinical trials suggest that ZOL treatment may reduce circulating and disseminated tumor cells, reduce recurrence and reduce residual invasive tumor size in breast cancer patients; it is not surprising that the overall effect is improved disease-free survival\textsuperscript{52, 54, 55}.

\textit{iii. Paget’s disease}

The main treatment of Paget’s disease is the prescription of potent bisphosphonate drugs, such as zoledronic acid\textsuperscript{56}. Biochemical markers of bone turnover, including serum alkaline phosphatase and urine hydroxyproline, radiographic signs of disease, pain and quality of life scores have all been studied after treatment with bisphosphonate medication\textsuperscript{57}. These indicators of remission have helped refine treatment regimens for patients with Paget’s disease.
III. BISPHOSPHONATE-RELATED OSTEONECROSIS OF THE JAWS (BRONJ)

A. Definition and Diagnosis

The description of exposed bone in 36 patients receiving IV bisphosphonate therapy by Dr. Marx in 2003 is the first widely-recognized report of BRONJ in the dental literature. The first reports of this condition engendered comparisons to earlier reports of the 19th century condition known as “phossy-jaw” or phosphorous necrosis of the jaw and to some this represents a similar 21st century disease. The diagnosis of BRONJ is based on clinical observation clinical and relies on the exclusion of head and neck radiation and the inclusion of a history of bisphosphonate therapy. The AAOMS guidelines typically required a history of exposed bone for at least eight weeks although there is disagreement regarding the duration of exposed bone required for diagnosis among clinicians and with the advent of the AAOMS inclusion of “Stage 0 BRONJ”, a diagnosis without frank bone exposure is recognized. Some consider a diagnosis of BRONJ with any duration of exposed bone or a mild-stage of BRONJ without bone exposure in the setting of other obscure findings, such as radiographic changes, in the context of a positive bisphosphonate and negative radiation history. The severity of BRONJ varies and is determined by clinical presentation. This ranges from mild, ill-defined radiographic findings to a larger volume of affected bone or more severe, infected, or fractured picture. For the purpose of this thesis, the 2009 AAOMS definition of BRONJ will be used.
B. Disease staging

In order to describe BRONJ, which has a broad clinical presentation, stages have been described in the literature to communicate severity and correlate acceptable treatment options (Table 2). The widely referenced classification system, established and most recently reviewed in 2009 by Ruggiero et al. for the American Academy of Oral and Maxillofacial Surgeons, has four stages: 0-3. This establishes the foundation for guidelines of therapy. The Spanish group led by Dr. Bagan\textsuperscript{61}, has aimed to evaluate the newest classification by Ruggiero et al.\textsuperscript{60} and further refine the descriptions as to not exclude a particular clinical subset based on a review of 120 cases. They have found that the limitation presented by the most popular updated staging system was in cases where there was no exposed necrotic bone, but the patients had other signs and symptoms that exceeded a Stage 0 classification (Table 2)\textsuperscript{62}.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Staging System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruggiero et al. 2009 Staging classification for the American Association of Oral and Maxillofacial Surgeons</td>
<td></td>
</tr>
<tr>
<td><strong>At-risk</strong></td>
<td>Patients that have been treated with PO or IV BPs, no apparent exposed/necrotic bone</td>
</tr>
<tr>
<td><strong>Stage 0</strong></td>
<td>No exposed bone, non-specific signs and symptoms</td>
</tr>
<tr>
<td><strong>Stage 1</strong></td>
<td>Exposed and necrotic bone, asymptomatic and have no evidence of infection</td>
</tr>
<tr>
<td><strong>Stage 2</strong></td>
<td>Exposed and necrotic bone with infection with pain and erythema in the area of exposed bone with or without purulence</td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td>Exposed and necrotic bone in patients with pain, infection, and ( \geq 1 ) of: osteolysis extending beyond the alveolus to Mx or Md border, pathologic fracture, extraoral fistula, oral antral/oral nasal communication</td>
</tr>
<tr>
<td>Bagan et al. 2012 Stage 3 from Ruggerio et al. is modified:</td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td>Exposed and necrotic bone or an oral fistula <strong>without</strong> exposed bone in patients with pain, infection, and one or more of the following: exposed necrotic bone extending beyond the alveolus (mandibular inferior border/ramus or maxillary sinus/zygoma), pathologic fracture, extraoral fistula, oral-antral/oral-nasal communication, or osteolysis extending to the inferior mandibular border or sinus floor</td>
</tr>
</tbody>
</table>
C. Incidence and risk factors

The incidence of BRONJ varies with mode of delivery, which is available in intravenous (IV) and oral (PO) formulations. Estimates of incidence range from 0.8% - 30% for IV vs. approximately 0.1% of PO recipients. Approximations of 1-10% have been sited after IV therapy and a traumatic insult.

i. Mode of administration and patient populations

With the ease of administration and solidly established clinical efficacy of oral bisphosphonate in the treatment of osteopenia/osteoporosis, PO bisphosphonate use has become very common (>5.4 million in the United States, 2007 data). In 2003, alendronate was listed as the 19th most commonly prescribed drug with 17 million prescriptions. The mode of administration affects the risk of developing BRONJ: the overall risk of developing BRONJ while under treatment with oral bisphosphonates is considered lower in comparison to IV administration and is reported to be 0.01% - 0.06%. In a retrospective study by Lo et al., the prevalence of BRONJ associated with PO bisphosphonate use was 0.10% among 8,572 survey respondents.

Intravenous administration is the route of choice for the treatment of patients with metastatic disease and malignancies with osseous events; consequently, these patients are at increased risk. Intravenous administration accounts for greater that 90% of reported cases and prevalence varies with treatment duration (1-5%). Bisphosphonates
administered as a component of oncologic treatment is associated with a 1-10% prevalence according to Khosla et al\textsuperscript{4}.

Approximately 95\% of osteonecrosis cases reviewed by Woo et al. and Marx et al. occur in cancer patients undergoing high-dose IV treatment cycles for prevention or treatment of their associated osseous disease (such as metastases to bone, osseous defects and hypercalcemia)\textsuperscript{68 3}. Marx et al. reviewed 119 cases of patients with BRONJ associated with either PO or IV therapy and report that 52.1\% of cases were undergoing IV bisphosphonate treatment for multiple myeloma\textsuperscript{3}. Woo et al. report that 85\% of the 94\% of patients with BRONJ associated with IV bisphosphonate therapy had a diagnosis of multiple myeloma or metastatic breast cancer\textsuperscript{68}.

Furthermore, in a prospective assessment by Bamias et al., 252 patients prescribed bisphosphonates for bone metastases were followed; 6.7\% developed BRONJ. The 6.7\% represented 111 patients, of which, 10\% percent had a diagnosis of multiple myeloma, 3\% percent with breast cancer, 7\% percent with prostate cancer and 4\% with other neoplasms\textsuperscript{63}. In another study of 124 patients with myeloma or breast cancer, 4 and 9 patients with myeloma and breast cancer, respectively, developed BRONJ (prevalence 10\%)\textsuperscript{69}.

In 2004, the International Myeloma Foundation conducted a web-based survey: of 904 respondents with a diagnosis of myeloma, 13\% reported osteonecrosis or lesions suspicious for osteonecrosis related to their intravenous therapy\textsuperscript{6}.
**ii. Duration**

Duration of bisphosphonate treatment is also related to the risk of developing BRONJ and the severity of its manifestation. Administration of PO bisphosphonates for $\geq 4$ years was associated with the highest prevalence of BRONJ in comparison to those on BP for $<4$ years (0.21% vs 0.04%, respectively) and no cases were associated with a treatment time less than 2.5 years $^{64}$. The duration of the PO bisphosphonate treatment was exponentially related to the size of the osseous exposure in this group of patients. Marx et al. report a range of 14.3 months (pamidronate) to 9.4 months (zoledronate) for the mean duration of drug therapy from onset of therapy to recognition of primary oral exposure; the few cases associated with alendronate occurred after a mean duration of treatment of 3 years $^{3}$. Bamias et al. found similar results in a subset of 252 myeloma patients on intravenous zolendronic acid in which the incidence of BRONJ increased from 1.5% in the first 4-12 months to 7.7% after treatment for 37-48 months.

**iii. Polypharmacy**

Other factors may contribute to the risk, including concomitant medication use with antiangiogenic capacity. This is not limited to the use of glucocorticoids, thalidomide and bortezomib in patients with multiple myeloma $^{7}$. However, the 2004 study by the International Myeloma Foundation did not note an increased risk with concurrent therapies such as corticosteroids or thalidomide $^{6}$. Maerevoet et al. in NEJM 2005 note
that main risk factors include dental procedures, poor dental hygiene and corticosteroid therapy \(^{70}\).

\begin{itemize}
  \item **iv. Patient health factors**
  
  Patient factors play a role as diabetes mellitus, peripheral vascular disease and hyperviscosity syndromes may also limit periosteal blood flow. This was also noted by the International Myeloma Foundation’s 2004 study found that a history of oral health issues was present in 81\% of patients with myeloma and 69\% of patients with breast cancer who had BRONJ in contrast to 33\% of patients without BRONJ. Diabetes has a multitude of effects on cellular function, including endothelial cell dysfunction \(^{71}\) and delayed wound healing \(^{72}\).
\end{itemize}

**D. Pathogenesis**

The current understanding of the pathogenesis of BRONJ is unclear. Several theories have been purported including the theory of decreased bone turnover, ischemia and avascular necrosis, tissue toxicity, and bacterial colonization and infection \(^{73}\).

BP medications interrupt the normal osteoclast resorption and feedback cycle. Normally, osteoclasts resorb bone and in doing so release bone morphogenic proteins and other growth factors and cytokines. This provides signaling to osteoblasts to induce bone formation and pluripotential cells to differentiate into osteo-competent cells, facilitating new bone formation. The effects of BPs are two-pronged: a reduction in the bone...
resorption by osteoclasts and a decrease in replenishment of bone by osteoblasts. The tissue that remains is then hypovascular and hypocellular.\(^{68,74}\)

\textit{i. Bacterial colonization/infection}

The role for microbial colonization in BRONJ pathogenesis is supported by many studies\(^ {73}\). These biofilms present on pathological specimens from patients with active BRONJ may represent a mixed population of 2-15 species with or without yeast (Candida) including Fusobacterium, bacillus, actinomyces, staphylococcus, Selenomonas and several treponemes\(^ {75}\). Actinomyces are often reported to be present on examined specimens\(^ {59,75,76}\), which is not surprising since they are primary colonizers of the oral cavity and are the most prominent in the oral cavity\(^ {77}\). More severe cases of actinomycosis of the jaws, a rare condition, has been described in patients with BRONJ. This may be related to the general condition of the patient and represent a subset of cancer patients who are either immunosuppressed or compromised by their primary condition\(^ {78}\). While it is presumable that bacterial stress may occur after the development of BRONJ, bacterial adhesion has been shown to increase on bisphosphonate-coated hydroxyapatite\(^ {79}\) which further supports the possibility that BRONJ may occur secondarily.

The common presence of bacterial colonies present on biopsied bone in BRONJ patients suggests that bacteria may play a role and whether the bacterial infection occurs primarily or secondarily in the pathogenesis of BRONJ is unclear. Over 500 microorganisms are present in the oral cavity and the obligate anaerobic \textit{Actinomyces} species are the most
prevalent. Likewise, this species, especially *A. israelii*, is consistently found in BRONJ sites. Further adherence of the *Actinomyces* to the BRONJ sites facilitates heterogeneous colonization from other bacterial species favouring infection. In a molecular profiling project of 12 sequestrectomy specimens from infected jaws, Wei et al. note differences between a control infection group (no BP exposure) and a BRONJ-related infection group. Here, the top three bacterium noted for BRONJ are *Streptococcus* (29%), *Eubacterium* (9%) and *Pseudoramibacter* (8%). Kaplan and others (2009) noted *Actinomyces* to be present in BRONJ - but the Wei group counters that they did not notice a prevalence of *Actinomyces* among the diversity of bacteria profiled and attribute previous accounts of this bacteria to the method of observation, namely, microscopy.

The colonization of *Actinomyces* in the oral cavity depends on the presence of Staphylococci and Streptococci strains. In prosthetic joints made of hydroxyapatite, increased bacterial adhesion, specifically *Staphylococcus aureus*, was demonstrated in those prostheses coated with BP (especially pamidronate); similarly, exposed BRONJ sites in patients show increased bacterial adhesion and the creation of an environment that favours chronic infection.

This increased adhesion to host surface proteins is mediated by the bacterial adhesins “microbial surface components which recognize adhesive matrix molecules” (MSCRAMM), which contain an amino-terminal domain. The binding of gram positive bacterial strains to these host surface molecules appears to be mediated by this amino domain. In the case of previous experiments involving S. aureus, it has been found that
the binding of the amino terminus is key in the pathogenesis of infections with this strain. The amino side chain in pamidronate may mimic host-proteins on the bone surface, thus binding the bacterial MSCRAMM. This is evidenced as non-amino containing BP have significantly less bacterial adherence. This increased MSCRAMM interaction may create a local propensity for infection.

Furthermore, multiplying bacteria in the oral cavity may lead to a host response that creates an environment that favours osseous resorption. Host inflammation is stimulated and matrix metalloproteinases can contribute to this destructive process.

The contribution of the localized infection to the pathophysiology of BRONJ may be explained via the osteoclast-independent bone resorption model.

a. Bacterial role in osteoclast-independent bone resorption

Bacteria may directly destroy bone via the release of proteases and acids which may explain the radiolucent areas of bone loss noted clinically in this otherwise resorption-impaired condition. In addition, bacteria may instigate a cascade of osteoclast and osteoblast processes that lead to bone destruction. The lipopolysaccharide (LPS) component of bacteria is hypothesized to act through the stimulation of osteoblasts; these osteoblasts release factors, which then recruit osteoclasts or activate them. This is supported by the work of Ishihara et al. who demonstrate that LPS-induced bone destruction can be mitigated with anti-inflammatory drugs, such as indomethacin or anti-IL-1.
antiserum. Furthermore, bacteria may inhibit bone matrix synthesis altering the normal course of healing and repair. There are several important mediators of host inflammation and subsequent osseous resorption, which are induced by LPS, including: IL-6, chemokine CCL-5 and PGE2. The interleukin IL-6 has been shown to promote bone resorption and is involved with differentiation of osteoclasts, a large CCL-5 response can lead to an exaggerated inflammatory cell infiltrate and harm to the alveolus and PGE2 can induce the formation of osteoclasts and PGE2 may also attenuate fibroblast-mediated tissue repair and therefore potentially soft tissue healing response after an insult. In terms of BRONJ pathogenesis, these factors become interwoven when considering the BRONJ site as a bacterially-laden necrotic zone, replete with host-mediated inflammatory factors and a propensity toward bone destruction and soft tissue repair latency.

**ii. Trauma**

Trauma is considered to be one of the major determinants of the development of BRONJ. Patients who undergo a traumatic dentoalveolar intervention after prolonged IV treatment of bisphosphonates with an aminoterminal group, such as pamidronate, or a nitrogen cyclic side chain, such as zoledronate, are challenged with the greatest risk of developing BRONJ. Trauma resulted in greater areas of exposure than those who did not have any intervention. Novartis, the manufacturer of pamidronate and zoledronate, released guidelines in 2004 which highlighted the risk of developing BRONJ after traumatic
Trauma is generally associated with the onset of mucosal breakdown and chronic bone exposure, however, spontaneous cases have been reported. In their case series of 36 patients, Marx et al. report a 50% occurrence of spontaneous cases of BRONJ in a nitrogen-containing bisphosphonate (alendronate or risedronate) therapy group. These findings are nearly reproduced in the retrospective analysis of 35 patients with BRONJ by Pozzi et al. who report that 51% of the cases were spontaneous compared to 49% which were the sequelae of dental procedures.

iii. Anti-angiogenesis and matrix necrosis

Bisphosphonates have been shown to inhibit angiogenesis, which may play a part in the development of BRONJ.

iv. Microdamage

Bisphosphonate therapy is associated with decreased bone remodeling and a change in osseous biomechanical properties. The bisphosphonates risedronate and alendronate have been shown in an experimental beagle dog rib cortex model (treated for one year) to allow accumulation of microdamage and suppress targeted and non-targeted bone remodeling. Where osseous cracks would normally initiate the repair process, this phenomenon was blunted in alendronate-treated subjects. This property was also
observed to be diminished in the stochastically distributed zones of remodeling resulting in widely dispersed decreased osseous toughness. A similar high-dose BP model examining vertebral, iliac and femoral regions under mechanical duress demonstrated significantly suppressed trabecular remodeling after 12 months of administration of either drug. Microdamage at each site tested was observed to be significantly greater in the BP groups vs. the control population again showing decreased bone toughness. The suppression of trabecular bone turnover is, however, associated with augmented strength in vertebral sites in spite of the microdamage and the reduction in the intrinsic energy absorption of trabecular bone. This is further supported by research which also showed increased osseous fragility in dog rib cortex. Prolonged or high-dose therapy may yield biochemical properties similar to osteopetrosis with increased bone density and impaired remodeling.

v. Soft tissue toxicity

While bisphosphonate drugs target osteoclasts, they affect cells globally and may affect local oral soft tissue health. When discs of bone treated with bisphosphonates are plated with overlying human epithelial or Chinese hamster ovary cells, growth inhibition in the cell lineage is remarkable after 2-3 days with decreased cell adhesion and reduced cell population numbers. The loss of effective prenylation was notable in the cell lineages, which is a known effect of inhibition in the mevalonate pathway. Furthermore, zoledronate given to mice have been show to inhibit both endothelial cell function and angiogenesis. Moreover, NBPs have been shown to affect oral mucosal soft tissue. In
an *in vitro* study of normal human oral keratinocytes (NHOK) and fibroblasts (NHOF), Kim *et al.* demonstrate the deleterious effect of pamidronate. The BPs applied to the cell cultures inhibited proliferation and migration of both cell groups by triggering apoptosis in NHOF cultures and leading to senescence in NHOK\textsuperscript{101}. The authors note that senescence is inversely related to the speed of wound closure\textsuperscript{102}: these factors may work in concert to impair the soft tissue healing of BP recipients.

**E. Treatment**

**i. Discontinuation of bisphosphonate therapy**

If the condition of the patient allows, a discontinuation of bisphosphonate administration may be recommended. A three month pre- and post-treatment holiday may be suggested to decrease the risk of BRONJ associated with a traumatic oral intervention. Longer-term discontinuation (6-12 months) may help resolve symptoms and can lead to spontaneous exfoliation of sequestrated necrotic bone and stabilization of other sites\textsuperscript{60}.

**a. Effects of discontinuation of bisphosphonate therapy**

Discontinuation of BP treatment does not yield an acceleration in bone loss as noted by Tonino *et al.* in 2000. In a clinical trial cohort of postmenopausal women on alendronate for 5 years for anti-resorptive therapy, there was no significant decrease in BMD at the spine or hip after discontinuation of their medication and
replacement with a placebo. A small but significant decrease was noted in the total body and forearm regions. Continuous treatment, however, did show improved skeletal outcomes in comparison to abbreviated treatment. Black et al. report that a decrease in fracture risk is notable early on in BP pharmacologic intervention.

**ii. Conservative and Invasive Treatments**

The treatments used vary according to the clinical stage of BRONJ. Patient education and impeccable oral hygiene and health is enforced early on, while antimicrobial rinses, such as chlorhexidine suspension, and antibiotics taken PO are used in stages I and II. If antibiotics do not offer resolution, or the patient presents with Stage III BRONJ, surgical debridement may be indicated. Mobile necrotic segments are removed while minimizing trauma to the adjacent sites.

**F. Biomarkers and BRONJ**

**i. The definition of a biomarker**

In order to monitor a patient’s disease status or response to an intervention, a biological marker (such as “a chemical, its metabolite, or the product of an interaction between a chemical and some target molecule or cell” in the body may serve as an indicator or indirect measure. The Center for Biomarkers in Imaging at the Massachusetts General Hospital defines biomarkers as “…anatomic, physiologic, biochemical, or
molecular parameters associated with the presence and severity of specific disease states. Biomarkers are detectable and measurable by a variety of methods including physical examination, laboratory assays and medical imaging. The context of the biomarker in question may change the accepted definition (such as in environmental or industrial research) and will be limited to the aforementioned definition in the context of this thesis focused on human medicine. In medicine, they may help to diagnose, prognosticate or predict outcomes, such as patient response to a drug therapy. There are several types of biomarkers, including molecular or biochemical biomarkers (eg. proteins), physiologic biomarkers (eg. basal metabolic rate), and anatomic biomarkers (eg. size of an anatomic structure).

**ii. Biomarker testing in BRONJ**

In order to help guide clinicians’ and patients’ decision-making for cases involving a history of BP use, research has turned to the possibility of using biomarkers. Specifically, the morning fasting serum C-terminal telopeptide (CTX) level has garnered the most attention in the dental literature, although investigators have also looked at other markers, such as alkaline phosphatase, as potential biomarkers in this application. In spite of the attention and support from Dr. Marx to employ CTX values as a reference for assessment and decision-making based on his observations of trends in a sample of 30 patients, CTX has not been validated as marker for BRONJ and the interpretation of the results of this test remains to be solidified. Furthermore, The American Society for Bone and Mineral Research Task Force on Osteonecrosis of the Jaw stresses that CTX is an established marker for bone resorption and that it’s serum level may serve as an indicator for therapeutic efficacy of bisphosphonate medications, so it is expected that
its level would be decreased in patients on a bisphosphonate medication and warn against adopting the practice of using CTX as a therapeutic guide\textsuperscript{114}.

\section*{IV. NEUTROPHILS}

\subsection*{A. Neutrophil function in the oral cavity}

Neutrophils are the most numerous and critical cellular elements of the innate immune system. They are important surveyors in the oral cavity and protect against bacterial and fungal invasion, helping to maintain a healthy equilibrium by recognizing, phagocytosing and killing microorganisms\textsuperscript{115, 115, 116}. Some patients who are taking bisphosphonate medications may have neutrophils that have become altered by the drug\textsuperscript{117}. The neutrophils in bisphosphonate-exposed mice have demonstrated dampened chemotaxis and reactive oxygen species-killing\textsuperscript{117}. This alteration in neutrophil function may help to explain why a subset of patients on bisphosphonate medications have chronic soft-tissue defects that may develop into BRONJ.

\subsection*{B. Neutrophil and epithelial cell cooperation}

Epithelial cells play an essential role in the physical barrier against microbes, but their function in primary innate defense is more dynamic than is often given credit for. The generation of antimicrobial peptides by epithelial cells occurs in response to infection\textsuperscript{118, 119} and in response to growth factors released after the epithelial integrity is breeched\textsuperscript{120}. The arrival of neutrophils from blood vessels into this layer results in neutrophil cytokine release, further supporting the aforementioned epithelial cell functions. The regional
epithelial cell changes (mucosal breakdown) recruit and activate otherwise quiescent neutrophils circulating in blood. Other molecules, such as urokinase plasminogen activator, spur the proliferation, migration and adhesion of keratinocytes, fibroblasts and endothelial cells in skin wounds\textsuperscript{121}. The production of such molecules is upregulated during inflammation and wound healing. These essential components of innate immunity work in concert to provide defense: one such peptide in this cooperative effort is a member of the cathelicidin family of antimicrobial molecules (LL-37) which is released from neutrophil granules to perform antimicrobial, chemotactic (for more neutrophils, monocytes and T-lymphocytes) and epithelial cell signaling functions\textsuperscript{122-125}. The epithelial interaction of neutrophil-sourced LL-37 results in epithelial gene activation. This type of interaction may extend to the keratinocyte subset where local neutrophils donate LL-37-related hCAP-18 for epithelial cell – and keratinocyte- stimulation and angiogenesis.\textsuperscript{126} More directly, neutrophils may secrete stimuli that activate this cell lineage, such as IL-8 and IL-1B, and VEGF, which stimulates angiogenesis, promoting wound healing and infection defense\textsuperscript{127}.

C. Neutrophil stimulation via receptors

Neutrophils are able to hone in on foreign targets via cell-surface receptors, including those for leukotriene B\textsubscript{4}\textsuperscript{128} and CXC\textsuperscript{129}, leading to ligation, phagocytosis and oxidative responses. Other cell-surface receptors, such as the fMLP receptor, function as chemotactic receptors by binding bacterial N-formylated peptides, which are potent pro-inflammatory mediators\textsuperscript{116}. Upon target identification and phagocytosis, the neutrophil’s enzyme-filled lysosome fuses with the phagosome, leading to pathogen destruction. Part
of the killing mechanism involves activation of the NADPH oxidase complex, which yields a toxic respiratory burst of hydrogen peroxide (H2O2), the superoxide anion (O2-) and nitric oxide (NO)\textsuperscript{116}.

The number of neutrophilic receptors for the lipid PMA ligand (which is soluble at the level of the cell membrane and may enter the cell to exert its effects) which are bound and occupied by PMA governs the extent of activation of the respiratory burst\textsuperscript{130}. This, in turn, sets off a signaling cascade that results in chemotaxis, phagocytosis, and respiratory burst.

In samples from BRONJ sites, bacterial colonization and the presence of \textit{Actinomyces} species are common\textsuperscript{74}. While other factors, such as microtrauma, alteration in bone biomechanics\textsuperscript{47} and tissue toxicity\textsuperscript{99} may contribute in the pathogenesis of BRONJ, bacterial challenge and inadequate immune response are important players among the host of influences in BRONJ development.

\textbf{D. Oral neutrophil activity reflects blood neutrophil activity}

Oral neutrophils are sampled in this study since this technique has proven useful in monitoring neutrophil mobilization to peripheral sites and functionality after transplantation of hematopoietic stem cells\textsuperscript{131}.

Neutrophil migration throughout the body is an essential part of effective innate immunity. Consequently, neutrophils are able to migrate into the oral cavity, where they may be collected by a simple oral rinse, counted and tested. Research from the Glogauer laboratory has bolstered the place for a simple oral rinse sample by demonstrating that it
may be used to survey a patients’ neutrophil count in the setting of periodontal health, resilience to infection, and neutrophil population after hematopoietic stem cell transplantation (HSCT).

This simple non-invasive test has been shown to monitor neutrophil counts in patients being treated with chemotherapeutic agents. Side effects of these cytotoxic agents includes profound neutropenia and monitoring of it’s recovery or onset is essential in patient care. This research actually demonstrated that while neutrophils collected from the oral cavity echoed the blood neutrophil trends, they reflected the patient’s clinical status more precisely than blood neutrophils. Similar findings by Cheretakis et al. as part of the Glogauer laboratory showed that a non-invasive oral rinse may also effectively survey the success of HSCT in a pediatric population. They echoed circulating blood neutrophil counts and clinical findings reflecting susceptibility to infection. The oral rinse samples showed that oral neutrophils reappeared almost a week earlier than those collected from the venous circulation. Here, the test is both reflective of the blood neutrophils and clinical presentation while being slightly more precise than the blood neutrophils. In 2012, Forster et al. have shown the same in an adult population of 29 patients 6 months post-HSCT.

E. Steroid effects on neutrophil function

Steroids are known to have a depressive effect on neutrophil function. In a study of post-graduate students experiencing high systemic cortisol levels in the context of an approaching final examination, increased plasma cortisol levels were associated with a diminished superoxide generation. These findings were reinforced with an in vitro
study that demonstrated that human neutrophils exposed to hydrocortisone yielded less superoxide\textsuperscript{136}. In addition, some sex steroids (estradiol, testosterone, progesterone) and cortisol may decrease superoxide yield from neutrophils, which has been demonstrated in vitro by Bekesi et al.\textsuperscript{137}. Dexamethasone was found to suppress superoxide production (32\% inhibition, P<0.001) but was reported to not have approached the depressive levels that the investigated sex hormones (estradiol, progesterone) achieved in simultaneous experiments\textsuperscript{138}. Systemic glucocorticoids may be taken to mitigate inflammatory disease (such as rheumatoid arthritis or systemic lupus erythematosus) by suppressing proinflammatory genes\textsuperscript{139, 140} and that much of this action works at the level of the macrophage and neutrophil\textsuperscript{140}. More specifically, Tsuji and Shioya have shown that a glucocorticoid (methylprednisolone) decreases the neutrophil superoxide production in vivo\textsuperscript{141}. 
Hypothesis and Objectives of Thesis

Bisphosphonate medications offer promising treatment for many disease states including malignant processes when administered intravenously. While these drugs offer the promise of quiescence or recovery, the complication of jaw osteonecrosis that results can range from 1-10% of patients who then undergo dental treatment\textsuperscript{142}. This results as normal osseous healing and remodeling cannot occur in the setting of osteoclast inhibition. The pathophysiology of bisphosphonate-related osteonecrosis of the jaws is not completely understood, however, a common denominator among this group of patients is the presence of bacterial colonies on necrotic bone biopsies\textsuperscript{60}. This suggests that the innate immune defense may be playing an insufficient role in this subset of patients. In order to evaluate if innate immunity may be decreased in patients with BRONJ, this thesis evaluates the function of patient neutrophils, the major players in the maintenance of health and healing. The hypothesis of this thesis is that neutrophil function is diminished in patients with a diagnosis of BRONJ and in patients who have begun IV bisphosphonate therapy, potentially serving as a biomarker for the subset of patients on bisphosphonate therapy who will develop the condition.
The objectives of this thesis were:

**Determine if there is a functional impairment in neutrophils in patients on bisphosphonate medication**

i) **Analyze blood and oral neutrophil function in patients with a diagnosis of BRONJ**

Patients attending an out-patient clinic with a diagnosis of BRONJ were asked to supply a history related to BRONJ risk-factors and a small sample of venous blood and saliva. The neutrophils were collected from the whole blood, counted and processed with a reactive oxygen species (ROS) assay and the oral neutrophils were filtered from the saliva. These were compared to healthy controls. This assay provides insight into the neutrophil’s capacity to provide bacterial killing at the BRONJ sites.

ii) **Analyze blood and oral neutrophil function in patients before and after bisphosphonate treatment**

In order to analyze if the bisphosphonates affected the neutrophils from patients without BRONJ once they had begun therapy, whole blood and salivary samples were collected from bisphosphonate-naïve patients before and after beginning IV pamidronate therapy. These patients all share a recent diagnosis of multiple myeloma and were initiated on similar chemotherapeutic and identical bisphosphonate regimens.
Study Design

In order to satisfy objective i):
Patients with BRONJ are recruited to Mt. Sinai from the OMFS clinic and the GTA for oral and blood sampling in the clinic and oxygen radical assay testing of these samples in the laboratory.

In order to satisfy objectives ii):
Patients new to BP were recruited from dentistry clinic for testing pre- and post-pamidronate via oral and blood sampling and oxygen radical assay testing of these samples in the laboratory.

In order to satisfy objectives i and ii)
Control samples will be collected each day patient samples are collected and processed that day in the laboratory. Those eligible for sampling include those who can provide informed consent and deny taking any medications, health problems, smoking, drug-use or heavy alcohol consumption.
CHAPTER 2

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 Study population 1: Patients with BRONJ

Patients attending regular follow-up appointments with the Oral and Maxillofacial Surgery Clinic at the Mount Sinai Hospital in Toronto, Canada, with a diagnosis of BRONJ were enrolled in this pilot study between December 2010 and August 2012. A medical history was collected for all participants and clinical histories were reviewed for staging of BRONJ. The severity was staged according to the classification system outlined in the AAOMS 2009 BRONJ Position Paper. These patients with a diagnosis of BRONJ met the following criteria: 1) were receiving or had received a bisphosphonate medication; 2) did not have a history of radiotherapy in the head and neck region; and 3) presented with non-specific clinical or radiographic changes with or without exposed and necrotic bone in the maxillofacial region. Blood and oral rinse samples were collected from subjects with an additional twenty-two volunteers serving as healthy controls. The healthy control subjects denied prior exposure to bisphosphonate treatment or concomitant medications and denied significant social history (smoking, drug use, heavy alcohol consumption). The healthy controls were not age-matched; neutrophil functions including phagocytosis, ROS-generation, and SOD activity are not significantly affected by ageing as shown by Niwa et al. This study was approved by both the Research Ethics Board at Mt. Sinai Hospital REB#19-311.
2.2 Study population 2: Patients without BRONJ new to BP therapy

Patients referred to the Princess Margaret Hospital Dental Clinic for pre-pamidronate dental evaluation were recruited to participate in the study. Patients who met the inclusion criteria had a recent diagnosis of multiple myeloma, no previous bisphosphonate treatment, no history of radiation to the jaw and consent to participate. A healthy control oral rinse and blood sample was processed in concert with the patient samples mentioned in 2.1 Study population 1. This study was approved by both the Research Ethics Board at Princess Margaret Hospital REB#10-09-36-ae.¹

¹ Note about random neutrophil collection.
It is notable that count, along with many other hematological parameters (melatonin, iron, transferrin, transferrin saturation, ferritin, cobalamin, folate, red blood cells and white blood cells), shows diurnal variation¹⁴⁸ however neutropenia was not an issue in this study.

It has also been shown that the enzyme G6PD, which is involved in the production of NADPH, a key element in the pathway to ROS production in neutrophils, fluctuates diurnally. Interestingly in this study, even in patients with <1% of control levels of this enzyme (G6PD-deficient patient group. 50 with p) the bactericidal capacity of these neutrophils was normal, which agree with other literature. The authors suggest that there may be enough NADPH present to support adequate ROS production or that other compensatory mechanisms may exist to support adequate NADPH¹⁴⁹.

In addition, the diurnal variation of G6PD activity in the control patients in Wolach’s study is not statistically significant (8 am vs. 1300h p = 0.493, 1300h vs 1800h p = 0.446, and 8 am vs. 1800h eve p = 0.448)¹⁴⁹. Actual ROS generation was not measured for diurnal fluctuation, although it is known to be positively correlated with G6PD activity¹⁴⁹. Again, there may be other compensatory mechanisms involved in spite of fluctuating levels of elements in ROS-generation pathway. Actual variation of ROS production diurnally has not been measured.
2.3 Blood sample neutrophil preparation

Blood samples were collected and immediately transported to the Glogauer laboratory for evaluation of reactive oxygen species (ROS) formation using a bench-top assay. The samples were coded and blinded to the evaluator. A blood sample from a healthy control volunteer was collected and processed each day that samples were collected from a patient. In order to isolate neutrophils, the 10 cc blood samples were drawn into a citrate-containing vacutainer. Neutrophils were isolated using a one-step neutrophil isolation solution. A 4.0 cc aliquot of blood was layered carefully over 4.0 cc of this solution (polymorphoprep; sodium metrizoate, 13.8% [weight/volume (wt/vol)]; and dextran 500, 8.0% [wt/vol]), and the mixture then centrifuged at 1,600 revolutions per minute (rpm) for 30 minutes at 4°C. The lower of the two translucent bands containing neutrophils was collected and washed in Hanks balanced salt solution centrifuged at 2,500 rpm for 5 minutes. Finally, a wash in 1 cc distilled water at 2,500 rpm for 5 minutes served to lyse remaining erythrocytes and concentrate the neutrophil pellet. The pellet was resuspended in 1 cc Hank’s balanced salt solution (HBSS) and neutrophils were counted with a hemocytometer. This method yields neutrophils with >95% cell viability as determined by hematoxylin and eosin staining.

2.4 Oral rinse neutrophil collection and isolation

Oral neutrophils were collected using a rinse of 3 cc of sterile 0.9% normal saline. The patients were instructed to rinse with this solution for one minute and expectorate into a
collection tube. This was repeated three times with 3-minute intervals separating the rinses to allow for oral neutrophils to repopulate the mouth. The samples were processed via sequential filtration starting with a 40 µm Nylon Net Filter (Millipore®), then a 20 µm and finally a 10 µm filter. The collected cells were then washed in HBSS and centrifuged for 10 minutes at 3,500 rpm. They were re-suspended in 0.5 mL of HBSS and neutrophils counted with a hemocytometer as with the peripheral blood neutrophils.

2.5 Neutrophil stimulation and reactive oxygen species formation

A neutrophil suspension at 1x10^7/cc concentration in phosphate buffered saline (PBS)* with 10 mM D-glucose™ was made and kept on ice. Aliquots of 0.1 cc (1 x 10⁶ cells) of neutrophil stock were combined with 1.76 cc of PiCM-G buffer¶¶ and 10 µL of equine horse ferricytochrome c§§ (0.1 mM final) in the sample cuvettes. The reference cuvette was prepared in the same fashion and the PiCM-G buffer was replaced with 10 µL (total 100 µg) of 5 mg/mL concentration of superoxide dismutase (SOD)|||. The cuvettes were then incubated at 37 °C for 10 minutes on a shaker prior to neutrophil stimulation. For each sample, one cuvette was stimulated with 10⁻⁵ M phorbol myristate acetate (PMA)** and another with 10⁻⁶ M formyl-met-leu-phe (fMLP)***. The stimuli were added simultaneously to the cuvettes (10 µL) and the time of the addition was noted. The rate of reactive oxygen species formation was quantified using a spectrophotometer set to 550 nm with a head-on photomultiplier tube; the absorbance of the reduced cytochrome c was measured at 5 minutes and then at 30 minutes when the rate of increased absorbance tapered off substantially.
2.6 **Statistics**

To measure the burst capacity of the neutrophils, the spectrophotometric value measured after the neutrophils were exposed to a stimulus (either fMLP or PMA) and compared to the value after the neutrophils were exposed to no stimulus (sham). By dividing the ROS yield after stimulation with one of the ligands by the unstimulated (sham) ROS quantity, a fold-change in reactive oxygen species is established. This number used for comparison between groups. Statistical significance was obtained using a Student’s T-test. A $P$ value of $<0.05$ was considered to be significant. Data are expressed as mean ± standard error of mean unless otherwise noted.
CHAPTER 3

RESULTS

Acknowledgements: We thank Dr. Donna Reece at the Princess Margaret Hospital Multiple Myeloma Clinic and the nurses at Princess Margaret Hospital for their assistance.
3. RESULTS

3.1 Photographs of BRONJ

Figure 3.1a Example of exposed bone in the left maxilla.
Figure 3.1b Example of exposed bone in the left maxilla as seen on a panoramic radiograph.
3.1c Example of exposed bone in the right mandible

3.1d Example of exposed bone in the right mandible as seen on panoramic radiograph
3.1e Example of BRONJ in the maxilla on CT scan, axial slice
3.1f Example of BRONJ in the mandible on CT ccan, axial slice (BRONJ 0)
3.2 Study population 1: Patients with BRONJ

i. Patient population

Of the Mt. Sinai Hospital population, twenty-three patients were candidates for the study. Eighteen patients were recruited for the study with a mean age of 71.2 years (Table 3a, 3b) and five patients declined to participate. Eleven out of the eighteen were female patients and the remaining seven were male. Seven patients had a history of treatment for multiple myeloma and had been on intravenous bisphosphonate medication (5 pamidronate, 2 zoledronic acid). In spite of one such patient’s six-year bisphosphonate
therapy, his stage of BRONJ was determined to be 1; likewise, one female patient had been on pamidronate for one year but also presented with stage 0 BRONJ and a male patient on zolendronic acid for a year and a half had stage 1. The remaining patients on intravenous medication presented with more advanced BRONJ, where four had stage 2. Of the remaining eleven patients, all on medication administered po, four were stage 0, two were stage 1, three were stage 2 and two were stage 3 with both oral-antral involvement and osteonecrosis to the inferior border of the mandible.

The risk factors identified, as outlined in the AAOMS Position Paper on BRONJ\textsuperscript{2}, included history of steroid use (7 patients, 39%; prednisone), diabetes mellitus (4 patients, 22%), and smoking (3 patients, 17%). Two patients had a history of more than one risk factor; one with a history of steroid therapy and previous smoking and the other patient reported diabetes mellitus and a remote smoking history.
Table 3a. Summary of Study population demographics (neutrophil reactive oxygen species assay): Patients with BRONJ

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Stage (0-3)</th>
<th>Drug type and Duration</th>
<th>Last BP Dose</th>
<th>Concomitant Risk Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 71.2</td>
<td>11 F (61%)</td>
<td>9 mild (0,1)</td>
<td>7 IV (39%)</td>
<td>Mean 1.5 years</td>
<td>7 systemic corticosteroids 3 smoking 4 diabetes mellitus</td>
</tr>
<tr>
<td>Range 57-90</td>
<td>7 M (39%)</td>
<td>9 advanced (2,3)</td>
<td>11 PO (61%)</td>
<td>Range ongoing – 6.8 years</td>
<td></td>
</tr>
</tbody>
</table>

Table 3b. Study population demographics (neutrophil reactive oxygen species assay)

Patients with BRONJ

<p>| Patients with bisphosphonate-related osteonecrosis of the jaws |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Stage</th>
<th>Drug Type and Duration</th>
<th>Last BP Dose</th>
<th>Concomitant Risk Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>F</td>
<td>0</td>
<td>Pamidronate IV 1 year</td>
<td>3 mos</td>
<td>Previous Smoking (quit)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Steroid</td>
</tr>
<tr>
<td>59</td>
<td>M</td>
<td>2</td>
<td>Risedronate PO 10 years</td>
<td>9 mos</td>
<td>Previous Smoking (quit)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aledronic acid PO 1 year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>F</td>
<td>0</td>
<td>Risedronate PO 5 years</td>
<td>6 mos</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>F</td>
<td>1</td>
<td>Alendronic acid PO 6 years</td>
<td>1.8 years</td>
<td>Steroid</td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>2</td>
<td>Alendronic acid PO 6 years</td>
<td>8 mos</td>
<td>Steroid</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>1</td>
<td>Pamidronate IV 7 years</td>
<td>6 years</td>
<td>-</td>
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<tr>
<td>67</td>
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<td>ongoing</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Diabetes Mellitus</td>
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<td>4 years</td>
<td></td>
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<tr>
<td>70</td>
<td>F</td>
<td>2</td>
<td>Alendronic acid PO</td>
<td>6 mos</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>Steroid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>5.5 years</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>M</td>
<td>2</td>
<td>Pamidronate IV</td>
<td>2.8 years</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Diabetes Mellitus</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>4 years</td>
<td></td>
</tr>
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<td>74</td>
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<td>1</td>
<td>Risedronate PO</td>
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<td></td>
<td></td>
<td></td>
<td>5 years</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>F</td>
<td>0</td>
<td>Alendronic acid PO</td>
<td>1 year</td>
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<td></td>
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<td></td>
<td>10 years</td>
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<td>75</td>
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<td>3</td>
<td>Risedronate PO</td>
<td>1 mo</td>
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<td></td>
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<td></td>
<td></td>
<td>Previous Smoking (quit)</td>
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<td>6.5 years</td>
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<tr>
<td>77</td>
<td>F</td>
<td>3</td>
<td>Alendronic acid PO</td>
<td>1.8 years</td>
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<td></td>
<td>5.5 years</td>
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<tr>
<td>77</td>
<td>M</td>
<td>1</td>
<td>Zolendronic acid IV 1.5 years</td>
<td>10 mos</td>
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<td>Steroid</td>
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<td>F</td>
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<td>Pamidronate IV</td>
<td>1 week</td>
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<td></td>
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<td>Steroid</td>
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<td>8 months</td>
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<td>Zolendronic acid IV</td>
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<td></td>
<td>7 months</td>
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<tr>
<td>84</td>
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<td>Alendronic acid PO</td>
<td>6.8 years</td>
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<td>3 years</td>
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<td></td>
<td>Steroid</td>
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<td>3 years</td>
<td></td>
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<td>90</td>
<td>M</td>
<td>2</td>
<td>Pamidronate IV</td>
<td>2 years</td>
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<td></td>
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<td>-</td>
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<td></td>
<td>4 years</td>
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</tr>
</tbody>
</table>
ii. Peripheral blood neutrophils: respiratory burst

The ROS production was measured revealing that the neutrophils isolated from the bisphosphonate-treated patients’ blood yielded lower levels of oxygen radicals as measured by cytochrome c reduction and spectrophotometry ($\lambda = 550$ nm) compared to healthy controls after stimulation with fMLP or PMA. The quantification of the fold change in ROS formation was obtained by dividing the mean channel fluorescence of neutrophils stimulated with fMLP or PMA (stimulated) divided by the mean channel fluorescence of neutrophils without stimulator (sham).

The bisphosphonate-treated patients’ samples showed lower levels of oxygen radicals after stimulation with $10^{-6}$M fMLP or when stimulated with $10^{-5}$M PMA in standard PBS in comparison to the respective control groups (Figure 4). In comparison to the healthy bisphosphonate-naïve controls, ROS generation in peripheral blood neutrophils was an average of 47% and 34% lower for the BRONJ samples stimulated with fMLP and PMA, respectively. This difference is statistically significant for the fMLP stimulus.
Figure 4. Fold change in the reactive oxygen species generated by the peripheral blood neutrophils from patients with BRONJ after stimulation with fMLP (14 control, 12 BRONJ patients) or PMA (15 control, 15 BRONJ patients). Blood neutrophil ROS response is decreased in patients with BRONJ. In order to determine if peripheral blood neutrophil respiratory burst response was impaired in patients with BRONJ, blood neutrophils were isolated and stimulated with fMLP or PMA and the ROS yield quantified. As shown in this figure, less reactive oxygen species were generated by the blood neutrophils from patients with BRONJ after stimulation with fMLP or PMA than by controls.
Grossly, two severities of BRONJ exist in the patient pool sampled: mild-moderate (stages 0-1) and moderate-severe (stages 2-3). There was no statistically significant difference in ROS generation between the mild-moderate and the moderate-severe groups (Figure 5).

Figure 5. Blood neutrophil ROS yield in mild vs. advanced BRONJ. Neutrophils were isolated from peripheral blood and stimulated with fMLP or PMA to generate a respiratory burst, which was evaluated by spectrophotometry. The results were grouped by the corresponding patient’s severity, where stages 0-1 were considered mild and stages 2-3 were considered advanced. There was no notable difference in the results from the samples from patients with BRONJ stages 0-1 (6 stimulated with fMLP, 8 with PMA) and stages 2-3 (7 stimulated with fMLP, 9 with PMA.
Figure 6. Blood neutrophil ROS yield from patients with BRONJ from IV vs PO BP medications. Neutrophils were isolated from peripheral blood and stimulated with fMLP or PMA to generate reactive oxygen species. The results were compared in the group taking oral BP medications (10 stimulated with PMA, 8 stimulated with fMLP) and intravenous BP medications (7 stimulated with PMA, 6 stimulated with fMLP). There is no statistically significant difference between the ROS yield in patients with BRONJ with different routes of BP delivery.

iii. Oral neutrophils: respiratory burst

The average ROS yield from the healthy bisphosphonate-naive oral neutrophils was greater than that of the BRONJ oral neutrophils when tested with both fMLP and PMA (15% and 40% greater response, respectively). These findings echo the corresponding patients’ blood neutrophil results (Figures 4 and 6) and were statistically significant for
the neutrophils stimulated with PMA. There was no statistically significant difference between the ROS response from the two severities of BRONJ (Figure 8).

Figure 7. Fold change in the reactive oxygen species generated by the oral neutrophils from patients with BRONJ after stimulation with fMLP (12 control, 9 BRONJ) or PMA (16 control, 15 BRONJ).
Figure 8. Oral neutrophil ROS yield in mild vs. advanced BRONJ. Neutrophils were isolated from the oral cavity and stimulated with fMLP or PMA to generate a respiratory burst, which was evaluated by spectrophotometry. The results were grouped by the corresponding patient’s severity, where stages 0-1 were considered mild and stages 2-3 were considered advanced. There was no notable difference in the results from the samples from patients with BRONJ stages 0-1 (6 stimulated with fMLP, 7 with PMA) and stages 2-3 (3 stimulated with fMLP, 8 with PMA).
Figure 9. Fold change in ROS yield from oral neutrophil samples from patients with BRONJ from PO vs. IV routes of BP administration. Oral neutrophils were collected, isolated and stimulated. The patients with a diagnosis of BRONJ and a history of oral bisphosphonate therapy (9 stimulated with PMA, 6 stimulated with fMLP) did not show a significantly different yield in ROS than that of the patients with a history of intravenous bisphosphonate therapy (6 stimulated with PMA, 3 stimulated with fMLP).
The study population: Patients without BRONJ new to BP therapy

i. Patient population

A total of eleven patients were identified as potential candidates to participate in this study. Two patients revealed that they would be continuing chemotherapeutic treatment at another institution and were therefore not eligible to participate. The remaining three were missed due to logistical reasons. Six patients met the inclusion criteria and were enrolled (Table 4), however, one patient was removed from the study after he went into remission. Five male patients were enrolled between November 2011 and July 2012 (mean age 65.2 yrs, range 53-83 yrs). Four patients provided pre-pamidronate saliva samples and four provided post-pamidronate saliva samples, one of which was processed but had inadequate viable neutrophils for testing. Two patients provided pre-pamidronate blood samples, which reflected their concurrent medications (notably dexamethasone and bortezomib chemotherapy), and four patients provided post-pamidronate blood samples. All patients were on the same chemotherapy (bortezomib 2.42-2.84 mg IV q4 weeks; pamidronate 90 mg IV q4 weeks) regimen and dexamethasone 40 mg PO/week. No patient had any symptoms of neutropenia and no neutropenia was detected by neutrophil counts obtained in the laboratory prior to sample testing.
### Table 4. Patients without BRONJ new to BP therapy

<table>
<thead>
<tr>
<th>Age (mean 65.2 years)</th>
<th>Gender (5M:0F)</th>
<th>EtOH or smoking</th>
<th>General oral health</th>
<th>Concomitant multiple myeloma-specific medications</th>
<th>Number of pamidronate treatments at sampling (90 mg IV q4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>M</td>
<td>Neither</td>
<td>Generalized moderate horizontal bone loss, no acute conditions</td>
<td>* Bortezomib (2.42 mg x 2 doses at baseline) * Dexamethasone 40 mg/week</td>
<td>None</td>
</tr>
<tr>
<td>57</td>
<td>M</td>
<td>Neither</td>
<td>Healthy</td>
<td>* Bortezomib (2.81 mg x 2 doses at baseline) * Dexamethasone 40 mg/week</td>
<td>1 treatment (6 days since last dose)</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>Neither</td>
<td>Healthy</td>
<td>* Bortezomib (2.84 mg x 4 doses at baseline) * Dexamethasone 40 mg/week</td>
<td>2 treatments (35 days since last dose)</td>
</tr>
<tr>
<td>70</td>
<td>M</td>
<td>Neither</td>
<td>Healthy</td>
<td>* Bortezomib (2.84 mg x 3 doses at baseline) * Dexamethasone 40 mg/week</td>
<td>1 treatment (21 days since last dose)</td>
</tr>
<tr>
<td>83</td>
<td>M</td>
<td>Neither</td>
<td>Healthy</td>
<td>* Bortezomib (2.55 mg x 1 dose at baseline) * Dexamethasone 40 mg/week</td>
<td>2 treatments (27 days since last dose)</td>
</tr>
</tbody>
</table>

**ii. Peripheral blood neutrophils: respiratory burst**

Whole blood was collected from two patients on bortezomib and dexamethasone medications prior to pamidronate treatment and from five patients after they had received pamidronate. When comparing healthy control neutrophils to those after pamidronate exposure, significantly less ROS resulted both fMLP- and PMA-stimulated neutrophils in the post-pamidronate samples (Figure 10) (fMLP p = 0.001 one-tailed, p = 0.002 two-tailed; PMA p = 7.0E-05, p = 0.0001). No significant change occurs for the pre- and post-pamidronate samples for both stimuli (Figure 11). Less ROS production (20% and
38% less in the fMLP -and PMA- stimulated neutrophils) was noted in the post-pamidronate group compared to baseline values, however, this was not statistically significant.
Figure 10. Peripheral blood neutrophil ROS response is reduced in patients exposed to pamidronate compared to control responses. Fold change in the reactive oxygen species generated by the peripheral blood neutrophils after stimulation with fMLP (12 control, 4 post-pamidronate) or PMA (16 control, 4 post-pamidronate). A significant decrease in ROS yield is seen in the post-pamidronate group in comparison to control for both fMLP and PMA-stimulated neutrophil samples.
iii. Oral neutrophils: respiratory burst

The salivary neutrophils yielded similar results, with a lower ROS yield after both fMLP and PMA stimulation in comparison to healthy controls and no change in ROS response after pamidronate exposure (Figures 12 and 13).
Figure 12. Fold change in the reactive oxygen species generated by the oral neutrophils after stimulation with fMLP (12 control, 3 post-pamidronate) or PMA (16 control, 3 post-pamidronate). A significant decrease in ROS yield is also seen in these oral neutrophils stimulated with PMA.
Figure 13. Fold change in the reactive oxygen species generated by the oral neutrophils after stimulation with fMLP (2 pre-pamidronate, 3 post-pamidronate) or PMA (4 pre-pamidronate, 3 post-pamidronate). There is no significant change from the ROS yield pre- vs. post-pamidronate.
CHAPTER 4

DISCUSSION
4.1 Discussion of results

This study presents evidence of a potentially novel biomarker for BRONJ susceptibility; neutrophil functional impairment. Among patients exposed to bisphosphonate drugs, some may develop a neutrophil impairment, which may predispose them to developing this jaw condition. Should these results prove reproducible in a larger clinical study with access to BP-treated, BRONJ-free controls, this data will allow for the development of a simple oral rinse test to screen patients with a history of bisphosphonate treatment for their susceptibility to develop BRONJ. The concept of impaired immunity in BRONJ was first demonstrated via in vitro and in vivo neutrophil studies using a murine model. Zolendronate and pamidronate were demonstrated to have inhibited neutrophil chemotaxis and ROS generation both in vivo and in vitro117. The potential exists that the same pattern of hypoactive neutrophil ROS generation in a subset of patients with BRONJ will occur in a larger sample of patients.

The bisphosphonate class is engineered to target calcium phosphate in bone31, potentially affecting the neutrophils within the local bone marrow. More generally, bisphosphonates interrupt the mevalonate pathway, preventing proper prenylation of Rho GTPases downstream144 where prenylation is required for normal small GTPase targeting to the plasma membrane where it carries out its signaling functions; zoledronate specifically has been shown to depress RhoA activity, potentially explaining the impaired migration and comparatively poor respiratory burst response, for which the protein plays a role117.

Blood and oral neutrophil sampling
Blood and oral sources for neutrophils were used in this study to determine whether the bisphosphonate-mediated effects were restricted to the oral environment or were in fact systemic. As demonstrated in this study, the bisphosphonate effects appear to be systemic or certainly impact the differentiating neutrophils in the bone marrow. Previous work from our laboratory has shown that oral neutrophil sampling has proven useful in monitoring neutrophil mobilization to peripheral sites and neutrophil functionality after transplantation of hematopoietic stem cells, where oral neutrophil counts reflected engraftment earlier than circulating peripheral blood neutrophils. Similarly, the oral neutrophils sampled in the bisphosphonate-exposed patients echoed the results from the blood neutrophils, providing insight into neutrophil function in the general circulation.

Prolonged bisphosphonate effect
As in the murine study by Kuiper et al., this study demonstrates longer-term evidence of neutrophil impairment after a drug holiday. The majority of BRONJ patients in this study discontinued taking the bisphosphonate medication an average of 1.5 years prior to sampling and neutrophil testing, which demonstrates a prolonged inhibitory effect on the neutrophil population. This is not surprising as the offset of action of these drugs is quite long.

Route of bisphosphonate delivery
It is notable that in the patient population with a previous exposure to BP, eleven of the eighteen patients (61%) were exposed to po bisphosphonate therapy. Contrary to the common belief that BRONJ is a result of intravenously-administered BP medications, the
oral route of administration represented the majority of BRONJ patients sampled from the outpatient clinic\textsuperscript{63, 68, 145}. This supports the relationship between oral bisphosphonate therapy and BRONJ that has been discussed by Sedghizadeh’s team in their survey of 208 patients taking po alendronate at the University of Southern California\textsuperscript{146}. While the results of their study supports a higher incidence of BRONJ (4\%) than is often reported for a po-bisphosphonate population, this may still underestimate the incidence as the survey included patients being treated for ONJ, rather than patients diagnosed with ONJ and would therefore miss Stage 0 patients. Similar findings were reported by Kim and Kwon in their study examining clinic patients diagnosed with BRONJ between 2005-2010; the authors noted that 9\% of the patients in the study were on oral bisphosphonate therapy\textsuperscript{147}.

Diminished respiratory burst

The group with previous exposure to BP has an overall decrease in neutrophil-mediated superoxide yield in comparison to the control population. Both the fMLP- and PMA-stimulated neutrophils from patients with BRONJ showed decreased superoxide production. Since the fMLP pathway is ligand-receptor directed, it may suggest that the inhibition lies somewhere in receptor number or configuration. However, the PMA stimulatory pathway is direct and a decrease in superoxide production was seen in both groups. This soluble molecule is able to pass directly through the cell membrane and into the cell setting off a cascade of signaling events, which, in turn, results in superoxide production\textsuperscript{148, 149}. The BRONJ neutrophils are likely to have a defect in one of the regulatory components of NADPH oxidase complex, most likely Rac which is a small
GTPase\textsuperscript{150}. It is possible that small GTPase activity is altered secondary to BP-mediated disruption of the mevalonate pathway, which is required for small GTPase localization to the plasma membrane compartment\textsuperscript{151}.

The preliminary data from these trials supports the concept of neutrophil impairment in a subset of bisphosphonate-exposed patients. This notion bolsters an earlier experiment by C. Forster\textsuperscript{152}, which showed that neutrophil chemotaxis is also perturbed in patients with BRONJ, where neutrophils isolated from their blood did not migrate as well as control blood neutrophils (Appendix 1). As a positive control, three patients diagnosed with osteoradionecrosis (ORN) were included in order to serve as a model for background chronic inflammation. Forster reports that no difference in chemotaxis between control and ORN samples, however, neutrophils from patients with BRONJ had compromised chemotaxis compared to neutrophils form patient with ORN (p<0.05).

Upon spectrophotometric analysis of both peripheral blood- and salivary-neutrophil respiratory burst capacities, the pre- vs. post-pamidronate exposure comparisons (Figures 11 & 13) did not show a significant change; this may be because this study was underpowered, or may represent a true lack in impairment after initiation of a bisphosphonate medication without a BRONJ setting.

In comparison, the healthy naïve controls vs. post-pamidronate exposure comparisons showed less ROS production in the bisphosphonate-exposed groups (Figures 10 & 12). The ROS yield from the peripheral blood neutrophils subsequent to pamidronate exposure was found to be significantly less than the ROS yield in the control group.
(Figure 10), however, this compares healthy patients to patients with a cancer diagnosis on multiple medications. This trend of compromised neutrophil function subsequent to bisphosphonate-exposure in a subset of recipients supports the other data in this study and in previous studies\textsuperscript{117,135}.

Study limitations

Limited data points and sample size

The data presented in this study is confined to a small sample size, which is due to the small population of eligible patients at the parent hospitals. In addition, patients with milder stage BRONJ can be seen less frequently with longer intervals between appointments, which may mean that patients in this category may be underrepresented. The patients beginning iv bisphosphonate treatment represented a group with many appointments to manage for therapy, phlebotomy and assessment. Rescheduled phlebotomy appointments were not always coordinated with the study and rushed appointments led to refusal to provide saliva samples. This left gaps in data collection for this particular group. Challenges in patient scheduling translated into missed appointments and therefore missed baseline sampling. Logistics presented a significant challenge in the Princess Margaret Hospital group due to both the sensitivity of the recent diagnosis of multiple myeloma, patients overwhelmed with appointments and a lack of continuity between the hematology clinic and the dentistry clinic.

Patient variables

Neutrophil function is affected by various environmental agents including smoking\textsuperscript{115,153-156}. In this sample of patients, smoking as a potential inhibitor of neutrophil function
was not a confounding variable since none of the patients reported active smoking in their histories at the time of sampling $^{157,158}$.

In terms of data comparison, the control group for the patients with a recent cancer diagnosis is not ideal as the patients beginning pamidronate therapy are not only sick, they are also on other medications. A similar limitation exists in the population of patients with BRONJ that were studied. Some of these patients were also on other medications, have comorbidities or have cancer, which could account for the observed neutrophil effects. In this context, the effects observed may serve as a biomarker for BRONJ and does not mean the physiologic effect on the cells is due to BP use or due solely to BP use. Healthy controls were also not age- or sex-matched. The argument could be made that there is potential for neutrophil ROS production to diminish with ageing and may differ between genders and that an age and sex-matched population should be used for comparison. In this study, this was not performed as the recruitment was in a clinical specialist setting where well-patient visits are rare. In this regard, age- and gender-matching an older population by recruiting patients who present with healthy mouths, an absence of health conditions, a negative social history and no drug therapies is not possible. In addition, the study by Niwa et al$^{143}$ examines ROS production in a range of ages and found that, in terms of ROS yield, neutrophils display negligible senescence.

4.2 Purpose of Thesis
The purpose of this thesis examined the potential to use innate neutrophil immune response as a biomarker of BRONJ susceptibility, where neutrophil ROS generation was examined in BRONJ patients and subsequent to BP therapy in patients without BRONJ. This diminished immune capacity increases the stressful bacterial load at exposed sites, which may contribute to the pathogenesis of BRONJ; pathogenesis aside, this finding may present a surrogate for BRONJ. This idea originates from literature indicating that bisphosphonates are related to decreased neutrophil counts and neutropenia\textsuperscript{159}, neutrophil enzymes\textsuperscript{160} and reactive oxygen burst\textsuperscript{135, 161, 162}.

### 4.3 Future Directions

Two major weaknesses in this study were population size and patient organization. It would be helpful in the next portion of the study to include a larger population base through multicenter involvement to increase sample size and to better coordinate the patient drug schedule via partnership with an assigned set of clinical nurses. Ideally, patients without a history of or planned head and neck radiation about to begin bisphosphonate therapy would be referred by their general practice physicians for baseline oral and blood neutrophil analysis. A medical history from their referring physician would accompany the patient as many patients are not optimal historians. The patients would be tested again following the administration of their bisphosphonate before osteonecrosis develops. They would be tested again 6 months later, and again at 1 year intervals or upon the diagnosis of BRONJ or whichever occurred first. The tests
would be repeated upon change in BRONJ stage status and/or resolution of the disease for 12 months or greater.

With regard to the bench-side challenge of diminished yield from the oral neutrophil samples after filtration, we have subsequently learned that two passes through a 40 μm filter not only make the subsequent filtrations through the 20 μm filter and 10 μm filter faster, this also prevents neutrophil loss.

It may be relevant to examine the microarray profiles of patients before and after pamidronate exposure to pinpoint which areas of neutrophil function may be hampered. This may elucidate another mechanism through which neutrophils are impaired, such as via compromised epithelial-cell signaling, further contributing to soft tissue breakdown and bone exposure.

It is essential that this information not only further our insight into the pathogenesis of BRONJ, but that it transfers into clinical application. This means that a correlation needs to be drawn between the clinical picture of BRONJ and BRONJ-susceptible patients and oral rinse studies. To determine if the less invasive oral rinse serves as a good predictor of BRONJ, the next step would be to prospectively follow patients from pre-bisphosphonate into their pre-BRONJ, BRONJ or remission states through oral neutrophil sampling similar to the plan described above. Sampling an adequate number of patients at different stages of BRONJ in an attempt to correlate laboratory findings with severity of disease would be built into this experimental model.

It is essential that there is, among these patients, a group who serve as control patients who have exposure to bisphosphonate drugs but do not develop disease.

The long-term goal is to further develop the oral rinse component for non-invasive
predictive testing of patients with a history of bisphosphonate therapy who require invasive oral treatment. The dampened respiratory burst seen in neutrophils exposed to bisphosphonate therapy\textsuperscript{117} may account for the continual soft tissue breakdown and ongoing osseous exposure. The mechanism that results in this neutrophil hypoactivity remains to be established.

4.4 Conclusions

The data presented here support that oral sampling is a useful tool in the evaluation of neutrophil function and suggest that neutrophil function may be an important biomarker to identify patients at risk of developing BRONJ.
Bisphosphonates are key medications in the resolution and quiescence of several disease processes and can represent not only an improved quality of life but an extended life. They are, however, associated with osteonecrosis of the jaws, which can be debilitating and refractory. The exact mechanism by which this occurs is unknown and no objective measure of patient risk of developing BRONJ exists. This has led to both patient and clinician concern in the context of oral surgical intervention. Both improved knowledge of the pathogenesis of BRONJ and a minimally invasive objective test of BRONJ-susceptibility are required.

This thesis suggests that neutrophil function may be impaired in patients with BRONJ. Patients with BRONJ may have a compromised killing response in comparison to a healthy control population. This decreased respiratory burst capacity is also seen in patients after pamidronate therapy when compared to the average capacity of a control pamidronate-naïve population but not when compared to pre-pamidronate baseline values. In both the BRONJ and pamidronate-groups, a correlation is seen between oral neutrophil test results and peripheral blood neutrophil test results.

This supports the idea that there may be a role for the immune system in providing a biomarker for BRONJ and perhaps in the monitoring of patients receiving bisphosphonate therapy.

The dampened respiratory burst seen in neutrophils in patients with BRONJ may, in part, account for the continual soft tissue breakdown and ongoing osseous exposure. The mechanism that results in this neutrophil hypoactivity remains to be established.
This further supports previous research findings that neutrophils are impaired after bisphosphonate treatment and that oral neutrophil testing is reflective of systemic neutrophil function. This bolsters the idea that a non-invasive oral rinse test may be developed to predict the BRONJ-risk status of patients for whom invasive oral treatment is indicated.

Future research should include a larger population base through multicenter involvement to increase sample size and a system for improved coordination of the patient drug schedule with sample collection dates as well as improved control population sampling. To determine if the less invasive oral rinse serves as a good predictor of BRONJ, the next step would be to prospectively follow patients from pre-bisphosphonate into their pre-BRONJ, BRONJ or remission states through oral neutrophil sampling. This could also be tested by running ROS assays for post-bisphosphonate patients without BRONJ and patients with BRONJ to elucidate whether a detectable deficit in neutrophil function occurs in the subpopulation of bisphosphonate-exposed patients with BRONJ and not in those without the disease. Microarray and proteomic profiling of patient neutrophils may pinpoint the mechanism through which neutrophil impairment may factor into the pathogenesis of BRONJ.
1. Forster experiment: Neutrophil chemotaxis to fMLP stimulation in BRONJ blood neutrophils.

Forster chemotaxis experiment results. Neutrophils collected from control, BRONJ patient and ORN patient peripheral blood neutrophils were isolated. Their migration capacity through a membrane in response to fMLP stimulation was evaluated. Significantly less neutrophils from the BRONJ patients migrated in the experiment in comparison to control and ORN patient neutrophils (p<0.05)\textsuperscript{152}
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