Pamidronate does not affect cortical porosity in transgenic mice overexpressing osteoblastic $\text{G}_{\alpha}$

by

Antonella Poon

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Pharmacology and Toxicology

University of Toronto

© Copyright by Antonella Poon 2019
Pamidronate does not affect cortical porosity in transgenic mice overexpressing osteoblastic Gαs

Antonella Poon

Master of Science

Department of Pharmacology and Toxicology

University of Toronto

2019

ABSTRACT

G-protein coupled receptors are involved in many signal transduction pathways. The Gαs protein becomes activated following ligand binding, to stimulate downstream adenylyl cyclase. Previously, Zhang et al. generated a transgenic murine model with osteoblastic Gαs overexpression, which led to increased bone mass. μCT showed a marked increase in closed cortical porosity, which histologically showed intracortical pores lined with osteoclasts.

Pamidronate is clinically used for excessive bone loss. It inhibits farnesyl pyrophosphate, an enzyme involved in synthesis of regulatory proteins, leading to osteoclastic apoptosis. Our objective was to use pamidronate to ameliorate intracortical porosity. Low dose pamidronate led to increased trabecular bone mass in wild-type mice only. High dose pamidronate led to increased trabecular bone mass in both wild-type and Gs-Tg mice. Cortical porosity was not affected at both dosages. There is evidence to suggest that pamidronate unsuccessful in affecting intracortical porosity due to lack of penetration into the intracortical space.

Word Count: 150/150 words
ACKNOWLEDGEMENTS

In Loving Memory of Charlie Yang

The journey to complete my graduate degree has been extremely difficult. Although it has not been an overwhelmingly positive experience, I have gained invaluable knowledge in the field of Pharmacology that will allow me to achieve my career goals. Over the past two years I learned that the most important thing is to have a good support network around you, to help weave through any challenge that may arise. I have reached the finish line only due to the support and encouragement of my family. To my parents who have supported me through eighteen years of consecutive education, I will continue to make you proud. To my best friend and life partner Jonathan Liu, we finally made it and I look forward to our future with Hooni. To my brothers Shane Tingting, Derek Ho, Bernardo Cardona, and Jason Li, thank you for being my family for the past ten years, and forever to come.

There are some amazing individuals that have been made this thesis possible. To David Murray, Adele Changoor, and Richard Cheung, my lab family, I will never forget the amazing working environment that you created for me. David, you are a pure and kind soul. Rich, you are always the voice of reason. Adele, I aspire to be like you in the future. To Gilberto Li Feng, I will always be around to support you, don’t ever forget that. To Jesse Rentz, Jordan Winberg, and Oladipo Oladapo, thank you for always trying to help, and for making me laugh. To Dr. Kim Sugamori, you are an amazing researcher, and I hope to see you continue and advance in our field. To Dr. Michelle Arnot, thank you for always giving me a chance, and for your efforts to support mental health for the students. To Dr. Peter McPherson, thank you for not only being a mentor, but a great friend. Your support has been vital to me over the past four years.

To Dr. Marc Grynpas, thank you for welcoming me into Grynpasland. I am honoured to have worked at the Lunenfeld-Tanenbaum Research Institute. I have made amazing memories and lifelong friendships. To Dr. Jane Mitchell, I was surprised when you accepted me into your lab. I did not think I was worthy or a good enough student to do so. I have always tried my best to produce good results. I have learned how to navigate a tough work environment and have emerged as a stronger student and researcher. I am grateful that I was able to work in two different research institutes and experience different environments.

I would like to specially thank members of the Salmena Lab and Dr. Leonardo Salmena for always welcoming me into the lab when I need to use the pH meter and to let off some steam. To the friends who have offered me continued support and encouragement, I thank you and will never forget the memories we have made together. Once again, thank you to everyone in my life who has helped me make it this far.
# Table of Contents

1. **CHAPTER 1: Introduction** ............................................................................................................. 1

1.1 **Bone Biology** .............................................................................................................................. 1

1.1.1 **Bone Structure** ..................................................................................................................... 1

1.1.2 **Bone Composition** ............................................................................................................... 2

1.1.3 **Bone Cells** ........................................................................................................................... 3

1.1.4 **Bone Development and Maintenance** .................................................................................... 6

1.1.5 **Bone Modeling and Remodeling** .......................................................................................... 6

1.1.5 **Bone Disease** ....................................................................................................................... 7

1.1.6 **Bone Therapeutics** ............................................................................................................... 8

1.2 **Bone Quality and Assessment** ................................................................................................. 9

1.2.1 **Bone Microarchitecture** ....................................................................................................... 10

1.2.2 **Bone Histomorphometry** ..................................................................................................... 11

1.3 **G Protein Coupled Receptors** .................................................................................................. 12

1.3.2 **G Protein Subtypes** .............................................................................................................. 14

1.3.3 **Gαs and Bone Disease in Humans** ...................................................................................... 15

1.3.4 **Gα and Bone Disease in Murine Models** ............................................................................. 19

1.4 **Project Rationale and Aims** .................................................................................................... 20

1.4.1 **Project Background** ............................................................................................................ 20

1.4.2 **Project Rationale** ............................................................................................................... 21

1.4.3 **Project Objectives** .............................................................................................................. 22
1.4.4  Project Hypotheses ......................................................................................................... 22

2  CHAPTER 2: Materials and Methods ................................................................................. 23

2.1  Animals ............................................................................................................................. 23

2.2  Pamidronate and Calcein Green Preparation ................................................................. 23

2.3  Experimental Design ....................................................................................................... 23

   2.3.1  Pilot Study: Investigation of age relative to cortical porosity ................................. 23

   2.3.2  Study 1: Investigation of preventative pamidronate administration in Gs-Tg mice 24

   2.3.3  Study 2: Investigation of reparative pamidronate administration in Gs-Tg mice 24

2.4  Sample Collection ........................................................................................................... 25

2.5  Assessment of Bone Microarchitecture ......................................................................... 25

   2.5.1  Scanning Specifications ............................................................................................. 25

   2.5.2  Trabecular Bone ........................................................................................................ 26

   2.5.3  Cortical Bone ............................................................................................................. 27

2.6  Assessment of Bone Histomorphometry ....................................................................... 27

   2.6.1  Sample Preparation .................................................................................................... 27

   2.6.2  Sectioning .................................................................................................................. 28

   2.6.3  Staining ....................................................................................................................... 28

   2.6.4  Trabecular Bone Analysis .......................................................................................... 28

   2.6.5  Cortical Bone Analysis ............................................................................................... 29

   2.6.6  Statistical Analyses .................................................................................................... 29

3  CHAPTER 3: Results ............................................................................................................ 30
3.1 *Pilot Study:* Investigation of cortical porosity relative to age ............................................ 30

3.2 *Study 1:* Investigation of preventative pamidronate administration in Gs-Tg mice ................ 31
   3.2.1 Assessment of trabecular bone: µCT and histomorphometry ........................................ 31
   3.2.2 Assessment of cortical bone: µCT and histomorphometry ........................................... 34

3.3 *Study 2:* Investigation of reparative pamidronate administration in Gs-Tg mice ............. 38
   3.3.1 Assessment of trabecular bone: µCT and histomorphometry ........................................ 38
   3.3.2 Assessment of cortical bone: µCT and histomorphometry ........................................... 41

4 *CHAPTER 4: Discussion* ........................................................................................................ 45

4.1 Effects of treatment on trabecular bone ............................................................................. 45
   4.1.1 Study 1 - trabecular bone microarchitecture ............................................................... 45
   4.1.2 Study 1 – trabecular bone histology .............................................................................. 47
   4.1.3 *Study 2* – bone microarchitecture .............................................................................. 47
   4.1.4 *Study 2* – histology ..................................................................................................... 49

4.2 Effects of treatment on cortical bone ................................................................................ 50
   4.2.1 *Study 1* – bone microarchitecture .............................................................................. 50
   4.2.2 *Study 1* – histology ..................................................................................................... 50
   4.2.3 *Study 2* – bone microarchitecture .............................................................................. 51
   4.2.4 *Study 2* – histology ..................................................................................................... 52

4.3 Limitations ......................................................................................................................... 52

4.4 Summary and Conclusions ............................................................................................... 53

4.5 Future Directions .............................................................................................................. 54
CHAPTER 5: References........................................................................................................................................... 59

CHAPTER 6: Appendix............................................................................................................................................... 68

6.1 Study 1 trabecular halftone view and thresholding ................................................................. 68

6.2 Study 1 cortical bone halftone view and thresholding ............................................................. 69

6.3 Study 2 trabecular halftone view and thresholding ................................................................. 70

6.4 Study 2 cortical halftone view and thresholding ................................................................. 71
LIST OF FIGURES

Figure 1.1. Representative long bone anatomy................................................................. 1
Figure 1.2. Visualization of trabecular bone and cortical bone, with functional osteon units ........ 2
Figure 1.3. Osteoblastogenesis pathway showing Cbfa1/RUNX2’s role ................................. 4
Figure 1.4. Osteoclastogenesis pathway showing M-CSF and RANKL’s role in differentiation ........ 5
Figure 1.5. Rates of osteopenia and osteoporosis in the United States.................................. 7
Figure 1.6 Skyscan 1174.................................................................................................. 11
Figure 1.7. Canonical TRAP stain – dark red indicates TRAP+ staining.............................. 11
Figure 1.8. Schematic of a canonical GPCR with 7TM helices............................................. 12
Figure 1.9. General schematic of GPCR action.................................................................... 13
Figure 1.10. Schematic of mechanism of homologous desensitization................................. 14
Figure 1.11. Schematic of the Ga subtypes and their interactions with their effectors ............... 15
Figure 1.12. Examples of café-au-lait lesions...................................................................... 17
Figure 1.13. X-ray image showing polyostotic FD in the pelvis and femur ............................. 18
Figure 1.14. Qualitative tibial sections comparing WT and Gs-Tg cortical bone at 26 weeks of age...... 20
Figure 2.1. Timeline for Study 1: Investigation of preventative PAM administration in Gs-Tg mice..... 24
Figure 2.2. Timeline for Study 2: Investigation of reparative PAM administration in Gs-Tg mice........ 24
Figure 2.3. Sampled view of raw CT scan and reconstructed images ..................................... 26
Figure 2.4. Sample of selected trabeculae for analysis on a reconstructed cross-sectional image........ 26
Figure 2.5. Sample of outlined cortical bone for analysis on a reconstructed cross-sectional image ....... 27
Figure 3.1. Graphical representation for Piló Study cortical bone microarchitecture..................... 30
Figure 3.2. Graphical representation for Study 1 trabecular bone microarchitecture.................... 32
Figure 3.3. Graphical representation for Study 1 trabecular bone histology.............................. 33
Figure 3.4. Representative images for Study 1 TRAP-stained trabecular bone ............................ 34
Figure 3.5. Graphical representation for Study 1 cortical bone microarchitecture ....................... 35
Figure 3.6. Graphical representation for Study 1 cortical bone histology..............................................36
Figure 3.7. Representative images for Study 1 TRAP-stained cortical bone.............................................37
Figure 3.8. Graphical representation for Study 2 trabecular bone microarchitecture..............................39
Figure 3.9. Graphical representation for Study 2 trabecular bone histology.............................................40
Figure 3.10. Representative images for Study 2 TRAP-stained trabecular bone......................................41
Figure 3.11. Graphical representation for Study 2 cortical bone microarchitecture.................................42
Figure 3.12. Graphical representation for Study 2 cortical bone histology.............................................43
Figure 3.13. Representative images for Study 2 TRAP-stained cortical bone........................................44
Figure 4.1. Histological sample showing the difference between primary and secondary osteons...........56
Figure 4.2. Examples of secondary osteons in Study 1.................................................. Error! Bookmark not defined.
Figure 4.3. Examples of secondary osteons in Study 2..........................................................58
Figure 6.1. Halftone and thresholding for Study 1 trabecular bone ......................................................68
Figure 6.2. Halftone and thresholding for Study 1 cortical bone ..........................................................69
Figure 6.3. Halftone and thresholding for Study 2 trabecular bone ......................................................70
Figure 6.4. Halftone and thresholding for Study 2 cortical bone ..........................................................71
### LIST OF TABLES

Table 3.1. Numerical values for *Pilot Study* cortical bone microarchitecture. .................................................. 31

Table 3.2. Numerical values for *Study 1* trabecular bone microarchitecture. ..................................................... 33

Table 3.3. Numerical values for *Study 1* trabecular bone histology. ................................................................. 34

Table 3.4. Numerical values for *Study 1* cortical bone microarchitecture. ......................................................... 35

Table 3.5. Numerical values for *Study 1* cortical bone histology. ................................................................. 37

Table 3.6. Numerical values for *Study 2* trabecular bone microarchitecture. ..................................................... 39

Table 3.7. Numerical values for *Study 2* trabecular bone histology. ................................................................. 41

Table 3.8. Numerical values for *Study 2* cortical bone microarchitecture. ......................................................... 42

Table 3.9. Numerical values for *Study 2* cortical bone histology. ................................................................. 44
LIST OF PUBLICATIONS AND ABSTRACTS


AWARDS AND HONOURS

2018: University of Toronto Fellowship, Department of Pharmacology and Toxicology

2019: Best Poster, Collaborative Specialization in Musculoskeletal Sciences Annual Research Day
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>µCT</td>
<td>micro-computed tomography</td>
</tr>
<tr>
<td>7TM</td>
<td>seven-transmembrane</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AHO</td>
<td>Albright hereditary osteodystrophy</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic proteins</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>core binding factor a1</td>
</tr>
<tr>
<td>Gs-Tg</td>
<td>Gas transgenic</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>APD</td>
<td>anterior-posterior diameter</td>
</tr>
<tr>
<td>B. Pm</td>
<td>bone perimeter</td>
</tr>
<tr>
<td>BP</td>
<td>bisphosphonate</td>
</tr>
<tr>
<td>BS/BV</td>
<td>bone surface/bone volume</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>BV/TV</td>
<td>bone volume/tissue volume</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotropic factor</td>
</tr>
<tr>
<td>Cs. Th</td>
<td>cross-sectional thickness</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>Ct. B. Ar</td>
<td>cortical bone area</td>
</tr>
<tr>
<td>DXA</td>
<td>dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>Ec. Ar</td>
<td>endocortical area</td>
</tr>
<tr>
<td>Ec. Pm</td>
<td>endocortical perimeter</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>B. Ar</td>
<td>bone area</td>
</tr>
<tr>
<td>FD</td>
<td>fibrous dysplasia</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FPP</td>
<td>farnesyl pyrophosphate synthase</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAS</td>
<td>McCune Albright Syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MLD</td>
<td>mediolateral diameter</td>
</tr>
<tr>
<td>MMI</td>
<td>polar moment of inertia</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>N. Oc</td>
<td>number of osteoclasts</td>
</tr>
<tr>
<td>N. Oc/BS</td>
<td>number of osteoclasts/bone volume</td>
</tr>
<tr>
<td>N. Oc/TV</td>
<td>number of osteoclasts/tissue volume</td>
</tr>
<tr>
<td>Oc. N</td>
<td>osteoclast number</td>
</tr>
<tr>
<td>Oc. S/BS</td>
<td>osteoclast surface/bone surface</td>
</tr>
<tr>
<td>Oc. S/PS</td>
<td>osteoclast surface/pore surface</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>Osf2</td>
<td>osteoblast specific factor 2</td>
</tr>
<tr>
<td>OSM</td>
<td>oncostatin M</td>
</tr>
<tr>
<td>PAM</td>
<td>pamidronate</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Po (cl)</td>
<td>closed porosity</td>
</tr>
<tr>
<td>Po. Ar</td>
<td>pore area</td>
</tr>
<tr>
<td>Po. N</td>
<td>pore number</td>
</tr>
<tr>
<td>Ps. Pm</td>
<td>periosteal perimeter</td>
</tr>
<tr>
<td>qCT</td>
<td>quantitative computed tomography</td>
</tr>
<tr>
<td>RANK</td>
<td>RANK ligand</td>
</tr>
<tr>
<td>RUNX2</td>
<td>runt-related transcription factor</td>
</tr>
<tr>
<td>SMI</td>
<td>structure model index</td>
</tr>
<tr>
<td>Tb. N</td>
<td>trabecular number</td>
</tr>
<tr>
<td>Tb. Pf</td>
<td>trabecular pattern factor</td>
</tr>
<tr>
<td>Tb. Sp</td>
<td>trabecular separation</td>
</tr>
<tr>
<td>Tb. Th</td>
<td>trabecular thickness</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>βarr2</td>
<td>beta-arrestin-2</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
</tbody>
</table>
1 CHAPTER 1: Introduction

1.1 Bone Biology

The skeletal system performs a myriad of roles within the human body, and its importance can sometimes be overlooked. It is involved in support and acts as a scaffold for internal organs, and other tissues. It is involved in protection, mechanically protecting the heart, lungs, and brain amongst others. It is involved in mineral storage, with bone acting as a vessel to store and regulate homeostasis of calcium and phosphate. It is involved in blood cell production, bone marrow being the origin of production for red and white blood cells, and platelets. Lastly, it is involved in the musculoskeletal system to act in conjunction with skeletal muscles to change allow diverse movements.

1.1.1 Bone Structure

Bones at the gross anatomy level can be categorized based on shape. There are cylindrical bones with bulbous ends, called long bones. Long bones include limb, hand, and foot bones. Other shapes are flat and tabular bones, or block-like irregular bones. The anatomical terminology in reference to long bones will recur throughout this dissertation and is detailed in Figure 1.1. The shaft of the bone, or diaphysis, is separated from the bulbous ends, or epiphyses, by a small area of bone called the metaphyses. The cylindrical area inside the shaft is called the medullary cavity.

However different the shapes may be, they are all made of the same basic structural components: trabecular bone, and cortical bone. It is important to note that the molecular and cellular components of both trabecular and cortical bone remain identical; the degree of porosity determine their distinction. Trabecular bone, also known as spongy or cancellous bone, exists in the medullary cavity and epiphyses in
long bone. It is composed of trabeculae, which are plate and rod-like structures of bone. The network of shapes provides an increase in maximal load support, without increasing the weight of the bones, which in turn facilitates movement. Cortical bone, also known as compact bone, exists as a dense layer that envelops the inner components. In humans, cortical bone is made up of functional units named osteons, or Haversian systems, which exist in a parallel orientation to the bone. The osteon is composed of a central canal, housing blood vessels, lymph, and nerve fibers. This canal is crucial in its role to provide blood to cells that are deep in cortical bone. In Figure 1.2 below, the interwoven trabeculae can be visualized, adjacent to the external cortical bone which is made up of functional osteon units.

![Figure 1.2. Visualization of trabecular bone and cortical bone, with functional osteon units](image)

1.1.2 Bone Composition

The molecular structure of bone is of a composite nature, which parallels the multiple roles that bone is involved in. Bone is composed of a combination of inorganic and organic components. The inorganic component of bone is made up of calcium phosphate (Ca₅(PO₄)₂) and calcium hydroxide (Ca(OH)₂), which combine to form hydroxyapatite (HA) crystals. The crystals also contain other ions and calcium moieties that adhere during their process of formation. The inorganic component of bone itself provides strength in rigidity and can resist simple compression forces but will shatter if twisted or bent. The organic component (~90% type 1 collagen, ~5% non-collagenous proteins) provides flexibility, tensile
strength, and allows bone to withstand a variety of different forces. The most abundant form of collagen in bone is type 1 collagen, and its triple helix structure is made of two α1 helices (COL1A1), and one α2 helix (COL1A2). Type 1 collagen’s unique crosslinking ability contributes to its many structural capabilities, including elasticity and acting as a scaffold for mineralization. The combination of the inorganic and organic components is what give bone its characteristic strength and enough flexibility to work in conjunction with muscles, to allow fluid and light movement.

1.1.3 Bone Cells

Osteoclasts, osteocytes, and osteoblasts are the three primary cell types that play different roles in the formation and maintenance of the highly complex bone. Osteoblasts and osteoclasts work closely with each other, but they originate from different cellular origins. Osteoclasts originate from hematopoietic stem cells, and osteoblasts originate from multipotent mesenchymal stem cells. Despite their differences, maintenance of normal physiological conditions in bone depends on the precise balance between osteoblastic and osteoclastic activity. When this balance is abnormally disturbed, alterations in bone quality could arise, such as osteoporosis or osteopetrosis.

1.1.3.1 Osteoblasts

Mature osteoblasts, commonly known as bone-forming cells, are cuboidal single-nuclear cells that sit in a single layer on bone surfaces. Osteoblastogenesis from mesenchymal stem cells occurs due to the presence of specific growth factors, cytokines, and adhesion molecules to regulate cell interactions. Briefly, osteoblastogenesis is initiated by bone morphogenetic proteins (BMPs). BMPs stimulate transcription of osteoblast specific factor 2 (Osf2) or core binding factor a1 (Cbfa1). These are known as osteoblast-specific transcription factors. Cbfa1, also known as runt-related transcription factor (RUNX2), is of utmost importance in osteoblastogenesis as it is the first step in differentiating the stem cells into preosteoblasts, as shown in Figure 1.3. RUNX2 also activates the further downstream osteoblast-specific genes like osteopontin (OPN), bone sialoprotein (BSP), type 1 collagen, and osteocalcin (OCN). The importance of RUNX2 was confirmed in knockout (KO) studies that ablated osteoblastogenesis and bone formation. Other
transcription factors or cytokines that are involved include transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and fibroblast growth factor (FGF) family members.

The process in which new bone is laid down is osteogenesis. Osteoblasts secrete osteoid or pre-bone tissue, which is collagen-rich organic bone matrix that has not been calcified. The osteoid eventually becomes mineralized following HA deposition and becomes a new layer of bone. The enzyme alkaline phosphatase (ALP) has been shown to be involved in the mineralization process, as ALP knockdowns lead to non-mineralized bone deposition. The exact mechanism has not been elucidated. ALP is often used as a histochemical or biochemical marker for osteoblasts, as it is highly expressed by osteoblasts.

1.1.3.2 Osteocytes

When osteoblasts secrete enough matrix to become enveloped in osteoid, they become stellate-shaped osteocytes. The spaces that they occupy are called lacunae. Embedded osteocytes maintain bone integrity through their mechanosensory abilities, relaying differential osteogenesis requirements caused by adaptive needs or injury, to osteoblasts. Cell-cell communication is maintained through connected lacunae extensions called canaliculi, which act as information tunnels. Most importantly, osteocytes maintain their surrounding matrix by monitoring their mineral content. Minerals like calcium salts are brought into the matrix through recycling or from blood if needed, as directed by osteocyte signaling.
1.1.3.3 Osteoclasts

Mature osteoclasts, commonly known as bone-resorbing cells, are large multinucleated cells commonly present at sites where bone matrix is being actively removed\textsuperscript{1,3}. Osteoclastogenesis from hematopoietic stem cells occurs due to presence of cytokines and colony-stimulating factors (CSFs). Specific examples include interleukin (IL) 1, 3, 6, and 11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotropic factor (CNTF), tumor necrosis factor (TNF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), and c-kit ligand\textsuperscript{6}. Of most importance is M-CSF, and RANK ligand (RANKL), as they are essential to initiate and carry out osteoclastogenesis as can be seen in Figure 1.4. They must act together to activate osteoclast specific genes like tartrate-resistant acid phosphatase (TRAP)\textsuperscript{10}.

The process in which bone is resorbed is called resorption, which means removal of bone\textsuperscript{2}. Osteoclasts adhere to bone surfaces using their finger-like projections\textsuperscript{6}, secreting acids and enzymes to achieve the breakdown of bone matrix\textsuperscript{3}. This process causes the matrix minerals to be released and can be an important physiological response when calcium or phosphate levels are low in blood\textsuperscript{1}. Multiple enzymes are involved in the process of matrix degradation. Matrix metalloproteinases (MMPs) break down the organic component including collagen, alongside cathepsins K, B, and L which also are secreted into the resorption area\textsuperscript{6}.

Figure 1.4. Osteoclastogenesis pathway showing M-CSF and RANKL’s role in differentiation\textsuperscript{10}.
1.1.4 Bone Development and Maintenance

Osteogenesis begins in the embryo and can continue up until an individual is 25 years old. Until an embryo reaches eight weeks of age, the skeletal components of the fetus is composed of mesenchyme or hyaline cartilage. Ossification is a term that describes replacement of mesenchyme or hyaline cartilage, with bone tissue. In the context of embryonic growth and development, there are two types of ossification: intramembranous and endochondral. Simply, intramembranous ossification occurs when bone is created from a fibrous membrane prior to cartilage development, and endochondral ossification occurs when bone replaces developed hyaline cartilage scaffolds.

In the context of this dissertation, endochondral ossification will be of more importance; bones that are formed from endochondral ossification are weight-bearing, such as the limbs and vertebrae. Endochondral ossification begins in the second trimester of pregnancy and continues through to adulthood. Bone deposition in the context of growth occurs through deposition of layers. This is because osteocytes cannot divide and are trapped within their respective calcified areas. The point of initiation of growth, where bone first replaces cartilage, is called the nutrient foramen. Continued deposition of bone layers is called appositional growth, for circumferentially directional growth. Lengthwise growth is carried out by the growth plate, or epiphyseal plate, which is a layer of cartilage that continues to push away from the shaft. As the rate of epiphyseal plate movement slows and ossification continues to be carried out, all epiphyseal cartilage will be converted to bone. This is called epiphyseal closure and signifies mature bone.

1.1.5 Bone Modeling and Remodeling

Bone modeling and remodeling are terms that frequently arise in bone and mineral research. They both refer roughly to bone maintenance. Bone modeling is maintenance of bone during growth, and remodeling refers to maintenance of mature bone throughout life in response to physiological changes. Bone that is present before it becomes modeled is called immature or woven bone, as the collagen fibres appear randomly ‘woven’. Woven bone will be replaced during modeling by mature or lamellar bone, which features organized mineralized matrix. As newly formed bone is mineralized during embryogenesis,
modeling can occur to simultaneously alter its architecture. For example, the modeling process for bulbous ends of long bones occurs when osteoblasts build periosteal bone and osteoclasts remove endosteal bone.

Remodeling occurs when existing bone is resorbed, and new bone is placed down. A simple example of this in humans is when adolescents get corrective braces. Teeth and tooth sockets will reposition due to remodeling that is initiated by mechanical forces. Bone in the old socket position will be resorbed and new bone will be placed down. The process of remodeling is carried out by basic multicellular units (BMUs) and is executed in a specific stepwise fashion. The first step is activation which requires newly differentiated cells to reach the site of remodeling. The second step is resorption, carried out by osteoclasts. The third step is reversal, with osteoclastic activity slowly transitioning to osteoblastic activity. Lastly, the formation step involves osteoblasts laying down new osteoid to be mineralized and become new bone.

1.1.5 Bone Disease

Osteoporosis is one of the most common bone diseases, with a prevalence of 1.5 million Canadians aged ≥40. In the United States, 10 million people ≥50 and older have hip osteoporosis, and 33.6 million people aged ≥50 are at risk of progressing from osteopenia to osteoporosis. The numbers can be quite drastic and with a largely aging population, the number of instances is projected to increase; by 2020 it has been projected that one in two Americans would be at risk of developing osteoporosis, seen in Figure 1.5.

Osteoporosis, a condition simply defined as reduced bone mass, can be divided into two categories: primary and secondary. Primary is the result of bone degradation over time, usually due to age. Age-related osteoporosis has higher incidence rates in women due to an acute spike of bone loss at the time
of menopause. It is postulated that progressively decreasing amounts of estrogen in both men and women is a contributor to age-related osteoporosis, and there are well documented effects of estrogen on bone loss. Secondary osteoporosis is when osteoporosis occurs as a result of a different disease or medication. This occurs frequently in conditions like Duchenne Muscular Dystrophy, where treatment plans are comprised of chronic glucocorticoid (GC) treatment. GCs have direct and indirect effects on bone resorption including induction of osteoclasts and suppression of osteoblast activity.

Unfortunately, there is no ‘cure’ for osteoporosis. The best approach to osteoporosis is prevention and early detection. Prevention and early detection rely heavily on measurement of bone mineral density (BMD). This can be achieved in humans through dual-energy x-ray absorptiometry (DXA), or quantitative computed tomography (qCT). DXA is non-invasive and has low radiation exposure levels, whilst qCT is costly and requires a high exposure to radiation. Whether this technology is regularly applied in clinic determines the patient outcomes; if primary care physicians do not regularly prescribe BMD measurement, progression to osteoporosis can occur. It is particularly important that peak bone mass in early adult life is maximized by adequate calcium intake, vitamin D levels, and regular bone-loading exercise during childhood. All of these contribute to mitigation of the effects of bone loss later in life. In the context of non-medicinal preventative measures, exercise, vitamin D, and calcium supplements are recommended. These are simple steps that every individual can have access to and can greatly benefit bone health. If not detected early and upon diagnosis, the best course of action for osteoporosis is treatment to prevent fractures.

1.1.6 Bone Therapeutics

A major focus of osteoporosis treatment is the concept of anti-resorptive therapy. This plays off the concept that a contributing mechanistic factor could be osteoclastic activity outracing osteoblastic activity. A major class of anti-resorptives are the bisphosphonate (BP) compounds. Bisphosphonates are pyrophosphate analogues with a central carbon atom instead of an oxygen atom. This confers a P-C-P moiety which is responsible for BPs affinity to HA, causing BPs to bind to bone. BP affinity to calcium moieties can be increased through structural modifications such as a hydroxyl group on the R1 side chain.
of the central carbon atom. The general encompassing mechanism for anti-resorptive activity of BPs is thought to come from their inhibition of osteoclasts by inducing apoptosis upon ingestion. A variety of things can happen to cause this including cytoskeletal disruption, loss of the ruffled border, and inhibition of lysosomal enzymes\textsuperscript{16,17}. Second-generation bisphosphonates were created in attempts to increase the potency of BPs and was achieved by insertion of a primary nitrogen atom. This increases the affinity of BPs by 10 to 100-fold when compared to the first-generation BPs, etidronate and clodronate. Addition of another nitrogen atom further increased potency and produced the nitrogenous BPs, such as ibandronate and olpadronate\textsuperscript{16}.

Pamidronate (PAM) is a second-generation bisphosphonate containing a single nitrogen atom. It is primarily used in conditions that present with excessive loss of bone or excessive resorption. Such conditions include Paget’s disease of bone, tumor-induced hypercalcemia, or osteolytic bone metastases arising from cancer\textsuperscript{18}. Excessive doses of PAM have been reported to cause features of osteopetrosis\textsuperscript{16}, further emphasizing that PAM is one of the stronger anti-resorptives. PAM exerts its anti-resorptive effects through direct inhibition of osteoclasts, by inducing apoptosis upon uptake into the cell. PAM inhibits the key regulatory enzyme farnesyl pyrophosphate synthase (FPP), which inhibits production of major lipids like cholesterol and isoprenoids. This in turn leads to inhibition of post-translational modification of critical proteins such as Ras and Rho, leading to disruption in osteoclast survival\textsuperscript{19}. Current BPs used in treatment for osteoporosis are risedronate and alendronate, as they have shown not only to reduce osteoclast activity but also reduce BMU activity and enhance mineralization\textsuperscript{16}.

1.2 Bone Quality and Assessment

Techniques to assess bone quality are integral in the clinic and on the bench. As mentioned previously, technology such as DXA and qCT allow clinicians to detect decreasing BMDs. Different methods of assessment of bone quality are frequently used in preclinical research. These methods are not always feasible in human populations. However, they allow researchers to scrutinize underlying molecular features of diseases in question, to hopefully be able to develop better and more effective treatments.
1.2.1 Bone Microarchitecture

Micro-computed tomography (µCT) is similar to qCT and comes with some improvements. It is less costly and does not have high radiation exposure\textsuperscript{20}. It allows users to see quantitatively and qualitatively, 2D and 3D structure and geometry of bones with a resolution of the micrometre range\textsuperscript{21,22}. However, it is most commonly used as an \textit{ex vivo} tool, as the higher spatial resolution limits feasible specimen sizes to wrist bones or vertebrae\textsuperscript{23,24}. In some cases, machine sizes require human bone samples to be biopsied\textsuperscript{22}. µCT works by using X-ray attenuation data through a sample, compared to the attenuation data through an HA standard. Images are taken while a sample is fully rotated, which are then reconstructed into a set of 3D cross-sectional images\textsuperscript{24}. Analysis is initiated by binarizing the greyscale raw scan images. A threshold for discrimination is set during analysis by the user to compensate for machine fluctuation, sensitivity, and sample differences. Rationales for thresholds should be provided with subsequent data as the thresholding can be subjective. Typically, a ‘uniform threshold’ is applied, meaning that the same threshold exists for a single batch, samples scanned sequentially\textsuperscript{20}.

There are several advantages to the use of µCT over its \textit{in vivo} human-based qCT counterpart. Both allow imaging of internal structures in a non-destructive manner; non-destruction of samples allows for specimens to be rescanned or used for other analyses without data loss. However, with µCT, much smaller objects can be imaged, and researchers can observe bone morphology and microarchitecture in the micrometre range. Specially, trabeculae can be modeled in a 3D manner, to observe parameters such as trabecular thickness, separation, and number. In addition, bone mineral density data can be used to create models for preliminary bone mechanics assessment\textsuperscript{24}. A major disadvantage in the use of µCT, especially in relation to human samples, is that larger samples can increase scan and loading times to a point where it is not feasible to run depending on the system in use. Reduction of voxel sensitivity, thus resolution, can lead to serious loss of accuracy\textsuperscript{21}. It remains to be seen whether future advancements of µCT will allow more accessibility for human samples, hopefully leading to \textit{in vivo} possibilities whilst retaining its merits.
1.2.2 Bone Histomorphometry

One of the advantages of μCT is that it is a faster method of bone morphology and microarchitecture assessment than histomorphometry. Whole bones can be scanned for 3D representations. However, histological assessment has its own strengths. With μCT, visualization of cellular morphology and nuclei are not possible. Histology is defined as the study of tissues at the microscopic level, following sectioning and staining of samples. Histomorphometrics is the quantitative study of microarchitecture and structure of tissue. There are five steps to preparing the sample so that it can be analyzed under a microscope: fixation, processing, embedding, sectioning, and staining. Histology can be quite versatile as there are many different stains available that allow researchers to visualize specific things. For example, hematoxylin and eosin (H&E) stain allows users to visualize cells and nuclei in detail. Biopsies can be histologically analyzed in order to discern whether there are cancerous cells present, and to identify the cell type.

1.2.2.1 TRAP Stain

A common stain used in bone histology is staining for the tartrate-resistant acid phosphatase (TRAP) enzyme. TRAP is an enzyme that is highly expressed in osteoclasts, thus it can be used as a histochemical marker for osteoclasts. Previous studies have shown that TRAP KO mice exhibit a mild osteopetrosis in the metaphyseal region. It has also been observed that individuals with elevated rates of bone resorption have increased serum TRAP levels. Kirstein et. al found correlations between resorptive activity and TRAP release, and that stimulation of resorption stimulated TRAP release. A proposed mechanism...
of action for TRAP activity in osteoclasts is that it leads to dephosphorylation of bone matrix proteins such as OPN and BSP, which are required for normal endochondral bone formation\(^2^9\). The canonical TRAP staining method is available in a kit, provided by Sigma Aldrich. This stain produces the gold-standard dark red staining of TRAP that can be seen and analyzed through light microscopy. Recently, there have been fluorescence-based innovations in staining TRAP, to allow combination of other fluorescent stains\(^3^0\).

1.3 G Protein Coupled Receptors

Pharmacology is defined as the study of substances that chemically interact with organisms, namely by binding to biologically relevant molecules. In many cases, these molecules tend to be receptors\(^3^2\). Physiologically there are four receptor classes, three of which are membrane proteins: channel-linked, enzyme-linked, G-protein-coupled, and intracellular\(^3^3\). G-protein coupled receptors (GPCRs) are the largest receptor family and are also referred to as seven-transmembrane (7TM) or serpentine receptors due to their shape, as seen in Figure 1.8\(^3^2\). The alternating transmembrane sections are composed of 7 \(\alpha\) helices, with intra and extracellular regions that connect the helices. Within the GPCR class, there are five subtypes roughly defined by structure and sequence homology. These are the rhodopsins (family A), secretins (family B), glutamate (family C), adhesion, and Frizzled/Taste2\(^3^4\).

The signal transduction partner for GPCRs are called G proteins, and they reside in the intracellular space beneath the receptor. G proteins are heterotrimeric, each with an \(\alpha\), \(\beta\), and \(\gamma\) subunit. As seen in Figure 1.9, the \(\alpha\) subunit is bound to guanine diphosphate (GDP) when inactive. When a ligand binds to the receptor, the receptor becomes activated, and the \(\alpha\) subunit will exchange the GDP for GTP. The activated \(\alpha\) subunit dissociates from its \(\beta\gamma\) partners and goes on to activate downstream effector molecules\(^3^7\). The \(G\alpha\) subunit has the innate ability to hydrolyze GTP back to GDP, as a mechanism to stop the signal transduction. However, the rate of GTP hydrolysis is relatively slow, which allows the post-receptor activation signal to be attenuated even after the receptor is no longer bound to its ligand\(^3^2\). In this way, the G protein can
influence the duration of the signal downstream from the receptor, not the receptor itself. However, reality is not as simple as this general pathway. There are multiple G protein subtypes of each subunit, who are coupled to different downstream effectors, and there are even G protein independent signaling pathways.\textsuperscript{34}

Desensitization is an extremely important concept when talking about GPCRs. It can be defined as loss of a response to continued agonist presence.\textsuperscript{35} There are two types of desensitization: homologous and heterologous. Homologous desensitization begins with phosphorylation of an agonist-occupied receptor, carried out by a GPCR kinase (GRK). Phosphorylated receptors have high affinity for proteins called arrestins, β-arrestin-1 and β-arrestin-2 (βarr-1 and 2). Arrestin binding sterically hinders the access that G proteins have to their receptor counterparts, disrupting signaling capacity.\textsuperscript{35,36} Once arrestin is bound, the receptor can be trafficked towards clathrin-coated pits. These pits either internalize, dephosphorylate, and recycle receptors, or target them to lysosomes for breakdown. The importance of β-arrestin mediated desensitization is emphasized in βarr-2 KO murine models. The mice show extensive attenuated response to morphine, indicating lack of desensitization of the µ opioid receptor. Heterologous desensitization does not require the receptor to be agonist-bound and phosphorylation of the receptor is carried out by kinases that become activated by second messengers, for example, protein kinase A (PKA). The phosphorylation of the receptor sufficiently impairs the ability of the receptor to interact with its G protein partners.\textsuperscript{36}
1.3.2 G Protein Subtypes

The Gα subunit dissociates from its βγ partners, to directly interact with the receptor and its downstream effectors for signal transduction. The signal that is transmitted is dependent on which α subunit is activated by the receptor, although there are some receptors that do not exclusively partner with a singular Gα subtype. 16 human gna loci encode all known human Gα proteins, including 35 subunits characterized by biochemical and cloning techniques. There are four major subtypes, or classes, of Gα, grouped by sequence homology and specific expression patterns: Gαs, Gαi, Gαq, Gα12. Each class interacts with downstream effectors in unique ways, leading to a variety of downstream signal transduction pathways.

The Gαs class contains Gαs and Gαolf, which interact with adenylyl cyclase (AC) in a directly stimulatory manner to increase intracellular levels of cyclic adenosine monophosphate (cAMP). The Gαi class contains Gαi, Gαo, Gαo, and Gαz, which act to decrease levels of intracellular cAMP. Gαi does this by...
directly interacting with adenylyl cyclase in a receptor-dependent inhibitory manner. The \( \text{G} \alpha_\text{q} \) class contains \( \text{G} \alpha_{11}, \text{G} \alpha_{14}, \text{G} \alpha_{15/16} \), which interact with and activate phospholipase C (PLC). The \( \text{G} \alpha_{12} \) class contains \( \text{G} \alpha_{12} \) and \( \text{G} \alpha_{13} \) and are slightly complicated in that they become activated by receptors coupled to \( \text{G} \alpha_\text{q} \) and \( \text{G} \alpha_{11} \), in addition to interacting with receptors themselves. The \( \text{G} \alpha_{12} \) class of G proteins can activate downstream effectors like phospholipase A, Na\(^+/\)H\(^+\) exchanger, or c-jun NH\(_2\) terminal kinases. They can also activate RhoA, which is a GTPase\(^{39,40}\). The \( \beta\gamma \) subunits also have several different subtypes, with 5 different \( \beta \) types and 12 different \( \gamma \) types. \( \beta\gamma \) subunits remain very tightly associated with each other under normal conditions when the subtypes are \( \beta 1-4 \). \( \beta 5 \) can be sometimes found in complex with other proteins\(^{39}\).

\[ \text{G} \alpha_\text{s} \]

Figure 1.11. Schematic of the \( \text{G} \alpha \) subtypes and their interactions with their effectors. A) \( \text{G} \alpha_\text{s} \) pathway showing downstream interaction with AC to increase cAMP. B) \( \text{G} \alpha_\text{i} \) pathway showing downstream interaction with AC to decrease cAMP. C) \( \text{G} \alpha_{12/11} \) pathway showing downstream interaction with PLC, and \( \text{G} \alpha_{12/13} \) pathway showing downstream interaction with GTPase RhoA\(^{39}\).

### 1.3.3 \( \text{G} \alpha_\text{s} \) and Bone Disease in Humans

As \( \text{G} \alpha_\text{s} \) is found in almost all cell types, it is implicated in many functions in the body. There is evidence to suggest that \( \text{G} \alpha_\text{s} \) not only partners with AC as a downstream effector, but also to calcium channels in the heart, and tyrosine kinases\(^{41}\). \( \text{G} \alpha_\text{s} \) is encoded by exons 1-13 of the GNAS gene in humans, on chromosome 20\(^{41,42}\), and is subject to a phenomenon called genetic imprinting\(^{43}\). Imprinting is an epigenetic event where expression of a parental allele is either dampened or lost completely. The imprinting of \( \text{G} \alpha_\text{s} \) usually occurs in endocrine tissues where the maternal allele is usually exclusively expressed. In
most other tissues, \( \text{G} \alpha \), is biallelically expressed\(^{43} \). Dysregulation of proper \( \text{G} \alpha \) function can lead to various disease states that implicate multiple physiological systems. \( \text{G} \alpha \) has been affected by activating and inactivating mutations, both of which lead to health defects.

1.3.3.1 Endocrine Tumors

Somatic activating mutations of the GNAS gene have been implicated in tumorigenesis of both endocrine and non-endocrine tumors, thus leading to the terminology \( gsp \) oncogene\(^{42} \). These mutations lead to constitutive activity of \( \text{G} \alpha \), and continuously increase intracellular levels of cAMP. Under certain cellular conditions, cAMP has been implicated in stimulation of cell proliferation and hormone secretion in the context of endocrine glands. Gain-of-function mutations were seen largely in human pituitary adenomas, mainly those that secrete growth hormone (GH) and adrenocorticotropic hormone (ACTH)\(^{41,43,44} \). With the GH-secreting tumors in particular, one-third of them were found to have activating \( \text{G} \alpha \), mutations\(^41 \). The large number of GH-secreting tumors containing GNAS mutations has allowed researchers to elucidate how the mutation leads to constitutive \( \text{G} \alpha \) activity. The intrinsic GTPase activity of \( \text{G} \alpha \) is disrupted by mutations on the Arg\(^{201} \) or Gln\(^{227} \) residues, which are crucial for normal GTPase activity. This allows \( \text{G} \alpha \) to remain in an active state, due to prolonged binding duration to GTP. The mutation is almost always on the maternal allele\(^{42,43} \). This same Arg\(^{201} \) residue is post-translationally modified by ADP-ribosylation by the cholera toxin, where the increased intracellular cAMP in intestinal cells leads to watery diarrhea seen with cholera infection\(^{41} \).

Moving away from endocrine glands, several hepatic tumors, renal clear cell carcinomas were also found to have activating GNAS mutations\(^{42} \). A study performed by Kalfa et al. identified activating \( \text{G} \alpha \), mutations to be present in a significant number of tumors with renal clear cell carcinoma\(^{44} \). In these cases, it is unclear whether the \( gsp \) oncogene initiates tumorigenesis\(^{42} \). It is also unclear whether genetic imprinting contributes to activating mutations and therefore tumorigenesis.
1.3.3.2 McCune Albright Syndrome

Somatic GNAS activating mutations can also lead to McCune Albright Syndrome (MAS), which occurs through the same missense mutations at the crucial GTPase site, at Arg^{201} or Gln^{227}. Subsequently constitutively active G{\alpha}_s causing increased amounts of intracellular cAMP. Disease severity is correlated with the extent of affected cells, or mosaicism^{42,45}. MAS patients typically have missense mutations in multiple tissues^{45}. These patients present with symptoms such as hyperpigmentation of skin (café-au-lait), sexual precocity, and/or fibrous dysplasia (FD)^{41}. The three symptoms together are called ‘the classic triad’^{42}. Café-au-lait skin pigmentation are the first manifestation of MAS^{46} and are asymmetric lesions or macules with jagged borders that arrange themselves in a segmental pattern. Examples of varying manifestation of café-au-lait lesions can be seen in Figure 1.12^{46}. Early sexual precocity or precocious puberty is more common in girls with MAS than boys. Girls present with early menstruation, development of mammary tissue, enlarged ovarian follicles, and large ovarian cysts that produce estrogen^{42,46}. In boys, enlarged testicles, increased spermatogenesis, and early development of secondary sex characteristics can be seen^{42}.

FD can be the most concerning outcome of MAS, as it can lead to bone deformities, significant pain, fractures, or compression of cranial nerves^{42}. FD occurs when normal trabecular bone is replaced with fibrous tissue, primarily composed of immature bone marrow stromal cells that appear fibroblastic^{41,45,47}. Simply, there is an inability to form mature lamellar bone^{47}. Poorly formed woven bone is also largely present and lined by cells with retracted cell bodies. The retraction is hypothesized to be caused by intracellular cAMP^{42}. FD lesions originate from the medullary cavity and slowly expand outwards as fibrous tissue is laid down^{42-47}. The immaturity of present mesenchymal cells is likely due to the fact that increased G{\alpha}_s and cAMP dually speeds up osteogenic commitment, whilst inhibiting osteoblastic
differentiation\textsuperscript{42}. Fracture risk is subsequently increased as there is low quality trabecular bone intermixed with fibrous tissue, in areas that require properly formed trabecular bone for weight bearing. FD lesions present in femurs tend to cause high levels of pain and can cause leg-length discrepancy along with gait issues. Typically, FD lesions involve multiple skeletal sites, with occurrences of 91% in femora, 81% in tibiae, 78% in pelvises, and 73% in feet.

![Figure 1.13. X-ray image showing polyostotic FD in the pelvis and femur. The femur is stabilized with an intramedullary rod.](image)

1.3.3.3 Albright Hereditary Osteodystrophy

Inactivating GNAS mutations can lead to Albright Hereditary Osteodystrophy (AHO), which is a term for a condition characterized by a specific collection of symptoms including short stature, obesity with round face, brachydactyly, subcutaneous ossification, and mental retardation\textsuperscript{41,42}. Fuller Albright first noticed that some patients presenting with symptoms of hypoparathyroidism had no response to injected parathyroid hormone (PTH). Instead, they had what is now known as pseudohypoparathyroidism (PHP). Patients with PHP were found to have diminished organ response to hormones. A subset of patients did not even have organ resistance to PTH whilst presenting with the constellation of physical symptoms. This caused Albright to coin a new term, pseudoPHP (PPHP), in order to be able to differentiate diagnoses, as the phenotypic presentation of disease was quite similar. In summary, patients who have the constellation of symptoms, with or without hormone resistance are said to have AHO and may also have PHP or P-PHP\textsuperscript{48}.

Depending on whether inheritance of AHO is maternal or parental, the resulting disease phenotype is very different. Maternal inheritance of the AHO trait is accompanied by multihormone resistance, PHP type 1a (PHP-1a). When PHP-1a patients are given PTH injections, their urine cAMP levels are not
elevated which indicates impaired cAMP response to activation of renal PTH receptors. This is consistent with the fact that PHP-1a patients have been shown to have around 50% reduction of $G_\alpha_s$ activity levels.

Post-cAMP release effects paired to the PTH receptor such as vitamin D synthesis, is also altered in these patients. Paternal inheritance of the AHO trait is not accompanied by multihormone resistance, and thus presents as PPHP. When PPHP patients are given PTH injections, their urine cAMP levels are normal with no hormone resistance. It is typically quite difficult to diagnose patients with paternal inheritance of AHO without a detailed family history, as some of the symptoms are non-specific to AHO.

1.3.4 $G_\alpha$ and Bone Disease in Murine Models

Murine model generation for MAS/FD has not been easy. As of 2014 the only in vivo model was based on transplantation of human skeletal cells containing mutated $G_\alpha_s$ into mice, which caused some issues of replicability and age-based tracking of the disease. In response, Saggio et al. generated a murine model with constitutively active $G_\alpha_s$ which they claim to be a direct replica of human FD. This was achieved by mutating Arg201, the previously mentioned residue crucial for innate GTPase activity, to a cysteine residue. The authors performed this mutation on two different murine backgrounds, FVB and C57BL/6 and concluded that the replication of FD was identical in both groups of mice. Skeletal lesions were observed, and the size of the lesions increased as time progressed followed by deformity of vertebrae and spontaneous fractures, all hallmarks of FD. In contrast with constitutive activity, overexpression of $G_\alpha_s$ in vivo has been previously done in hearts of transgenic mice, but not in a strictly skeletal context.

Biallelic or homozygous knockout of exon 2 of GNAS in murine models leads to unviability, indicating the importance of GNAS in global development. Germain-Lee et al. generated a mouse model for AHO through disruption of exon 1 of the GNAS gene. Supporting the different disease expression based on maternal or paternal inheritance, only progeny that inherit the targeted allele from their mothers express hormone resistance. Similar mice with maternally transmitted disruption of GNAS present with altered cAMP urine output, and hormone resistance. Huso et al. generated another AHO mouse model, which presented with subcutaneous ossification which occurs regardless of which parent AHO was inherited.
from\textsuperscript{53}. Both murine models were generated through targeted disruption of exon 1 of the GNAS gene\textsuperscript{52,53}. The emergence of subcutaneous ossification in Huso et al.’s mice was explained to be a product of age in the mice, rather than the generation itself, providing further evidence towards validity of models targeting exon 1 of GNAS, in studying AHO\textsuperscript{53}.

1.4 Project Rationale and Aims

1.4.1 Project Background

Clinically, there are established clinical conditions caused by mutations in G\textsubscript{\alpha}s. For example, somatic activating mutations causing constitutively active G\textsubscript{\alpha}s, leads to MAS which presents with fibrous dysplasia, and bone fractures. The development of an animal model for G\textsubscript{\alpha}s-related clinical presentations, is a crucial step in advancing patient care. Historically, there have been demonstrated attempts to achieve specific G\textsubscript{\alpha}s changes in murine models. G\textsubscript{\alpha}s knockout causes unviability, and knockdown of the protein disrupts proper bone development\textsuperscript{41}. Targeted osteoblastic downregulation of G\textsubscript{\alpha}s resulted in mice with decreased bone mass, with targeted upregulation showing the opposite phenotype\textsuperscript{54}.

Zhang et al. generated a transgenic mouse model to understand the \textit{in vivo} effects of G\textsubscript{\alpha}s overexpression specifically in osteoblasts. This was achieved by only overexpressing G\textsubscript{\alpha}s in osteoblasts, by expressing the transgene under the COL1A1 promoter. Increases in bone mass were found. The resulting HOM-G\textsubscript{s} mice were found to have an average 4.6-fold increase in expression of the G\textsubscript{\alpha}s protein in long bone\textsuperscript{55}. However, this increase was accompanied by a sacrifice in bone quality, with bone biomechanics data showing more brittle and weaker bones. Assessment of bone microarchitecture through uCT showed
increases in bone mineral density, in trabecular and cortical bone respectively. The underlying cause of this phenotype has not been elucidated. Upon examination of potential causes of weaker and more brittle bones, Zhang et al. found an exaggerated increase in cortical bone porosity. This was shown quantitatively in the femur uCT analysis, provided with additional qualitative support from tibial TRAP-stained sections shown above in Figure 1.1455.

1.4.2 Project Rationale

Osteoporosis has been defined by the International Osteoporosis Foundation as progressively porous and fragile bone, “a disease in which the density and quality of bones are reduced”56. The data published by Zhang et al. has indicated that despite Gs-Tg mice having increased bone density, the bones are of reduced quality, seen through microarchitectural and mechanical assessment. Increased cortical porosity was proposed to be a potential contributor to the fragility of Gs-Tg bones. Upon histological examination, it was clear that the intracortical pores were lined with osteoclasts, as seen in Figure 1.14.

The combination of porous bone, fragility, and osteoclast-lined pores led to the idea that existing osteoporosis treatments might be able to prevent or reverse the phenotype. Bisphosphonate compounds are a large class of compounds that are used to treat various bone conditions. They are sometimes known as bone strengthening or bone-hardening treatments, as they slow progression of bone breakdown57. In this case a bisphosphonate drug, pamidronate, was selected for its proposed inhibitory mechanism of action on osteoclasts16. The idea is that osteoclasts lining the pores would be inhibited and could alter the porous phenotype. Pamidronate previously showed unexpected results with its use in our lab, with Dela Cruz et al. not seeing significantly decreased osteoclast numbers following treatment in mice overexpressing osteoblastic Gα1183. The idea behind the selection of pamidronate is not only due to its proposed mechanism, but also due to the need to further investigate its ability to affect osteoclasts without its supposed main effect through induction of apoptosis.

The phenotype of Gs-Tg mice is progressive; more bone continues to get laid down as the mice age. However, there was no difference between cortical porosity between 9 and 26 weeks of age. The
timeline for progression of porosity is unclear. Early development is when bone is most rapidly laid down, which could be a crucial time of establishment for cortical porosity.

1.4.3 Project Objectives

Objective 1: To understand the timeline of cortical porosity development by assessing preliminary microarchitectural parameters in Gs-Tg at 3 and 6 weeks of age.

Objective 2: To assess whether PAM administration can be preventative by injecting PAM prior to establishment of pores. PAM effects on wild-type age-matched control mice will also be investigated.

Objective 3: To assess whether PAM administration can be reparative by injecting PAM after establishment of pores. PAM effects on wild-type age-matched control mice will also be investigated.

1.4.4 Project Hypotheses

We propose the following hypotheses:

➢ That PAM administration will inhibit osteoclasts and result in prevention of porosity when injected prior to pore establishment.

➢ That PAM administration will inhibit osteoclasts and result in reversal of porosity when injected after pore establishment.
2 CHAPTER 2: Materials and Methods

2.1 Animals

Gαs transgenic mice (Gs-Tg) on an FVB background were previously generated in our lab. A 1232-bp full length coding region from exons 1 to 13 from the GNAS gene (human Gαs long subunit) was inserted into a PIRES2-DSRed2 vector. The CMV-promoter in the PIRES2-DSRed2 vector was replaced with the Col3.6/1.6-kb promoter\(^5\). This promoter was used to achieve specificity of Gαs overexpression in osteoblasts. The Col3.6 promoter is active in early mesenchymal progenitor cells which include preosteoblasts and osteoblasts. It is also frequently used as a marker of osteoblast differentiation\(^5\). The pOBCol3.6-Gαs-long expression vector was created, and an 8.5-kb fragment was purified to be injected into FVB mouse embryos. FVB mice are frequently used for the generation of transgenic mouse lines due to their demonstrated superior fecundity to other inbred mice\(^5,6\). As previously mentioned, the resulting Gs-Tg mice were found to have an average 4.6-fold increase in expression of the Gαs protein in long bone\(^5\). The Gs-Tg mice are bred and housed in the Division of Comparative Medicine at the University of Toronto. The animal use protocol was reviewed and approved by the University of Toronto’s Animal Care Committee.

2.2 Pamidronate and Calcein Green Preparation

Pamidronate disodium salt hydrate (Sigma-Aldrich) was dissolved into phosphate-buffered saline (PBS), and filtered in a 0.2 μm syringe filter (Pall Corporation) under sterile conditions. Calcein green (0.6% calcein green; 30mg/kg; Sigma-Aldrich) was prepared under the same conditions.

2.3 Experimental Design

2.3.1 Pilot Study: Investigation of age relative to cortical porosity

A pilot study was conducted to assess the cortical porosity levels in younger mice, as Zhang et al. had only shown established porosity in 9 and 26-week old mice. Five 3-week and five 6-week old female Gs-Tg mice were sacrificed for preliminary assessment of bone microarchitecture through uCT.
2.3.2 Study 1: Investigation of preventative pamidronate administration in Gs-Tg mice

For Study 1, forty 3-week-old WT and Gs-Tg mice were randomly assigned to either SAL or PAM injections. This resulted in four groups of ten: wild type injected with SAL (WT-S), wild type injected with PAM (WT-P), Gs-Tg injected with SAL (GS-S), and Gs-Tg injected with PAM (GS-P). The mice were injected with either SAL or 3mg/kg of PAM at 3 weeks of age, at noon time, based on the pediatric dose administered in children with osteogenesis imperfecta\(^6\). Calcein green was injected s.c. 9 and 2 days before sacrifice. Mice were sacrificed at 6 weeks and 1 day of age.

![Timeline for Study 1](image)

**Figure 2.1.** Timeline for Study 1: Investigation of preventative PAM administration in Gs-Tg mice.

2.3.3 Study 2: Investigation of reparative pamidronate administration in Gs-Tg mice

For Study 2, forty mice were randomly assigned to either SAL or PAM injections. This resulted in four groups of ten: wild-type SAL (WT-S), wild-type PAM (WT-P), Gs-Tg SAL (GS-S), Gs-Tg PAL (GS-P). The dosing regimen was 3mg/kg/week. Subcutaneous (s.c.) injections were performed weekly at noon from 6 to 12 weeks of age. Calcein green was injected s.c. 9 and 2 days before sacrifice. Mice were sacrificed at 12 weeks and 5 days of age.

![Timeline for Study 2](image)

**Figure 2.2.** Timeline for Study 2: Investigation of reparative PAM administration in Gs-Tg mice.
2.4 Sample Collection

Cardiac punctures were performed on all fasted mice under isoflurane anesthesia to obtain blood samples, prior to tissue collection. Mice were sacrificed through overdose of anesthesia. Subsequently, serum samples were isolated from the blood samples following centrifugation and stored at -80°C. Cervical dislocations were not executed as the vertebrae were to be collected. Calvaria, humeri, femora, tibiae, and lumbar vertebrae L3-L6 were dissected and cleaned thoroughly of attached soft tissues. Calvaria and humeri were snap-frozen in liquid nitrogen and stored at -70°C. Femora along with L5 and L6 vertebrae were wrapped in saline-soaked gauze to prevent dehydration, then stored at -20°C. Tibiae were cut transversely for the distal end to be used in histological analysis of cortical bone. The right tibiae were paired with L3 vertebrae and fixed in 10% NBF for 7 days, before undergoing acetone dehydration and embedding in Spurr’s resin. Left tibiae were paired with L4 vertebrae and fixed in 70% ethanol for 7 days, before undergoing ethylenediaminetetraacetic acid (EDTA) demineralization and embedding in paraffin wax.

2.5 Assessment of Bone Microarchitecture

2.5.1 Scanning Specifications

Bone microarchitecture was measured using micro-computed tomography (μCT). Samples for scanning were wrapped tightly in fresh saline-soaked gauze to prevent dehydration and air pockets within spaces of the bone, as the scanning chamber can get quite warm. CT scans were obtained using Skyscan 1174 (Bruker, Kontich, Belgium), at 50 kV and equipped with a 25mm Aluminum filter. Scans were done daily to minimize differences due to source fluctuations. Calibration for BMD calculations was performed using 0.25 and 0.75 g/cm³ HA phantoms, scanned at the beginning of each day. All raw scans were then reconstructed into cross-sectional images using N.Recon 1.7.3.0 (Bruker, Kontich, Belgium) for analysis, as shown in Figure 2.3. Reconstruction parameters were optimized per scan batch, for beam-hardening correction, ring artifact reduction, smoothing, and post alignment. All reconstructed images were analyzed using CTAn 1.18.8.0 (Bruker, Kontich, Belgium). CTAn parameters for thresholding and despeckling were thoroughly researched and validated through several trials.
Trabecular Bone

Left femora were scanned at the distal femur metaphysis at a resolution of 6.2 µm³ voxels and 3700ms exposure time for trabecular bone analysis. Reconstructed images were loaded into CTAn. The region of interest was defined as 2mm below the first point of cartilage bridge formation, which represents 1mm in height. Trabeculae were segmented throughout the entire region of interest, as shown in Figure 2.4. BMD was calculated using calibration parameters based on the phantom. 3D parameters were obtained using the software’s built-in custom processing, which included a task list of thresholding and a series of despeckling (Appendix 1).
2.5.3 Cortical Bone

Left femora were scanned a second time at a resolution of 11.6 µm³ voxels and 3000ms exposure time for cortical bone analysis. Reconstructed images were loaded into CTAn. The region of interest was defined to be 50 cross-sectional images above and below the mid-diaphysis for a total of 100 images, equivalent to 0.619mm. Midpoints were identified following mechanical measurement of the femur length. Cortical bone was selected using the donut method to exclude the marrow space, as shown in Figure 2.5. BMD was calculated using calibration parameters based on the phantom. 2D parameters were obtained through the software’s built-in morphometry analysis.

![Image of outlined cortical bone for analysis](image)

Figure 2.5. Sample of outlined cortical bone for analysis on a reconstructed cross-sectional image.

2.6 Assessment of Bone Histomorphometry

2.6.1 Sample Preparation

Left tibiae collected at the time of sacrifice were fixed for 7 days in 10% NBF. Once fixed, the samples underwent decalcification in EDTA for 7 days at room temperature. Samples were then placed in an automated tissue processor, the Shandon Excelsior ES (Thermo Scientific), for 48 hours. Xylene was first used to remove ethanol and clear the tissue. The samples were then immersed in wax to allow infiltration into the sample, to prepare them for embedding. Embedding in paraffin wax was performed using the Shandon Histocentre 3 Embedding Center (Thermo Scientific), where samples were placed in molds of hot wax in the desired orientation, then cooled until the wax solidified. The proximal ends of the halved tibiae were embedded anterior-face down to be sectioned mediolaterally for trabecular bone
observation. The distal ends of the halved tibiae were embedded upright to be sectioned transversely for cortical bone observation. Cooled samples were kept at room temperature until sectioning.

2.6.2 Sectioning

A Leica Reichert Jung 2030 microtome (Leica) was used to section the samples embedded in paraffin wax. The sectioning procedure is identical for both trabecular and cortical bone. All blocks were first coarsely trimmed to remove excess wax and to expose the bone. Prior to fine trimming with a fresh blade, each sample was placed on Molliflex Tissue Softener (Millipore Sigma) for 20 minutes, then cooled on ice for 20 minutes. The Molliflex Tissue Softener facilitates the sectioning process through rehydration, which prevents the sample from getting fractured during sectioning. Uneven staining and loss of microanatomical details, as a consequence of improper processing, is also remedied through this process. The ice incubation re-solidifies the wax to allow thinner and more stable sections, acting as a scaffold for the sample. Finely trimmed sections of 5 um in thickness were obtained from all samples, placed on charged slides, and immediately placed in a 60° oven for 48 hours to melt excess wax around the bone.

2.6.3 Staining

Slides to be stained were de-waxed through a series of xylene washes, then rehydrated using 100%, 95% and 70% ethanol. This process allows subsequent aqueous staining to be able to penetrate the bone. Samples were incubated in 0.2M TRIS buffer for 60 minutes, to reactivate the fixed enzyme, prior to staining using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich). Slides were cover-slipped with CC/Mount (Sigma-Aldrich).

2.6.4 Trabecular Bone Analysis

Samples were imaged at 20X using the OsteoImager V1887-A2329-Q31180 (Applied Scientific Instrumentation). Samples were analyzed using BioQuant OSTEO 2018. The region of interest for trabecular bone analysis was defined as having a height of 1mm below the growth plate, with a border of 150 microns inwards of cortical bone. Bone and osteoclast parameters were analyzed based on an optimized version of BioQuant’s provided TRAP analysis protocol. The criteria for counting osteoclasts include: the
cell being TRAP positive, containing more than 3 nuclei, and appearing to be adherent to the bone surface. All osteoclasts were analyzed under a light microscope at 40X magnification for confirmation of the number of nuclei present. Intensity of the staining is not measured and is not considered as criteria for measuring osteoclasts. Examples of measured parameters include bone surface and osteoclast surface.

2.6.5 Cortical Bone Analysis

Cortical porosity is not a commonly observed histological parameter. A new protocol was designed and optimized for analysis using BioQuant 2018. Pores were defined as being in the intracortical space, and having a threshold of greater than 100 microns, to exclude artifacts. This threshold was applied by the program, ensuring accuracy of exclusions. To match an important trabecular analysis parameter, osteoclast surface/bone surface (Oc.S/B.S), a similar parameter was devised for cortical bone. As resorbing osteoclasts were lining the perimeter of the pores, the parameter osteoclast surface/pore surface (Oc.S/P.S) was developed. Nomenclature is consistent with ASBMR standards. The execution of analysis of this parameter on the software is identical to trabecular Oc.S/B.S analysis. The criteria for counting osteoclasts pore surface. All osteoclasts were analyzed under a light microscope at 100X magnification for confirmation of the number of nuclei present. Intensity of the staining is not measured and is not considered as criteria for measuring osteoclasts. Additional parameters included porosity, pore area, endocortical area, perimeter, periosteal area, and perimeter.

2.6.6 Statistical Analyses

All data was assessed using an implicit blinding method, with quantitative data analysis performed only after full collection of all raw data. All quantitative data is shown as mean ± standard deviation (SD). Statistical analyses were performed using in-program processes on Graphpad Prism 6 following entry of raw data points. Significance was assessed using one-way analysis of variance (ANOVA) with Tukey’s post-hoc multiple comparisons test between all groups for the Pilot Study. For Study 1 and Study 2, two-way ANOVA with Tukey’s post-hoc multiple comparisons test between all groups was used. All tests were two-tailed with a p value of <0.05 being indicative of statistical significance.
CHAPTER 3: Results

3.1 Pilot Study: Investigation of cortical porosity relative to age

A preliminary assessment of cortical bone microarchitecture through an age progression was performed. The left femora of mice aged 3, 6, and 12 weeks were scanned by μCT, and will be further referred to as 3WK, 6WK, and 12WK GS. All assessments were performed at the mid-diaphyses. Figure 3.1 shows significant increases with age for BMD, bone area, and cross-sectional thickness of cortical bone. Bone perimeter was only significantly different between 3 and 6 weeks of age, at 8.28 ± 0.521 and 8.52 ± 0.446 for 6WK and 12WK GS respectively. Cortical porosity showed establishment of porosity occurring gradually with time over the specified time period of 3 to 12 weeks of age, although significant differences were only seen between 3WK and 12WK Gs. All numerical means ± SD can be seen in Table 3.1.

Figure 3.1. Graphical representation for Pilot Study cortical bone microarchitecture. A) BMD measures for 3, 6, and 12-week-old GS mice. B) Bone Area measures for 3, 6, and 12-week-old mice. C) Bone Perimeter measures for 3, 6, and 12-week-old mice. D) Cross-sectional Thickness measures for 3, 6, and 12-week-old mice. E) Closed Porosity measures for 3, 6, and 12-week-old mice. Values are shown as means ± SD. Significance: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Table 3.1. Numerical values for Pilot Study cortical bone microarchitecture. Abbreviations: BMD, bone mineral density; B. Ar, bone area; B. Pm, bone perimeter; MMI, polar moment of inertia; Cs. Th, cross-sectional thickness; Po (cl), closed porosity; APD, anterior-posterior diameter; MLD, mediolateral diameter. Values are shown as means ± SD. a = vs. 3WK GS, b = vs. 6WK GS. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001. 3WK GS n=5, 6WK GS n=5, 12WK GS n=10.

3.2 Study 1: Investigation of preventative pamidronate administration in Gs-Tg mice

In order to observe whether the progressive establishment of cortical porosity could be prevented, forty 3-week old WT and Gs-Tg mice were randomly assigned to receive a bolus dose 3mg/kg of PAM or equivalent volumes of SAL. They were sacrificed at 6 weeks of age. Four groups were assessed, wild-type injected with SAL (WT-S), wild-type injected with PAM (WT-P), Gs-Tg injected with SAL (GS-S), and Gs-Tg injected with PAM (GS-P). All groups in study 1 will be referred to herein as: 6WK WT-S, 6WK WT-P, 6WK GS-S, 6WK GS-P, to represent their age at time of sacrifice. Bone microarchitectural assessment was performed using µCT on both trabecular and cortical bone. Bone histomorphometrical assessment was performed using BioQuant OSTEO software on both trabecular and cortical bone.

3.2.1 Assessment of trabecular bone: µCT and histomorphometry

µCT was performed on distal left femora of all mice. Shown in Figure 3.2, 6WK WT-P mice had statistically significant increases in bone mass parameters such as BMD, bone volume/tissue volume, and trabecular number, when compared to the 6WK WT-S group. This indicated increased amounts of bone in the medullary cavity and was accompanied by a very significant decrease in trabecular separation, 0.127 ± 0.0137 compared to 0.0934 ± 0.00791, p<0.0001. Notably, there were no significant differences in
trabecular thickness as shown in Table 3.2. Structure model index and trabecular pattern factor were significantly decreased, indicating increased plate-like structures, and trabecular connectivity. SAL-treated Gs mice showed significantly increased trabecular bone when compared to SAL-treated WT mice. There were no significant differences between PAM-treated Gs mice (6WK GS-P) and SAL-treated Gs controls (6WK GS-S) across all trabecular bone parameters. 6WK WT-P and 6WK GS-S showed differences only in structure model index, 1.09 ± 0.0777 vs. 0.867 ± 0.213, p<0.05. The two PAM treated groups showed significant differences across all parameters except bone surface/bone volume, and trabecular thickness. All numerical means ± SD can be seen in Table 3.2.

![Graphical representation for Study 1 trabecular bone microarchitecture.](image)

Figure 3

2. Graphical representation for Study 1 trabecular bone microarchitecture. The four groups are 6WK WT-S, WT-P, GS-S, GS-P. All measured using µCT, scanned at the distal femora. A) BMD assessment. B) BV/TV assessment. C) SMI assessment. D) Tb. Pf assessment. E) Tb. Sp assessment. F) Tb. N assessment. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
Histological assessment of the trabecular bone was performed in the proximal tibiae. Each group when compared to 6WK GS-S showed significant differences for number of osteoclasts. When normalized to the amount of bone, significance was ablated, shown in the number of osteoclasts/bone surface measure. The comparison of the sizes of the osteoclasts shown in the osteoclast surface/bone surface measure, also showed no significant difference between all groups. Representative images of each group are shown in Figure 3.4. All numerical means ± SD can be seen in Table 3.3.

Table 3.2. Numerical values for Study 1 trabecular bone microarchitecture. Abbreviations: BMD, bone mineral density; BV/TV, bone volume/tissue volume; BS/BV, bone surface/bone volume; Tb. PF, trabecular pattern factor; SMI, structure model index; Tb. Th, trabecular thickness; Tb. N, trabecular number; Tb. Sp, trabecular separation. Values are shown in means ± SD. a = vs. 6WK WT-S, b = vs. 6WK WT-P, c = vs. 6WK GS-S. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Figure 3.3. Graphical representation for Study 1 trabecular bone histology. A) Total number of osteoclasts in the region of interest, 1mm below the growth plate. B) N.Oc/BS. C) Oc.S/BS. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
Assessment of cortical bone: µCT and histomorphometry

µCT was performed on distal left femora of all groups, at the mid diaphysis. Seen in Figure 3.5, cortical BMD and bone area showed differences between groups 6WK WT-S and the two GS groups, 6WK GS-S and 6WK GS-P. 6WK WT-P was also increased compared to 6WK GS-S and 6WK GS-P. There were no significant differences between 6WK WT-S and 6WK WT-P for all parameters except polar moment of inertia, 0.3157 ± 0.04689 vs. 0.3255 ± 0.03117, p<0.05 (Table 3.4.). Cross-sectional thickness was not changed across all groups. There was increased cortical porosity with only 6WK GS-S, and not 6WK GS-P, when compared to the WT groups. There was no change in porosity between 6WK GS-S and 6WK GS-P at 0.3663 ± 0.4173 and 0.2675 ± 0.2057. All numerical means ± SD can be seen in Table 3.4.

<table>
<thead>
<tr>
<th></th>
<th>6WK WT-S</th>
<th>6WK WT-P</th>
<th>6WK GS-S</th>
<th>6WK GS-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.Oc/TV</td>
<td>179.5 ± 88.2</td>
<td>188.4 ± 22.4</td>
<td>271.4 ± 122.8</td>
<td>280.8 ± 35.5</td>
</tr>
<tr>
<td>N.Oc/BS</td>
<td>10.90 ± 4.89</td>
<td>12.01 ± 1.56</td>
<td>13.40 ± 6.07</td>
<td>16.07 ± 2.11</td>
</tr>
<tr>
<td>Oc.S/BS</td>
<td>0.1935 ± 0.0957</td>
<td>0.2392 ± 0.0538</td>
<td>0.2087 ± 0.0950</td>
<td>0.3033 ± 0.0737</td>
</tr>
<tr>
<td>N. Oc</td>
<td>147.40 ± 69.64</td>
<td>178.0 ± 12.3</td>
<td>255.3 ± 28.7</td>
<td>262.0 ± 31.4</td>
</tr>
</tbody>
</table>

Table 3.3. Numerical values for Study 1 trabecular bone histology. Abbreviations: N.Oc/TV, number of osteoclasts/tissue volume; N.Oc/BS, number of osteoclasts/bone surface; Oc.S/BS, osteoclast surface/bone surface; N.Oc, number of osteoclasts. Values are shown as means ± SD. A = vs. 6WK WT-S, b = vs. 6WK WT-P, c = vs. 6WK GS-S. Significance: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. 6WK WT-S n=8, 6WK WT-P n=6, 6WK GS-S n=7, 6WK GS-P n=6.
Figure 3.5. Graphical representation for Study 1 cortical bone microarchitecture. The four groups are 6WK WT-S, WT-P, GS-S, GS-P. All measured using µCT, at the femur mid-diaphysis. A) BMD assessment. B) B. Ar assessment. C) B. Pm assessment. D) Cs. Th assessment, no differences across groups. E) Po (cl) assessment. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Table 3.4. Numerical values for Study 1 cortical bone microarchitecture. Abbreviations: BMD, bone mineral density; B. Ar, bone area; B. Pm, bone perimeter; MMI, polar moment of inertia; Cs. Th, cross-sectional thickness; Po (cl), closed porosity; APD, anterior-posterior diameter; MLD, mediolateral diameter. Values are shown as means ± SD. a = vs. 6WK WT-S, b = vs. 6WK WT-P, c = vs. 6WK GS-S. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001. 6WK WT-S n=10, 6WK WT-P n=10, 6WK GS-S n=10, 6WK GS-P n=10.
Histological assessment of cortical bone in tibiae showed no differences between all groups for cortical bone area and periosteal perimeter. Osteoclast number was significantly increased between 6WK WT-S and the two GS groups, 6WK GS-S and 6WK GS-P. The same trends were repeated when comparing 6WK WT-P to the two GS groups (Figure 3.6). Pore area and pore number followed similarly, with significant differences between 6WK WT-S compared to 6WK GS-S and GS-P, as well as 6WK WT-P compared to 6WK GS-S and GS-P. Osteoclasts were significantly increased in Gs bone in comparison to WT bone. When normalized to the amount of bone, osteoclast surface/pore surface showed differences between 6WK WT-S and the two GS groups, and 6WK WT-P was only significantly different from 6WK GS-P, 0.07489 ± 0.06180 vs. 0.1733 ± 0.09074, p<0.05. Across all parameters, there were no differences detected between PAM vs. SAL treated mice, in both WT and Gs-Tg mice. All numerical means ± SD can be seen in Table 3.5. Representative images are shown in Figure 3.7.

Figure 3.6. Graphical representation for Study 1 cortical bone histology. The four groups are 6WK WT-S, WT-P, GS-S, GS-P. All measured following TRAP stain, with transverse sections obtained from the mid-diaphyses. A) Cortical bone area assessment. B) Periosteal perimeter assessment. C) Osteoclast number assessment. D) Pore area assessment. E) Pore number assessment. F) Osteoclast surface/Pore surface assessment. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
Table 3.5. Numerical values for Study 1 cortical bone histology. Abbreviations: Ct. B. Ar, cortical bone area; Ec. Ar, endocortical area; Ec. Pm, endocortical perimeter; Ps. Pm, periosteal perimeter; Po. Ar, pore area; Po. N, pore number; Oc. N, osteoclast number; Oc.S/PS, osteoclast surface/pore surface. Values are shown as means ± SD. a = vs. 6WK WT-S, b = vs. 6WK WT-P, c = vs. 6WK GS-S, d = vs. 6WK GS-P. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
3.3 Study 2: Investigation of reparative pamidronate administration in Gs-Tg mice

In order to observe whether the established porosity could be reversed, forty 6-week old WT and Gs-Tg mice were randomly assigned to a weekly dose of 3mg/kg/week of PAM or SAL control for 6 weeks. They were sacrificed at 12 weeks of age. Four groups were assessed, wild-type injected with SAL (WT-S), wild-type injected with PAM (WT-P), Gs-Tg injected with SAL (GS-S), and Gs-Tg injected with PAM (GS-P). All groups in study 2 will be referred to herein as: 12WK WT-S, 12WK WT-P, 12WK GS-S, 12WK GS-P, to represent their age at time of sacrifice. Bone microarchitectural assessment was performed using µCT on both trabecular and cortical bone. Bone histomorphometrical assessment was performed using BioQuant software on both trabecular and cortical bone.

3.3.1 Assessment of trabecular bone: µCT and histomorphometry

µCT was performed on distal left femora of all groups, as shown in Figure 3.8 and Table 3.6. Compared to all groups, 12WK WT-S had lower BMD, bone volume/tissue volume, trabecular thickness, and trabecular number, the bone mass parameters. 12WK WT-P had higher bone mass parameters than both the 12WK WT-S and 12WK GS-S groups, with means that surpassed the existing basal bone mass differences between 12WK WT-S and 12WK GS-S groups. There were no differences between 12WK WT-P and 12WK GS-P across all parameters, with both groups showing comparable results shown in each.

Focusing on the Gs-Tg mice, the bone mass parameters showed the basal difference between WT and SAL-treated Gs-Tg mice: BMD, bone volume/tissue volume, trabecular thickness, and trabecular number showed significantly higher values when compared to 12WK WT-S. Relative to PAM-treated WT mice, 12WK GS-S showed lower bone mass accrual. 12WK GS-P, was increased in comparison to 12WK WT-S, and 12WK GS-S respectively, in BMD, bone volume/tissue volume, and trabecular number. Bone surface/bone volume was decreased in 12WK GS-P when compared to 12WK WT-S, and unchanged between 12WK WT-P, and 12WK GS-S. PAM had positive effects on bone mass, as both PAM-treated groups were comparable and significantly increased compared to their SAL-treated counterparts. All numerical means ± SD can be seen in Table 3.6.
Figure 3.8. Graphical representation for Study 2 trabecular bone microarchitecture. The four groups are 12WK WT-S, WT-P, GS-S, GS-P. All measured using µCT, scanned at the distal femora. A) BMD assessment. B) BV/TV assessment. C) SMI assessment. D) Tb. Pf assessment. E) Tb. Sp assessment. F) Tb. N assessment. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Table 3.6. Numerical values for Study 2 trabecular bone microarchitecture. Abbreviations: Ct. B. Ar, cortical bone area; Ec. Ar, endocortical area; Ec. Pm, endocortical perimeter; Ps. Pm, periosteal perimeter; Po. Ar, pore area; Po. N, pore number; Oc. N, osteoclast number; Oc.S/PS, osteoclast surface/pore surface. Values are shown as means ± SD. a = vs. 12WK WT-S, b = vs. 12WK WT-P, c = vs. 12WK GS-S, d = vs. 12WK GS-P. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>12WK WT-S</th>
<th>12WK WT-P</th>
<th>12WK GS-S</th>
<th>12WK GS-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm³)</td>
<td>0.1945 ± 0.0230</td>
<td>0.5465 ± 0.0494</td>
<td>0.4209 ± 0.0484</td>
<td>0.5540 ± 0.0820</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>8.791 ± 1.895</td>
<td>44.58 ± 4.14</td>
<td>32.40 ± 4.29</td>
<td>44.14 ± 7.14</td>
</tr>
<tr>
<td>BS/BV (1/mm)</td>
<td>137.6 ± 7.2</td>
<td>106.7 ± 3.6</td>
<td>101.7 ± 6.0</td>
<td>101.0 ± 4.3</td>
</tr>
<tr>
<td>Tb. PF (1/mm)</td>
<td>37.10 ± 4.48</td>
<td>-4.640 ± 4.126</td>
<td>2.740 ± 4.157</td>
<td>-3.892 ± 7.187</td>
</tr>
<tr>
<td>SMI</td>
<td>1.613 ± 0.113</td>
<td>-0.2641 ± 0.2273</td>
<td>0.1586 ± 0.2443</td>
<td>-0.2311 ± 0.4184</td>
</tr>
<tr>
<td>Tb. Th (mm)</td>
<td>0.03292 ± 0.00153</td>
<td>0.03614 ± 0.00116</td>
<td>0.03969 ± 0.00204</td>
<td>0.03883 ± 0.00171</td>
</tr>
<tr>
<td>Tb. N (1/mm)</td>
<td>2.669 ± 0.565</td>
<td>12.35 ± 1.23</td>
<td>8.199 ± 1.269</td>
<td>11.43 ± 2.20</td>
</tr>
<tr>
<td>Tb. Sp (mm)</td>
<td>0.2519 ± 0.0399</td>
<td>0.06655 ± 0.01147</td>
<td>0.1252 ± 0.0183</td>
<td>0.07234 ± 0.02238</td>
</tr>
</tbody>
</table>

39
Histological assessment of trabecular bone for mice in *Study 2* was performed in the proximal tibiae, values shown in Figure 3. 9. When looking at the mean number of osteoclasts in the entire region of interest, 1mm below the growth plate, the number of osteoclasts was higher in 12WK GS-S, and both PAM-treated groups 12WK WT-P, and 12WK GS-P. Upon normalization for the increased amount of bone, the number of osteoclasts/bone surface showed that 12WK WT-P showed an overall decreased number of osteoclasts. The significance between SAL-control WT and Gs-Tg mice was also ablated upon normalization. In terms of osteoclast size, osteoclast surface/bone surface showed increased osteoclasts in both Gs-Tg groups compared to WT-S. For osteoclast surface/bone surface, the two PAM treated groups were also different, with 12WK WT-P showing lower osteoclast surface/bone surface, p<0.05. All numerical means ± SD can be seen in Table 3.7. Representative images are shown in Figure 3.10.

---

**Figure 3.9.** Graphical representation for *Study 2* trabecular bone histology. A) Total number of osteoclasts for all 12WK groups. B) N.Oc/BS. C) Oc.S/BS for all 12WK groups. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
Assessment of cortical bone: µCT and histomorphometry

µCT was performed on distal left femora of all groups, at the mid diaphysis. There were no changes between 12WK WT-S and 12WK WT-P across all parameters. Figure 3.11 shows that 12WK WT-S had significantly lower, bone area, cross-sectional thickness, and closed porosity compared to 12WK GS-S and 12WK GS-P. The BMD for 12WK GS-S was significantly higher than all other groups, including 12WK GS-P. There were no differences between SAL and PAM treated groups for bone area. Bone perimeter was only higher in 12WK GS-P. There were no differences between the two GS groups for Po (cl), nor between the two WT groups. PAM treatment did not change or reverse the establishment of porosity. All numerical means ± SD can be seen in Table 3.8.
<table>
<thead>
<tr>
<th></th>
<th>12WK WT-S</th>
<th>12WK WT-P</th>
<th>12WK GS-S</th>
<th>12WK GS-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm³)</td>
<td>1.152 ± 0.021</td>
<td>1.151 ± 0.016</td>
<td>1.176 ± 0.014</td>
<td>1.147 ± 0.017</td>
</tr>
<tr>
<td>B. Ar (mm²)</td>
<td>0.7892 ± 0.0530</td>
<td>0.8245 ± 0.0389</td>
<td>0.9157 ± 0.0743</td>
<td>0.9576 ± 0.0947</td>
</tr>
<tr>
<td>B. Pm (mm)</td>
<td>8.583 ± 0.475</td>
<td>8.520 ± 0.311</td>
<td>8.523 ± 0.446</td>
<td>9.452 ± 0.682</td>
</tr>
<tr>
<td>MMI (polar)</td>
<td>0.3157 ± 0.0469</td>
<td>0.3255 ± 0.0312</td>
<td>0.2980 ± 0.0370</td>
<td>0.3470 ± 0.0481</td>
</tr>
<tr>
<td>Cs. Th (mm)</td>
<td>0.1838 ± 0.0046</td>
<td>0.1936 ± 0.0078</td>
<td>0.2148 ± 0.0107</td>
<td>0.2026 ± 0.0133</td>
</tr>
<tr>
<td>Po (cl) (%)</td>
<td>0.05870 ± 0.10790</td>
<td>0.04136 ± 0.08643</td>
<td>0.5865 ± 0.4107</td>
<td>0.5851 ± 0.2904</td>
</tr>
<tr>
<td>APD (mm)</td>
<td>1.240 ± 0.035</td>
<td>1.258 ± 0.033</td>
<td>1.242 ± 0.052</td>
<td>1.278 ± 0.064</td>
</tr>
<tr>
<td>MLD (mm)</td>
<td>1.622 ± 0.085</td>
<td>1.625 ± 0.050</td>
<td>1.537 ± 0.040</td>
<td>1.627 ± 0.068</td>
</tr>
</tbody>
</table>

Table 3.8. Numerical values for Study 2 cortical bone microarchitecture. Abbreviations: BMD, bone mineral density; B. Ar, bone area; B. Pm, bone perimeter; MMI, polar moment of inertia; Cs. Th, cross-sectional thickness; Po (cl), closed porosity; APD, anterior-posterior diameter; MLD, mediolateral diameter. Values are shown in means ± SD. a = vs. 6WK WT-S, b = vs. 6WK WT-P, c = vs. 6WK GS-S. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.
Histological assessment of cortical bone in mice for Study 2 showed no changes between all groups for endocortical area, endocortical perimeter, and pore area. Cortical bone area was increased in 12WK GS-P compared to 12WK WT-S only. There was no effect of PAM on structural parameters when compared to SAL-treated mice in both WT and Gs-Tg mice. At the cellular level, osteoclast number in the intracortical space was significantly increased in both Gs-Tg groups when compared to both WT groups. Average pore area of each group was not significantly different, possibly due to the high variability within groups. Pore number shared the same trends as osteoclast number, being significantly higher in Gs-Tg mice compared to WT mice. Osteoclast surface/pore surface showed the same trends as well. Overall, there was no difference between SAL and PAM treated mice across all parameters. All numerical means ± SD can be seen in Table 3.9. Representative images are shown in Figure 3.13.

Figure 3.12. Graphical representation for Study 2 cortical bone histology. The four groups are 6WK WT-S, WT-P, GS-S, GS-P. All measured following TRAP stain, with transverse sections obtained from the mid-diaphyses. A) Cortical bone area assessment showing no differences. B) Periosteal perimeter assessment showing no differences. C) Osteoclast number assessment. D) Pore area assessment. E) Pore number assessment. F) Osteoclast surface/Pore surface assessment. Values are shown as means ± SD. Significance: *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001
<table>
<thead>
<tr>
<th></th>
<th>12WK WT-S</th>
<th>12WK WT-P</th>
<th>12WK GS-S</th>
<th>12WK GS-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct. B. Ar</td>
<td>0.5501 ± 0.0620</td>
<td>0.6195 ± 0.0483</td>
<td>0.6233 ± 0.0523</td>
<td>0.6856 ± 0.0677</td>
</tr>
<tr>
<td>Ec. Ar</td>
<td>0.1658 ± 0.0180</td>
<td>0.1335 ± 0.0266</td>
<td>0.1522 ± 0.0347</td>
<td>0.1691 ± 0.0585</td>
</tr>
<tr>
<td>Ec. Pm</td>
<td>1.586 ± 0.123</td>
<td>1.400 ± 0.148</td>
<td>1.570 ± 0.167</td>
<td>1.565 ± 0.293</td>
</tr>
<tr>
<td>Ps. Pm</td>
<td>3.276 ± 0.122</td>
<td>3.386 ± 0.129</td>
<td>3.464 ± 0.166</td>
<td>3.614 ± 0.137</td>
</tr>
<tr>
<td>Po. Ar</td>
<td>0.001461 ± 0.001391</td>
<td>0.005061 ± 0.008217</td>
<td>0.009392 ± 0.004325</td>
<td>0.01073 ± 0.00456</td>
</tr>
<tr>
<td>Oc. N</td>
<td>1.500 ± 2.380</td>
<td>2.333 ± 2.958</td>
<td>22.44 ± 11.77</td>
<td>29.22 ± 9.72</td>
</tr>
<tr>
<td>Oc.S/PS</td>
<td>0.04179 ± 0.07012</td>
<td>0.05209 ± 0.05072</td>
<td>0.1781 ± 0.0851</td>
<td>0.1917 ± 0.0519</td>
</tr>
</tbody>
</table>

Table 3.9. Numerical values for Study 2 cortical bone histology. Abbreviations: Ct. B. Ar, cortical bone area; Ec. Ar, endocortical area; Ec. Pm, endocortical perimeter; Ps. Pm, peristomal perimeter; Po. Ar, pore area; Po. N, pore number; Oc. N, osteoclast number; Oc.S/PS, osteoclast surface/pore surface. Values are shown in means ± SD. a = vs. 12WK WT-S, b = vs. 12WK WT-P, c = vs. 12WK GS-S. Significance: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3.13. Representative images of TRAP-stained cortical bone for Study 2 visualized at the mid-diaphyses of the tibiae. A) 12WK WT-S. B) 12WK WT-P. C) 12WK GS-S. D) 12WK GS-P.
4 CHAPTER 4: Discussion

The studies outlined in this dissertation surround the issue of increased trabecular bone and increased cortical porosity in transgenic mice that overexpress osteoblastic Gaα. Based on previously reported findings from our lab, these mice had significantly increased bone mass, but decreased mechanical properties. Cortical bone was porous, and the pores were lined with osteoclasts based on qualitative findings. The bisphosphonate pamidronate was chosen in attempts to treat the porous bone in young Gs-Tg mice as it has been used in children with osteogenesis imperfecta, a disease of high bone turnover. Our objectives were three-fold; first to identify a timeline of pore development in early growth of the mice, second to examine preventative treatment prior to pore development, and third to examine reparative treatment post-pore development. The pilot study showed that the pores developed with age, with a significant difference in porosity between 3 and 12 weeks of age, p<0.0001 (Figure 3.1). Based on these results we administered a single dose of pamidronate at 3 weeks of age to assess preventative treatment, and weekly doses from 6-12 weeks of age to assess reparative treatment.

4.1 Effects of treatment on trabecular bone

4.1.1 Study 1- trabecular bone microarchitecture

Mice were assessed at 6 weeks of age, following a single dose of 3mg/kg of pamidronate given at 3 weeks of age. As expected, anti-resorptive treatment led to increased trabecular bone mass, in the pamidronate-treated WT mice compared to their saline-controls (Figure 3.2). We observed a significant increase in bone mass through increased bone mineral density, and bone volume/tissue volume. This was further confirmed by increases in trabecular number with concomitant decreases in trabecular separation. However, we should equally be concerned about the quality of the accrued bone; previous studies in our lab have shown that more bone does not necessarily confer quality. While bone biomechanical properties were not measured in these studies, other parameters obtained from μCT allow inferences about the quality of the increased mass of trabecular bone. Lower structure model index in 12WK WT-P mice compared to 12WK WT-S mice indicated that the shape of trabeculae transitioned from cylindrical rods (SMI=3) to
parallel plates (SMI=0), making the bone stronger and less porotic. This is based on the concept that concavity is indicative of connectivity and connected structures called nodes, and convexity is indicative of isolated structures called struts. Plate-to-rod transition is correlated to increased fracture risk in humans and begins when osteoclast resorption cavities become so large that the plate becomes punctured. This is followed by continuous erosion and widening of the punctured region. Trabecular pattern factor can also be interpreted as a quality-based measure as lower connectivity between trabeculae has also been found in fracture patients and those with vertebral compressions. Hahn et. al, founder of the trabecular pattern factor concept, has said that it can be used as an early detector of bone loss; higher values indicate disconnected, perforated trabeculae, whereas lower values indicate increased connectivity. In this case, connectivity between trabeculae was not significantly changed between the two 6WK WT groups. All together, the effect of early pamidronate treatment in the WT mice resulted in presence of more plate-like trabeculae. Despite the lack of significant changes in connectivity, this could indicate that the newly formed bone was of good quality.

Previous findings in our lab have shown differences between basal phenotypes of WT and Gs-Tg mice at 9 and 26 weeks of age. Here we have shown that these basal differences also exist at 6 weeks of age, at least in the trabecular bone. Bone microarchitectural data from Study 1 showed increases in bone mass parameters such as bone mineral density, bone volume/tissue volume, and trabecular number with a decrease in trabecular separation, in the 6WK GS-S mice compared to 6WK WT-S (Figure 3.3). The structure model index value for 6WK GS-S was below 1, meaning that it is mostly composed of strong, plate-like structures. The connectivity measure, trabecular pattern factor, showed a significantly lower value compared to 6WK WT-S as well, meaning the trabeculae were well-connected, conferring strength. In the trabecular bone alone, it seems that the increased bone mass is well-structured and well-connected, for the 6WK WT-P and 6WK GS-S groups. In the case of 6WK GS-S compared to 6WK GS-P, there were no differences between the two groups. Despite this, there seems to be a non-significant trend towards increased bone mass in the pamidronate-treated Gs-Tg mice.
4.1.2 Study 1 – trabecular bone histology

Analysis of the TRAP-stained tibial sections showed what had occurred at the cellular level to the osteoclasts following pamidronate administration. Differences in the number of osteoclasts occurred only when comparing WT mice to Gs-Tg mice. These differences were all abolished when normalized to the amount of bone present as seen in number of osteoclasts/bone surface (Table 3.3). Osteoclast surface/bone surface, not to be confused with number of osteoclasts/bone surface, is a measure of osteoclast size normalized to bone surface. There were no differences between groups. This is consistent with findings from Zhang et al. who observed no difference in osteoclast surface/bone surface between basal WT and Gs-Tg mice at 9 and 26 weeks of age. The lack of difference between pamidronate treated and saline treated group means shown in both the number of osteoclasts/bone surface and osteoclast surface/bone surface, indicate that osteoclast presence and size were not diminished by pamidronate.

Histological parameters alone seem to indicate that the treatment was not successful. However, the µCT parameters showed that trabecular bone mass was increased in WT mice. Combined, these results indicate that the anti-resorptive action of PAM was indeed successful in the WT mice, despite continued presence of TRAP+ osteoclasts on the bone surface indicating that the cells have not apoptosed. A potential explanation for this is an alternate identified mechanism seen in alendronate, another nitrogen-containing bisphosphonate. Alendronate has been shown to lead to decreased osteoclast adhesion to bone surfaces and decreased ruffled border formation. This would lead to a decrease in bone resorption and increase in bone accrual, without affecting the osteoclast population. It is impossible to know at the level of histology used in this study, whether the TRAP+ osteoclasts are adherent to the bone surface, or whether they have ruffled borders. Further studies using electron microscopy would be required to answer these questions.

4.1.3 Study 2 – bone microarchitecture

Study 2 involved 6 weeks of pamidronate injections, with mice being sacrificed at 12 weeks of age. The effects of longer treatment and higher doses of pamidronate on the trabecular bone were much larger and more distinctive than those seen in Study 1. Both WT and Gs-Tg mice exhibited significantly increased
bone mass when given pamidronate, compared to their saline controls. For perspective, the relative increase of bone mineral density in 12WK WT-P mice compared to the saline-controls is 180%, whereas the relative increase of bone mineral density in the Study 1 mice, 6WK WT-P vs. 6WK WT-S, is 17%. Relative increases in the two pamidronate-treated groups compared to their saline-controls were similar across other parameters, and larger in magnitude compared to the 6WK counterparts. With the larger doses of pamidronate the trabecular thickness was changed between groups in Study 2, as opposed to no changes seen in Study 1. Trabecular thickness is measured at points where solid bone is detected, to give the output of mean thickness of all trabeculae. In the 12WK Study 2 group, trabecular thickness was increased in both pamidronate-treated groups relative to WT saline-control. Treatment with pamidronate in the context of WT mice, was able to increase the thickness of trabeculae specifically, as there was no difference in this parameter between 12WKGS-S and GS-P. In the 6WK old mice in Study 1 there was no difference in trabecular thickness across all groups. By 12 weeks of age, there was a significant difference existing between WT and Gs-Tg saline-controls, which indicated that the trabeculae in the Gs-Tg mice thickened at a faster rate with age, than WT mice.

The larger magnitude of difference in effects of PAM on the trabecular bone in 12WK mice vs. 6WK mice also can be seen in the other bone microarchitectural parameters. Notably, the structure model index and trabecular pattern factor of the PAM treated WT and Gs-Tg mice crossed into negative index values. The negative structure model indices and trabecular pattern factor values means there is an abundance of enclosed cavities and concave surfaces, or nodes, in both PAM-treated WT and Gs-Tg mice. Negative structure model index values tend to occur in regions with a relative bone volume above 50%, reiterating the presence of drastically increased bone mass in the PAM-treated groups. Overall, the PAM treatment led to increased trabecular bone mass that was well-connected and plate-like, which could be interpreted as of being good quality.
4.1.4 Study 2 – histology

The histological analysis of structural and cellular parameters for Study 2 were performed at the proximal tibia. The mean number of osteoclasts in the region of interest, 1mm below the growth plate, showed that each group had more osteoclasts compared to 12WK WT-S, and were not different from each other. When the number of osteoclasts was normalized to bone surface, the 12WK WT-P group showed lower number of osteoclasts/bone surface compared to 12WK WT-S. This indicated that pamidronate treatment had decreased the number of osteoclasts present in the trabecular bone region of interest, in the pamidronate-treated WT mice. This effect of pamidronate on decreasing osteoclasts in the trabecular region is consistent with its mechanism of action, of inducing apoptosis of osteoclasts. The decreased number of osteoclasts relative to bone surface was not seen in Study 1 where a single dose of pamidronate was given. This indicates that the larger dose of pamidronate in Study 2 was more effective in negatively affecting the osteoclasts in the trabecular bone region of wild-type mice. Number of osteoclasts/bone surface between the two Gs-Tg groups was unchanged. Therefore, pamidronate only had an effect on osteoclast number in the WT mice, and not the Gs-Tg mice.

When looking at osteoclast size, through the osteoclast surface/bone surface parameter, there were significant differences as expected between WT and Gs-Tg SAL-control mice. This is an indication that larger osteoclasts are present in 12WK GS-S mice, and larger osteoclasts are known to have enhanced resorptive activity over smaller osteoclasts\(^70\). There was no difference in osteoclast surface/bone surface between SAL and PAM treated groups, in either WT or Gs-Tg mice. This indicated that pamidronate did not have anti-resorptive effects through decreasing the size of osteoclasts, therefore not affecting the activity of the osteoclasts. In the case of the Gs-Tg mice, because there were significant increases in bone mass despite lack of cellular effects on osteoclasts, the disturbed adhesion and ruffled border hypothesis is again a likely contributor\(^69\). There were no significant effects on the osteoclasts at the cellular level except in the 12WK WT-P group, where PAM treatment led to a decrease the number of osteoclasts/bone surface. Size of the osteoclasts were not altered.
4.2 Effects of treatment on cortical bone

4.2.1 Study 1 – bone microarchitecture

There were no differences between 6WK WT-S and 6WK WT-P mice in cortical bone microarchitectural parameters, except for an increase in polar moment of inertia, and anterior-posterior diameter in the WT-P group. This is interesting as there were notable differences between these groups in the trabecular bone. The same positive effects on bone mass that PAM had in trabecular bone is absent in the cortical bone. The increase in polar moment of inertia is indicative of an increase in basic strength, as this parameter measures torsional resistance of a cross-section around a static axis. The measurement of polar moment of inertia assumes that material stress-strain properties are uniform. However, this does not indicate anything about the geometry or structure of the cortical bone. For example, if there was increased bone mineral density and increased cross-sectional thickness alongside the increase in polar moment of inertia, it could be an indication that increased strength was due to increased bone mass. In this instance, it is impossible to know what this increase in basic strength is caused by. The parameter of interest, closed porosity, showed the basal difference between control WT and Gs-Tg mice. There was no changed detected between 12WK GS-S and 12WK GS-P, indicating that the pamidronate treatment had not functioned the way it was expected to, and did not prevent formation of cortical pores.

4.2.2 Study 1 – histology

At the histological level, cortical bone area, endocortical area, endocortical perimeter, and periosteal perimeter were all unchanged. This meant that no structural differences were found in the cortical bone. Focusing on pores specifically, pore area and number shared the same trends. The expected differences between genotypes were conserved, with both Gs-Tg groups showing significant increases in pore area and number. No differences were found between saline and pamidronate-treated mice in either WT or Gs-Tg mice. At the level of the osteoclasts, there was also no difference between saline and pamidronate-treated mice in either WT or Gs-Tg mice. When osteoclast surface was normalized to pore surface, the difference between saline controls of WT and Gs-Tg mice was conserved. Again, there was no
difference between SAL and PAM treated mice in either genotype. Overall, as there was no difference between SAL or PAM treated mice in any parameter, it seems that the treatment was unsuccessful in preventing cortical porosity. Again, this is curious as there were definitive effects of PAM on trabecular bone. It seems that the anti-resorptive effects of PAM are functional in trabecular, but not cortical bone, in the WT mice of Study 1.

4.2.3 Study 2 – bone microarchitecture

Bone microarchitectural analysis for Study 2 showed expected changes in bone mineral density of 12WK GS-S compared to WT mice, with higher bone mineral density, bone area, cross-sectional thickness, and porosity. Interestingly, the bone mineral density for 12WK GS-P was significantly lower than that of 12WK GS-S, p<0.001. It is difficult to say why the bone mineral density is lower in pamidronate-treated compared to saline-treated Gs-Tg mice, despite increased bone perimeter and comparable bone area to 12WK GS-S. If the porosity in 12WK GS-P had been significantly increased compared to its saline-control counterpart, the trade-off between density and porosity could explain the lower bone mineral density. Also, unlike cortical bone microarchitecture from Study 1, the data presented in Study 2 show more differences between saline and pamidronate-treated Gs-Tg groups only, including bone mineral density, bone perimeter, and cross-sectional thickness. This is indicative of the pamidronate treatment having an effect in the Gs-Tg mice. It is likely that the increased dosing regimen was able to push pamidronate to have an effect in the cortical bone, despite its previous reported difficulties in targeting this region\cite{69,76,77}. However, these effects were not seen in the WT mice. Pamidronate is known to act in human cortical bone by suppressing intracortical remodeling. It is possible that there is increased intracortical remodeling in the Gs-Tg mice with their largely increased rates of bone formation and accrual of bone\cite{55}, therefore increasing the propensity of pamidronate to exert action\cite{69,77}. Additional evidence of intracortical remodeling in the mice involved in both Study 1 and Study 2 can be seen below, in Figure 4.1. Despite structural effects, there was no effect in any group of pamidronate on closed cortical porosity.
4.2.4  Study 2 – histology

The increase in bone perimeter seen in femur $\mu$CT was also seen in the histological analysis of transversely sectioned tibiae. Periosteal perimeter of 12WK GS-P was increased significantly compared to WT mice, although not different from 12WK GS-S. The osteoclast analysis showed the expected increase of osteoclast number in both Gs-Tg groups compared to both WT groups, which was expected and can be clearly seen in the representative images of TRAP-stained cortical bone. Surprisingly, pore area was not significantly different across all group, but this is likely due to the high intra-group variability. A common theme across both studies is that porosity measures such as closed porosity in $\mu$CT, and pore area or number in histology, show high intra-group variability. Pore number and osteoclast number both did not show any differences between saline and pamidronate-treated mice in both WT and Gs-Tg mice. Osteoclast surface/pore surface showed no difference in osteoclast size between saline and pamidronate-treated groups. The continued lack of significance in cellular parameters between saline and pamidronate treated groups across all studies have shown that pamidronate was not successful in ablating presence of osteoclasts. Again, it is unclear whether these TRAP+ osteoclasts that are present are indeed adherent, or whether they have ruffled borders.

4.3  Limitations

As the mice are bred in-house, many of the mice were born, injected, and sacrificed on different days. Although each injection, sacrifice, sample collection, and sample processing were done as identically as possible, it would have been best to be able to do all forty mice at once to enhance the quality of analysis. Inability to have all forty mice at once also directly interfered with the possibility of any parallel in vitro studies to look more closely at the cells. Each different preparation of cell cultures themselves already entail increased variability, but when each preparation has different n numbers, it is difficult to combine them into an n of 10 per group for statistical comparison.

There are also inherent limitations to histological analysis in that it is impossible to accurately say that the depth and location of sections taken between each histological sample analyzed is the same. We
can only make estimations of where the mid-diaphysis of a tibial shaft is by human eye. In addition, having data from biomechanical assessment and dynamic histomorphometry would have given more insight into these studies, but were not performed at this time.

4.4 Summary and Conclusions

Due to the differential effects of PAM on trabecular and cortical bone, conclusions must be drawn separately for the two bone regions. Following administration of a single preventative 3mg/kg dose of pamidronate at 3 weeks of age, prior to pore establishment:

➢ In the trabecular bone, pamidronate exerted its anti-resorptive effects and led to an increase in bone mass in WT mice only.

➢ In the trabecular bone, pamidronate did not lead to loss of TRAP+ osteoclast staining, histologically, in both WT and Gs-Tg mice.

➢ In the cortical bone, pamidronate did not show anti-resorptive effects, and bone mass was unchanged, in both WT and Gs-Tg mice.

➢ In the cortical bone, pamidronate did not lead to a loss of TRAP+ osteoclast staining, histologically, in both WT and Gs-Tg mice.

➢ Therefore, pamidronate was not successful in preventing the formation of cortical pores by 6 weeks of age, although it exerted positive effects on bone mass in WT mice as expected.

Following administration of PAM at 3mg/kg/week from 6 to 12 weeks of age, after pore establishment:

➢ In the trabecular bone, pamidronate exerted its anti-resorptive effects and led to a drastic increase in bone mass, in both WT and Gs-Tg mice.

➢ In the trabecular bone, pamidronate did not lead to loss of TRAP+ osteoclast staining, histologically, in both WT and Gs-Tg mice.
➢ In the cortical bone, pamidronate did not show anti-resorptive effects, and bone mass was unchanged in WT mice. In Gs-Tg mice, pamidronate administration led to decreased bone mineral density.

➢ In the cortical bone, pamidronate did not lead to a loss of TRAP+ osteoclast staining, histologically, in both WT and Gs-Tg mice.

➢ Therefore, pamidronate was not successful in reversing the formed cortical pores by 12 weeks of age, although it exerted positive effects on bone mass in both WT and Gs-Tg mice.

4.5 Future Directions

The work outlined in this dissertation examined whether pamidronate could alter the bone phenotype of Gs-Tg mice. There is evidence to suggest that the lack of effect of PAM in cortical bone was likely due to penetration complications. The anti-resorptive effects of PAM were well established in the trabecular bone of both 6 and 12WK mice. In humans, the most prescribed BPs specifically for osteoporosis are alendronate, zoledronic acid, ibandronate, and risedronate. The binding affinity to hydroxyapatite of BPs, from highest to lowest, is as follows: zoledronic acid > pamidronate > alendronate > ibandronate > risedronate > etidronate > clodronate. As PAM has the 2nd highest affinity to hydroxyapatite, it will preferentially bind to trabecular bone which has more metabolic needs and higher turnover than cortical bone. The concept that binding affinity affects penetration is clinically relevant, as increasing cortical porosity commonly seen in menopausal women. 80% of fractures are in fact cortical, and non-vertebral, thus a treatment that could better target cortical bone may be useful in those who have increased cortical fragility. In a human cohort study, Silverman et al. found that risedronate treatment resulted in lower hip and non-vertebral fractures rates compared to those who had been treated with alendronate. BP action in human cortical bone depends on reducing porosity through inhibiting BMUs and thus, remodeling. The appearance of new BMUs following risedronate administration is suppressed from 70-80%.

In 2012, Roelofs et al. designed an in-vivo proof-of-concept study looking at radiolabeled-BP distribution to bone, based on several studies showing preferential localization of BPs to areas of high bone
turnover. They used three different conjugates of risedronate, to represent high, medium, and low affinity binding. Their findings showed that the different affinities affected the penetration ability of BPs into cortical bone. High-affinity BPs instantly bound to the bone surface, the first mineral they encounter. This led to “trapping” of high-affinity BPs, and lack of penetration into the osteocyte network from the Haversian canals. Low-affinity BPs were able to penetrate further into intracortical space and bind to mineral surrounding osteocytes. Turek et al. proposed that the use of rodent models for BP effects in cortical bone is limiting, as rodents lack remodeling in the intracortical space. The authors showed differential penetration of high and low affinity BPs in skeletally mature female rabbits, with low-affinity BPs penetrating past the osteonal cement line in the cortical bone of the ribs. In the tibiae, Turek et al. were not able to see penetration past the cement line. This was likely due to the difference of metabolic needs between rib and tibial cortical bone, due to difference in remodeling rates.

A secondary osteon is defined as a Haversian canal surrounded by lamellar bone, with a clear encircling boundary called a cement or reversal line. The existence of secondary osteons is the result of remodeling as they replace primary bone. Most importantly, primary osteons do not have cement reversal lines. Anthropological analyses of extinct giant rats showed existence of properly organized Haversian remodeling, and intracortical secondary osteons. There have been studies that have shown the existence of intracortical secondary osteons in rodents. For example, an experiment in 1953 fed female rats a calcium free diet which led to increased cortical porosity. When the same rats were later given a normal diet, osteons with concentric lamellae were found in the intracortical bone. Most surprisingly, a 2009 study found presence of secondary osteons in the compact bone of rat tissue, although it was not a result of canonical Haversian remodeling. The same author found secondary osteons in their control groups of two different species of rodents: 12-week old Swiss mice, and one-month-old Wistar rats, in 2014 and 2018 respectively.

Overall, this is sufficient evidence to support the theory of lack of penetration of PAM into cortical bone resulted in lack of resolution of the cortical bone phenotype of Gs-Tg mice in these studies. In addition,
this information provides rationale for proposal of future studies, especially because some of our mice have secondary osteons. Presence of secondary osteons indicates that some remodeling could be occurring in the intracortical space\textsuperscript{3}, which could mean that if a BP was able to penetrate cortical bone, there may be some effects on cortical porosity in the Gs-Tg mice. I have observed some examples of osteons surrounded by clear cement or reversal lines below, in Figure 4.2. I must emphasize that not all the mice have these secondary osteons. I suggest this to be enough to propose a fluorescent penetration study of different affinity BPs in our mice. It is highly likely that pamidronate, a very high affinity BP was unable to penetrate to the cortical bone at all, and that is what shows the stark lack of effect in cortical versus trabecular bone.

The penetration issues are also sufficient evidence to propose the study of a non-bisphosphonate treatment such as the antibody denosumab. Denosumab binds to RANK ligand (RANKL) therefore inhibiting activation of the RANK receptor, a key receptor in osteoclast synthesis and activity. Previously, a randomized double-blind, placebo-controlled in vivo assessment of alendronate, a bisphosphonate, and denosumab has shown that the antibody treatment led to significantly improved cortical porosity outcomes over alendronate. Both treatments were shown to have similar effects on trabecular bone\textsuperscript{84}.

![Figure 4.1 Example of histological sample showing the difference between primary and secondary osteons. Note the cement reversal line surrounding the secondary osteon.](image)
Figure 4.2. Examples of secondary osteons. Arrows point towards cement lines surrounding osteons. The panels on the left show samples from WT mice at 6 weeks of age. The panels on the right show samples from GS mice at 6 weeks of age.
Figure 4. 3. Examples of secondary osteons. Arrows point towards cement lines surrounding osteons. The panels on the left show samples from WT mice at 12 weeks of age. The panels on the right show samples from GS mice at 12 weeks of age.
CHAPTER 5: References


11. What is the impact of osteoporosis in Canada and what are Canadians doing to maintain healthy bones? (2010).


59


CHAPTER 6: Appendix

Raw images from the midpoints of trabecular and cortical ROIs were obtained from WT-S samples. They were subsequently visualized in halftone view, comparing different threshold values. Bone is visualized as the lighter green color. The voxels that CTAn recognizes as bone appear in red and overlap the light green.

6.1 Study 1 trabecular halftone view and thresholding


The halftone view does not include the despeckling effect which gets rid of all clusters less than 100 voxels in size. This is done to reduce noise that is picked up by a more sensitive threshold. A threshold of 45 and despeckling <100 was applied to trabecular bone for Study 1.

6.2 \textit{Study 1} cortical bone halftone view and thresholding

There is no despeckling for cortical bone. A threshold of 70 was applied to cortical bone for Study 1.
6.3 Study 2 trabecular halftone view and thresholding

Figure 6.3. A) Raw image view of ROI midpoint. B) Halftone view of ROI midpoint, threshold 100. C) Halftone view of ROI midpoint, threshold 60. D) Halftone view of ROI midpoint, threshold 50. E) Halftone view of ROI midpoint, threshold 45. F) Halftone view of ROI midpoint, threshold 40.

The halftone view does not include the despeckling effect which gets rid of all clusters less than 100 voxels in size. This is done to reduce noise that is picked up by a more sensitive threshold. A threshold of 45 and despeckling <100 was applied to trabecular bone for Study 2.
6.4 Study 2 cortical halftone view and thresholding

There is no despeckling for cortical bone. A threshold of 80 was applied to cortical bone for Study 2.

![Figure 6.4](image_url) A) Raw image view of ROI midpoint. B) Halftone view of ROI midpoint, threshold 130. C) Halftone view of ROI midpoint, threshold 100. D) Halftone view of ROI midpoint, threshold 90. E) Halftone view of ROI midpoint, threshold 80. F) Halftone view of ROI midpoint, threshold 60.