The Effect of Bisphosphonate Therapy on Neutrophil Function and Delivery

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Dentistry
University of Toronto

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Abstract

Bisphosphonate-related osteonecrosis of the jaw is a significant complication of bisphosphonate administration and its pathogenesis has not been fully elucidated. Previous studies in mice have revealed that polymorphonuclear neutrophils develop impaired function following exposure to bisphosphonates. Our hypothesis is that there will be an impairment of oral and blood polymorphonuclear neutrophil reactive oxygen species production and chemotactic capabilities one month following the initial infusion of pamidronate in bisphosphonate-naïve patients relative to baseline testing. We observed an overall decrease in both oral and blood polymorphonuclear neutrophil reactive oxygen species production and chemotaxis. This finding suggests that the decrease in polymorphonuclear neutrophil function may play a role in the pathophysiological processes that contribute to the development of bisphosphonate-related osteonecrosis of the jaw. Furthermore, this suggests that reduced polymorphonuclear neutrophil function may serve as a potential biomarker in patients who might be susceptible to the development of bisphosphonate-related osteonecrosis of the jaw.
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List of Abbreviations

AAOMS: American Association of Oral and Maxillofacial Surgeons
AU: Absorption units
bALP: Bone-specific alkaline phosphatase
BRONJ: Bisphosphonate-related osteonecrosis of the jaw
CTX: C-terminal telopeptide of collagen I
df: Degrees of freedom
fMLP: N-Formylmethionyl-leucyl-phenylalanine
FPP: Farnesyl diphosphate
GGPP: Geranylgeranyl diphosphate
GTPase: Guanosine triphosphate-hydrolyzing enzyme
HBSS: Hanks’ balanced salt solution
IGF: Insulin-like growth factor
IL: Interleukin
MDCT: Multi-detector computed tomography
mg: Milligram
mL: Millilitre
mM: Millimole
NADPH: Nicotinamide adenine dinucleotide phosphate
NCE: Non-contrast enhanced
nM: Nanometre
OPG: Osteoprotegerin
PBS: Phosphate-buffered saline
PMA: Phorbol myristate acetate
PMN: Polymorphonuclear neutrophil

RANK: Receptor activator of nuclear factor kappa B

RANKL: Receptor activator of nuclear factor kappa B ligand

ROS: Reactive oxygen species

RPM: Rotations per minute

SEM: Standard error of the mean

SD: Standard deviation

SOD: Superoxide dismutase

μL: Microlitre

μm: Micrometre
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Chapter 1

1 General Introduction

1.1 Bone Physiology

The skeleton serves as a structure of support, locomotion, protection, storage for, and supply of, minerals and hematopoiesis. This structure is constantly remodeling after the cessation of growth, serving to repair and maintain its structural and functional integrity. The process of bone remodeling involves the coordinated activity of bone forming and resorbing cells; namely, osteoblasts and osteoclasts, respectively.

Osteoclasts, multinucleated giant cells derived from monocytic cell fusion, adhere to the bone matrix where they develop their characteristic ruffled borders. Once activated, these cells produce proteolytic enzymes and hydrogen ions, which dissolve the organic and inorganic matrices of the underlying bone. Osteoblasts arise from mesenchymal stem cells within the bone marrow and are responsible for producing the organic matrix upon which bone is mineralized.

The regulation of the actions of these cells in the process of bone remodeling necessitates a tight balance of their respective activities. Bone remodeling requires the recruitment and activation of osteoclasts, which results in osseous resorption and, subsequently, osteoclast apoptosis. Formation of new bone is accomplished by the production and mineralization of osteoid by osteoblasts. This process is orchestrated by several systemic and local modulators of bone resorption and formation, such as parathyroid hormone, calcitonin, and several cytokines such as insulin-like growth factor (IGF)-I and II and interleukin (IL)-1 and 6 that are secreted by osteoblasts and osteoclasts. One of the most important local mediators of bone metabolism is the receptor activator of nuclear factor kappa B (RANK)/RANK Ligand (RANKL)/osteoprotegerin (OPG) system. RANKL is responsible for osteoclast maturation and survival while OPG inhibits osteoclastic differentiation leading to an increase or decrease, respectively, in bone resorption. Several of the other systemic and
local mediators of bone turnover can either increase or inhibit the effects of RANKL and OPG\textsuperscript{8,9}.

1.2 Bisphosphonates

1.2.1 Structure and Function

Bisphosphonate medications are pyrophosphate analogues that act principally as inhibitors of osteoclast-mediated bone resorption\textsuperscript{10}. The substitution of the central oxygen in pyrophosphate with a carbon atom makes these compounds resistant to hydrolysis (Figure 1)\textsuperscript{11}. The side chains, designated R1 and R2, are bonded to the central carbon and determine the activity and potency of the bisphosphonate (Table 1)\textsuperscript{11,12}. Contemporary aminobisphosphonates, such as alendronate and pamidronate, possess a single hydroxyl group and a nitrogenous chain attached to the central carbon atom and are significantly more potent than the non-nitrogen containing bisphosphonates such as etidronate and clodronate\textsuperscript{13,14}. This potency is especially dramatic in those bisphosphonates which possess side chains containing nitrogenous heterocycles\textsuperscript{15}.

Whether they are administered orally or intravenously, bisphosphonates bind to hydroxyapatite and are internalized by active osteoclasts. Once located intracellularly, nitrogen-containing bisphosphonates act on the mevalonate biosynthetic pathway to inhibit the formation of farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), thereby disrupting the downstream post-translational prenylation of several GTPases (Rho, Ras, Rab and Rac) (Figure 2)\textsuperscript{16–20}. In the osteoclast, the inhibition of these vital cellular switches results in cytoskeletal alterations, inhibition of osteoclastogenesis, limited adhesion to osseous surfaces, and the induction of apoptosis\textsuperscript{21,22}. Non-nitrogenous bisphosphonates, in contrast, are internalized by osteoclasts, where they act as cytotoxic adenosine triphosphate (ATP) analogues resulting in cellular apoptosis\textsuperscript{18}.

1.2.2 Indications

In diseases such as osteoporosis, there is a gradual decoupling of the balance between osteoblastic and osteoclastic activity leading to a decreased bone mass and increased
Several pharmacologic-based strategies are available for the treatment of this condition including vitamin D and calcium supplementation, denosumab (a RANKL inhibitor), and raloxifene (a selective estrogen receptor modulator). Despite the wide array of available therapeutic agents, the most commonly prescribed medications for the treatment of osteoporosis are the bisphosphonates. With consistent long-term administration, bisphosphonates are effective inhibitors of bone resorption, reducing the risk of bone fractures and leading to an increase in bone mineral density.

Given their ability to inhibit the function of osteoclasts, bisphosphonates have also been prescribed in the treatment of malignancies associated with bone metastases. The bone marrow is an attractive site for metastases due to its rich, low-velocity vascular network. Once in the marrow, tumor cells can modulate this environment to favor osteoclastic activity and tumor growth. Bisphosphonates, in these cases, not only inhibit osteoclast-mediated bone resorption and prevent entry of tumor cells into bone, but also block adhesion of tumor cells, decrease local growth factor production and inhibit neoangiogenesis, thereby preventing tumor invasion and the establishment of a metastatic focus of disease. These mechanisms of action are employed in the management of patients with multiple myeloma, a neoplastic hematologic malignancy. Intravenous pamidronate or zolendronic acid are prescribed routinely to prevent the establishment of osseous metastatic lesions that lead to the development of bone pain and pathologic fractures in an effort to improve the quality of life for affected patients.

1.3 Bisphosphonate-Related Osteonecrosis of the Jaws

1.3.1 Definitions

Unfortunately, despite their therapeutic benefit, especially in the prevention of bone metastases, the continued administration of bisphosphonates predisposes these patients to the risk of developing bisphosphonate-related osteonecrosis of the jaw (BRONJ). BRONJ is a clinical diagnosis and, as per the most recent American Association of Oral and Maxillofacial Surgeons (AAOMS) guidelines, patients must have a history of bisphosphonate treatment,
exposed bone or bone that can be probed through a mucosal defect that has been present for more than eight weeks, and no history of radiation therapy or obvious metastatic disease involving the maxillofacial complex\textsuperscript{35}.

BRONJ is a morbid condition, which can drastically alter a patient’s quality of life\textsuperscript{36}. Clinical presentations range from non-specific pain without frank bone exposure to those individuals who have large regions of exposed necrotic bone, extra-oral fistulae, oro-antral communications, and jaw fractures (Plates 1 and 2)\textsuperscript{37}. Radiographic imaging, while not necessary for the diagnosis of BRONJ, is helpful in delineating the extent of osseous disease and involvement of surrounding soft tissue structures (Figures 3, 4 and 5)\textsuperscript{38,39}. Histopathologic evaluation of debridement specimens is useful to rule out the presence of a metastatic focus of disease should suspicions be elevated by an abnormal clinical appearance (Figure 6)\textsuperscript{40}. While there is a system to classify the severity of BRONJ and several suggested treatment options, there are no evidence-based guidelines for the management of this disorder\textsuperscript{35}. Treatment strategies for BRONJ vary widely from the application of conservative measures including analgesics and antimicrobial therapy to radical surgical options including debridement and jaw resection\textsuperscript{41–44}.

The prevalence of BRONJ varies widely and is largely dependent on the route of bisphosphonate administration. In those patients receiving oral agents, estimates from several groups report a prevalence significantly below 1\%, whereas those patients receiving intravenous agents range between 1.2% and 18.6\%\textsuperscript{45–48}.

There are multiple factors that contribute to the development of BRONJ. The first, and most fundamental, of these factors include previous exposure to a bisphosphonate medication. The extent to which this factor plays a role is also dependent on the route of administration, whether intravenous or oral, the type of bisphosphonate, whether it is nitrogen or non-nitrogen containing, as well as the dose and length of time the agent in question has been prescribed\textsuperscript{49,50}. Other factors, such as the concomitant use of chemotherapeutic agents and corticosteroids, invasive dentoalveolar procedures, and patient factors such as smoking, old age, and diabetes may also increase individual risk for the patient\textsuperscript{50–52}. 
1.3.2 Mechanism

The precise mechanisms by which BRONJ manifests are currently unknown. It is probable that the pathophysiological mechanisms underlying this condition are multi-factorial. First, bisphosphonates may induce jaw necrosis through a combination of over-suppression of bone turnover and local concentration of bisphosphonates which may lead to osseous microtrauma via daily physiologic function. Following maxillofacial trauma or exodontia, regions suffering from microtrauma may be more susceptible to BRONJ as osseous healing mechanisms may be impaired\(^{53,54}\). Second, anti-angiogenic mechanisms may increase patient susceptibility to the development of BRONJ as an impairment of the proliferation, adhesion, and migration of endothelial cells has been demonstrated following treatment with bisphosphonates, which may lead to osseous necrosis\(^{55-57}\). Bisphosphonates may also act negatively upon fibroblasts and keratinocytes which are found within the periodontal ligament and oral mucosa, respectively. This soft-tissue toxicity may predispose patients to the subsequent development and maintenance of soft tissue defects\(^{58-60}\). Interestingly, several bacterial species, especially members from the *Actinomyces* species, have been identified in histopathologic examination of specimens collected from patients with a diagnosis of BRONJ. This suggests the possibility that microbial infection could play an integral role with regard to the etiopathogenesis of BRONJ through the impairment of the immune system\(^{61,62}\). This impairment may allow for microbial colonization of affected sites with exposed bone, and could also contribute to the continued maintenance of soft tissue defects that can arise from bisphosphonate effects on the oral mucosa. Conversely, patients with impaired immunity that are also taking bisphosphonates may be at an increased risk of developing BRONJ, especially those with pre-existing dental infections.

1.4 The Neutrophil

Polymorphonuclear neutrophils (PMNs) are a member of the myeloid lineage and a critical member of the innate immune system\(^{63}\). Though short-lived with a half-life of approximately seven hours, PMNs are the hallmark cell of the acute inflammatory response\(^{64,65}\). Neutrophils are recruited from the vasculature to a site of inflammation by
tissue-resident leukocytes that release several inflammatory mediators\textsuperscript{66}. This results in the rolling and arrest of neutrophils on the activated endothelium mediated by selectins and integrins, respectively\textsuperscript{67–70}. Following their firm adhesion, neutrophils exit the vasculature by either paracellular or transcellular migration\textsuperscript{71–73}. After exiting the vasculature, PMNs follow a gradient of chemical signals mediated through several chemotactic cell surface proteins such as the N-formylmethionine-leucyl-phenylalanine (fMLP) and phorbol myristate acetate (PMA) receptors\textsuperscript{74}. To chemotactically localize to the site of the invading microorganism, the PMN undergoes a series of cytoskeletal alterations resulting in a polarized appearance demonstrating lamellipodium and the uropod, the leading and trailing ends of the PMN, respectively\textsuperscript{75}. Following the arrival of PMNs to the site of infection, pathogens are phagocytized, leading to the fusion of phagosomes and lysosomes\textsuperscript{76}. These lysosomes are filled with the reactive oxygen species (ROS) resulting from the activation of the NADPH oxidase complex, including hydrogen peroxide and the superoxide anion, enabling pathogen destruction\textsuperscript{77,78}.

Interestingly, while the administration of bisphosphonates does not necessarily predispose patients to an increased susceptibility of infection, they have been demonstrated to diminish the production of ROS and other enzymes necessary for wound healing\textsuperscript{79–81}. In a similar manner within the osteoclast, prenylated GTPases play a crucial role in the regulation of PMN function\textsuperscript{82}. The Rac GTPases, as members of the Rho GTPase family, are responsible for the activation of PMN NADPH oxidase as well as PMN chemotaxis via regulation of the actin cytoskeleton and the formation of lamellipodia\textsuperscript{83,84}. Furthermore, Cdc42 and RhoA, other members of the Rho GTPase family, participate in the regulation of neutrophil polarity during migration. Cdc42 participates in the generation the protrusive forces necessary for the formation of filopodia, smaller actin cellular projections, at the leading margin of the PMN while RhoA assists in the generation of the contractile and retractive forces within the PMN uropod\textsuperscript{85–88}. 
Chapter 2

2 Hypothesis and Objectives

2.1 Hypothesis

Recent murine research has demonstrated that bisphosphonates possess the ability to decrease PMN function\textsuperscript{89}. While their specific role in the etiopathogenesis of BRONJ is unclear, it has been shown that the GTPases (Rho, Ras and Rac) that regulate osteoclast function are also involved in the regulation of cellular chemotaxis and the respiratory burst in PMNs. It could be speculated that bisphosphonates alter the prenylation of these cellular regulators in PMNs in a manner similar to osteoclasts.

The hypothesis of this thesis that there will be an observable functional impairment with respect to PMN ROS production and chemotaxis in bisphosphonate-naïve patients diagnosed with multiple myeloma one month after receiving their first infusion of pamidronate. The functional impairment of blood PMNs will be reflected by the PMNs sampled from the oral cavity.

2.2 Objectives

2.2.1 Functional Impairment of PMNs

Blood and saliva will be collected from bisphosphonate-naïve patients to determine if there is functional impairment in PMNs with respect to ROS production and chemotaxis following the initial infusion of a single dose of pamidronate.

2.2.2 Correlation Between Oral PMNs and Blood PMNs

The function of PMNs derived from peripheral blood and the oral cavity will be compared. The rationale for this is to determine whether any changes seen in the peripheral blood PMNs are also reflected in PMNs harvested from the oral cavity.
Chapter 3

3 Materials and Methods

3.1 Study Population

Patients who were referred to the Department of Dental Oncology, Maxillofacial and Ocular Prosthetics at the Princess Margaret Cancer Centre for a dental evaluation prior to intravenous administration of pamidronate (90 mg/month) were eligible to participate in this study. Inclusion criteria mandated that patients must have a diagnosis of multiple myeloma with no prior history of exposure to bisphosphonates, had no history of therapeutic radiation to the maxillofacial region, and were able to provide informed consent. Patients were enrolled between September 2014 and July 2015. Samples of blood and saliva were obtained from the subjects prior to the administration of pamidronate and then one-month later so that the impact of bisphosphonate treatment on the function of blood and oral PMNs, respectively, could be assessed within subjects. As per routine protocol, all patients received dental treatment in order to assure that their oral health was optimal prior to the administration of pamidronate. Blood and saliva samples were processed for immediate assessment of chemotactic activity and oxidative burst by measurement of the production of ROS. In order to prevent bias, all samples were assigned a unique patient identifier number so that the evaluator and laboratory technologist were blinded to the source of the samples being analyzed. Blood and saliva samples collected from an external control were processed concurrently with study patient samples. This study was approved by the University Health Network Research Ethics Board (REB #10-0936-CE).

3.2 Blood Sample Collection and Preparation

Two 5 mL blood samples were drawn into sodium citrate-containing Vacutainers (Becton Dickinson, Rutherford, NJ, USA). PMNs were isolated by layering a 5 mL aliquot of blood over 5 mL of 1-Step® Polymorphs (Accurate Chemical and Scientific Corporation, Westbury,
NY, USA), and the mixture was centrifuged at 1500 RPM for 35 minutes at 18°C. The lower translucent band containing PMNs was isolated and then washed in Hanks’ balanced salt solution (HBSS, ThermoFisher Scientific, Waltham, MA, USA) and re-centrifuged at 2500 RPM for 5 minutes. Residual erythrocytes were lysed utilizing 1 ml of distilled water and the samples were centrifuged again at 2500 RPM for 5 minutes to concentrate the PMN pellet. This pellet was then resuspended in 1 mL phosphate buffered saline (PBS, Sigma-Aldrich Chemical Company, Oakville, ON, Canada), and PMNs were counted with a Beckman Coulter Z2 counter (Beckman Coulter Canada, Mississauga, ON, Canada).

3.3 Oral Rinse Collection and Preparation

Saliva samples were obtained utilizing a 3 mL sterile 0.9% normal saline rinse (Baxter, Deerfield, IL, USA) in accordance with collection techniques previously utilized by our laboratory. Patients were instructed to rinse with this solution for 30 seconds and expectorate into a 50 mL Falcon Tube (Becton Dickinson, Rutherford, NJ, USA). This process was repeated five times, with 1-min intervals separating each rinse cycle. The samples were processed immediately using sequential filtration beginning with a 40 μm nylon mesh sterile cell strainer (Fisher Scientific Company, Ottawa, ON, Canada), followed by a 20 μm nylon net filter (Millipore, Etobicoke, ON, Canada), and a 11 μm nylon net filter (Millipore, Etobicoke, ON, Canada). Samples were recovered and then centrifuged for 10 minutes at 2500 rpm. The cell pellets were reconstituted with 1 mL PBS and counted with a hemocytometer.

3.4 Assessment of PMN ROS Production

The PMNs were placed in a cuvette containing a solution of 100 μL of PBS with 10 mM D-glucose on ice at a concentration of 1 x 10⁶ cells/mL. A 0.1 mL aliquot of the PMN suspension was combined with 880 μL of PiCM-G buffer (University of Toronto Media Preparation Services, Toronto, ON, Canada) and 10 μL of equine ferricytochrome c (0.1 mM final concentration; Sigma-Aldrich Chemical Company, Oakville, ON, Canada). A reference cuvette was prepared in a similar fashion, with 10 μL of 5 mg/mL solution of superoxide
dismutase (SOD, Sigma-Aldrich Chemical Company, Oakville, ON, Canada) replacing the PiCM-G buffer. The cuvettes were incubated at 37°C for 10 minutes and stirred continuously prior to stimulation of the PMN oxidative burst. In order to stimulate the production of superoxides, 10 μL of PMA (1 μM final concentration; Sigma-Aldrich Chemical Company, Oakville, ON, Canada) or 10 μL fMLP (1 μM final concentration; Sigma-Aldrich Chemical Company, Oakville, ON, Canada) were added to the cuvettes and incubated for 30 minutes at 37°C. The concentration of reduced cytochrome c was assessed after 10 minutes using a spectrophotometer measuring the absorbance at 550 nm.

3.5 Assessment of PMN Chemotaxis

A PMN suspension of 1 x 10^6 cells/mL in 100 μL HBSS and 1% gelatin (Sigma-Aldrich Chemical Company, Oakville, ON, Canada) was layered on bovine serum albumin (BSA) coated glass coverslips (22 x 40 mm) for 10 minutes at 37°C. The individual coverslips were inverted onto a Zigmond chamber where 100 μL HBSS medium was added to the right chamber and 100 μL HBSS medium containing 1 μM fMLP was added to the left chamber. Time-lapse video microscopy was utilized to record PMN migration towards the fMLP concentration gradient within the chamber over a period of 15 minutes at 20 second intervals using a Nikon Eclipse E1000 microscope. Images were then analyzed using cell-tracking software (Retrac, Version 2.1.01 Freeware) in order to measure cellular chemotaxis.

3.6 Statistical Analysis

Normally distributed patient pre-bisphosphonate and post-bisphosphonate and control baseline and follow-up data were analyzed by paired t-tests. A P-value of less than 0.05 was considered to be significant. The collected data are expressed as the mean and standard deviation (SD) unless indicated otherwise.
Chapter 4

4 Results

4.1 Patient Population

A total of 14 patients were eligible to participate in this study. One patient did not wish to return for post-pamidronate sample collection. Another patient elected to not receive pamidronate therapy after pre-pamidronate sample collection had been completed. A final patient suffered a severe allergic reaction to trimethoprim and sulfamethoxazole (antibiotic agent) two days prior to collection of blood and saliva samples post-pamidronate administration. With regard to the latter patient, these events could elevate PMN function thereby confounding the study outcomes. Accordingly, data from these three patients were excluded from the analysis. Eleven patients (eight males, three females) with a mean age of 54.81 ± 13.00 years met the inclusion criteria and were enrolled (Table 2). Neutropenia was not detected in any of the patients at the time of both pre-treatment and post-treatment sample analysis.

4.2 PMN ROS Production

Exposure to pamidronate appeared to cause a significant reduction in PMN ROS production when compared to pre-treatment production (Figures 7 and 9, Table 3). Using fMLP stimulation, it was demonstrated that pamidronate-induced reduction of ROS production was 41.2% and 19.5% for patient blood and oral PMNs, respectively ($P < 0.05$). Similarly, when using PMA as the stimulant, pamidronate-induced reduction of ROS production was 45.5% and 41.4% for patient blood and oral PMNs, respectively ($P < 0.05$). There were no statistically significant differences between external control samples regardless of the stimulant used ($P \geq 0.05$) (Figures 8 and 10, Table 3)
4.3 PMN Chemotaxis

PMN chemotaxis as a response to fMLP stimulation was impaired in patient PMNs treated with pamidronate and this reduction in chemotactic function was observed for both oral and blood PMNs (Figure 11, Table 3). Overall, there was an 61.4% and 49.1% reduction in blood and oral PMN chemotaxis, respectively ($P < 0.05$). No statistically significant difference was noted in the control analysis ($P \geq 0.05$) (Figure 12, Table 3).
Chapter 5

5 Discussion

5.1 Findings

The findings from this work demonstrate that the intravenous administration of pamidronate results in a marked impairment of PMN ROS production and chemotactic capabilities which may hamper the robustness of the innate immune response. This is the first study that demonstrates the possibility of utilizing a non-invasive oral rinse to assess the effects of intravenous bisphosphonates on the activity of PMNs. In this regard, we observed that both oral and peripheral PMNs were affected in a similar manner by pamidronate. Impairment of the functional processes in PMNs would result in a decreased recruitment from the vasculature and a reduced lysosomal functionality, both factors having a significant effect on their role in the innate immune response. BRONJ has been proposed to have an infectious etiology, and these findings may, in part, lend further evidence to support this hypothesis. Therefore, one could extrapolate that patients with decreased PMN functionality would be at a higher risk for developing BRONJ, particularly after undergoing invasive dentoalveolar procedures. Thus, the assessment of PMN function may serve as a viable biomarker to screen for BRONJ susceptibility. Additionally, with the knowledge that the oral and blood PMNs demonstrate similar decreases in PMN functionality, the less invasive oral rinse could be used in an effort to obviate the need for a phlebotomy procedure.

Given that bisphosphonates act on the mevalonate pathway within the osteoclast, thereby inhibiting the prenylation of several families of GTPases, it would be reasonable to consider this same mechanism of impairment in PMNs. Similar GTPases are integral in the proper functioning of PMNs and may account for the observed decrease in ROS production and chemotaxis impairment. The process of inhibiting the prenylation of GTPases results in a loss of cellular function as the prenyl moiety is required to anchor GTPases to the cell membrane. In the absence of membrane bound GTPases, there is a loss of the regulatory
switch that may be required for cellular function, proliferation, and apoptosis\textsuperscript{94,95}. As previously discussed, the inhibition of GTPase prenylation within the PMN results in impaired chemotaxis and NADPH oxidase activity. With this notion in mind, while our data does not definitively implicate the participation of PMNs in the etiopathogenesis of BRONJ, it would be reasonable to suggest that, should a patient have an invasive dental procedure or suffer gnathic trauma, PMNs with impaired function will be unable to exert their ability to localize and clear gnathic infections. This may facilitate the development and continued maintenance of soft tissue and osseous wounds and thus, BRONJ.

5.2 Limitations

Limitations in this investigation are similar to those faced in most clinical investigations. While the sample size that we obtained is the largest reported to date, it is still a relatively small population with which to make generalizable conclusions about the observed bisphosphonate effects. Our cancer center treats a large number of patients suffering from multiple myeloma, therefore recruiting those that are completely bisphosphonate-naïve is difficult due to the frequent prescription of oral bisphosphonates for osteoporosis and intravenous bisphosphonates for the treatment of hypercalcemia of malignancy, which is the first presenting symptom in some of these individuals. Furthermore, patients who are being treated for multiple myeloma are generally also prescribed corticosteroids as well as several other cytotoxic agents which are also known to depress the function of PMNs\textsuperscript{96}. Finally, long-term follow-up on patients who develop skeletal metastases is difficult due to the poorer prognosis associated with this finding. Therefore, it cannot be stated without further investigation that the drug-induced impairments in PMN function are playing a causal role with respect to the development of BRONJ. In order to determine if pamidronate-induced reductions in PMN function directly predispose patients to the development of BRONJ, we suggest that additional investigation is required.

This study included individuals of varying ages (range: 34 to 77) which may bring to question the concept of immunosenescence, or age-related functional impairment. Though this concept is better understood as it relates to the adaptive arm of the immune system, it has
been demonstrated that the innate immune system may also be affected. More specifically, the PMN may suffer from reduced chemotaxis, phagocytosis, and ROS production\textsuperscript{97–99}.

### 5.3 Future Direction

Future work should be conducted to determine if there is a decrease in PMN functionality in the long-term. Therefore, continued surveillance of this patient population who commonly receive monthly intravenous pamidronate infusions should be completed. Ideally, this should be executed as a multi-centre study involving those institutions that treat patients diagnosed with multiple myeloma and are capable of and familiar with techniques of PMN isolation and functional testing. Following patients through their initial course of pamidronate as well as after its discontinuation will confirm whether or not impairment of the function of PMNs is a permanent or transient phenomenon associated with initial exposure to a bisphosphonate. In this regard, it is possible that following cessation of bisphosphonate treatment, PMN function could return to normal. However, if impaired PMN function truly plays an important role in the development of BRONJ, a condition that can develop well after drug exposure has been stopped, it would be expected that the effects of bisphosphonates on PMN function are prolonged. This would be feasible, as bisphosphonates are known to be stored in bone for several years and released in their active form during bone remodeling. The effects of the long biological half-lives of these medications have been noted in the context of observing no significant differences in bone mineral density following drug holidays of several years in length\textsuperscript{100}. Therefore, prolonged follow-up of those patients treated with intravenous bisphosphonates to observe whether there is a correlation between BRONJ development and depressed innate immune system function would be of great interest.

Although this investigation focused on the use of pamidronate in patients diagnosed with multiple myeloma, there is growing use of another bisphosphonate, zoledronate. This bisphosphonate can be infused more quickly than pamidronate and has reduced long-term effects on kidney function\textsuperscript{101,102}. The effects of zoledronate on the function of PMNs have not been elucidated, although the literature suggests that the use of this agent might
increase the risk for the development of BRONJ when compared to pamidronate\textsuperscript{103,104}. Therefore, the effects of this drug on PMN function will have to be investigated.

5.4 Clinical Relevance

Several attempts have been made to validate the use of biomarkers such as bone-specific alkaline phosphatase (bALP) and C-terminal telopeptide of collagen I (CTX) to assess BRONJ susceptibility with limited success\textsuperscript{105,106}. Biomarkers, in general, are objectively quantifiable indicators that aid in diagnosis and prognostication of disease states through the evaluation of biochemical, molecular, physiologic, and anatomic parameters\textsuperscript{107}. As we have only observed PMN dysfunction one month following the administration of pamidronate, our data cannot support the use of PMN function as a biomarker at this stage. In order to accomplish this, we suggest that it will be important to assess PMN function in those patients who develop BRONJ as compared to similarly-treated patients who do not develop BRONJ. In relation to this, we would expect that those who develop BRONJ would have significantly reduced PMN function in comparison to the PMNs derived from patients who do not develop BRONJ. This could lead to the identification of a minimum level of PMN functional impairment, below which the development of BRONJ becomes likely. In the cases where patients demonstrate markedly impaired PMN function, closer attention would be given to the prevention of dentoalveolar disease as well as the more aggressive treatment of prognostically questionable teeth to prevent the development of BRONJ in a proactive fashion. For those patients who have received or currently require intravenous bisphosphonate therapy, the development of a simple and non-invasive oral rinse to stratify BRONJ susceptibility using PMN impairment as the biomarker will aid with the selection of surgical approaches and informed consent from the patient. Furthermore, the identification of those individuals at significant risk of developing BRONJ may also remove some of the apprehension associated with treating patients who have previously been exposed to bisphosphonate medications\textsuperscript{108}. Thus, reduced PMN function may become an integral biomarker for patients receiving bisphosphonates due its potential ability to stratify the risk for developing BRONJ.
Chapter 6

6 Conclusion

The results from this study demonstrate that PMN chemotaxis and ROS production are impaired following the administration of pamidronate in previously bisphosphonate-naïve patients. As such, we accept our aforementioned hypothesis. Further studies, as outlined above, will be required to evaluate the potential of utilizing blood and oral PMN function as a biomarker for BRONJ susceptibility and elucidate the role of depressed innate immunity in the pathogenesis of BRONJ.
Appendix

![Chemical structures](image)

Figure 1: Inorganic pyrophosphate and bisphosphonate structure (Inorganic pyrophosphate [left]; generic bisphosphonate [right]).
Figure 2: The mevalonate pathway (Pharmacologic agents [*blue boxes*] which inhibit normal cellular activity; HMG-CoA: Hydroxymethylglutaryl-coenzyme A; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate).
Figure 3: Panoramic image of a patient diagnosed with BRONJ (Generalized sclerosis of the right and left mandible [blue arrows] and sequestration [red arrow] within the body of the right mandible).
Figure 4: NCE MDCT (axial slice, bone window) of a patient diagnosed with BRONJ (Sclerosis and persistent extraction sites [*blue and green arrows, respectively*] involving the right and left mandible and sequestration [*red arrow*] within the body of the right mandible).
Figure 5: NCE MDCT (coronal slice, bone window) of a patient diagnosed with BRONJ (Sclerosis [blue arrows] of the right and left mandible and cutaneous fistula [green arrow] and sequestration [red arrow] within the body of the right mandible).
Figure 6: Photomicrograph of a biopsy specimen from a patient with a diagnosis of BRONJ (Fragments of necrotic bone lacking osteocytes [blue arrow] with prominent reversal lines [green arrow], bacterial colonies [red arrow], scattered erythrocytes [yellow arrow] and neutrophils [orange arrow]; Hematoxylin and eosin staining, decalcified specimen; Magnification x50).
Figure 7: Boxplot of patient blood PMN ROS activity as measured by cytochrome c oxidation with PMA and fMLP stimulation (Statistically significant difference noted between pre-pamidronate [blue bars] and post-pamidronate [red bars] evaluation; **P < 0.05; AU: Absorption Units).
Figure 8: Boxplot of control blood PMN ROS activity as measured by cytochrome c oxidation with PMA and fMLP stimulation (No statistically significant differences \( P \geq 0.05 \) noted between baseline [yellow bars] and follow-up [green bars] evaluation; AU: Absorption Units).
Figure 9: Boxplot of patient oral PMN ROS activity as measured by cytochrome c oxidation with PMA and fMLP stimulation (Statistically significant difference noted between pre-pamidronate (blue bars) and post-pamidronate (red bars) evaluation (**P < 0.05; AU: Absorption Units).
Figure 10: Boxplot of control oral PMN ROS activity as measured by cytochrome c oxidation with PMA and fMLP stimulation (No statistically significant differences [$P \geq 0.05$] noted between baseline [yellow bars] and follow-up [green bars] evaluation; AU: Absorption Units).
Figure 11: Boxplot of patient blood and oral PMN chemotaxis within a Zigmond chamber with fMLP stimulation (Statistically significant differences noted between pre-pamidronate [blue bars] and post-pamidronate [red bars] evaluation [**$P < 0.05$]).
Figure 12: Boxplot of control blood and oral PMN chemotaxis within a Zigmond chamber with fMLP stimulation (No statistically significant differences \[ P \geq 0.05 \] noted between baseline [yellow bars] and follow-up [green bars] evaluation).
<table>
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<tr>
<th>Drug Name</th>
<th>Trade Name</th>
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<th>R2</th>
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Table 1: Common bisphosphonate agents.
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<td>Coronary Artery Disease</td>
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<tr>
<td>62</td>
<td>Sickle Trait</td>
<td>CyBorD</td>
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</table>

Table 2: Bisphosphonate naïve study patient information (CyBorD: Cyclophosphamide 300mg/m\(^2\), Bortezomib 1.5mg/m\(^2\), Dexamethasone 40mg chemotherapy).
Table 3: Paired t-test analysis (Comparison of pre-pamidronate and post-pamidronate chemotaxis and ROS results in patients and controls; §Migration speed [μm/minute], #Cytochrome C Oxidation Activity [Absorption Units]; *P < 0.05; NS Not Significant, P ≥ 0.05).
Plate 1: Facial photograph of a patient diagnosed with BRONJ (Two extra-oral fistulae [blue arrows] located inferiorly to the inferior border of the right mandible).
Plate 2: Intra-oral photograph of a patient diagnosed with BRONJ (Region of exposed bone [blue arrow] associated with the mesio-lingual surface of the right mandibular second molar).
References


Postmenopausal Women With Osteoporosis Treated With Raloxifene. Results From a 3-Year Randomized Clinical Trial. *JAMA.* 1999;282(7):637.


