McBride, Alexandra H., Craniofacial Bone Mineral Density in Mice with Osteogenesis Imperfecta (OI). Master of Science (Medical Sciences-Anatomy), May 2019

Osteogenesis imperfecta (OI) is a rare genetic disorder characterized by the abnormal synthesis and assembly of type I collagen, a major organic component of bone. Clinical manifestations of the severe OI type III include small body size, limb deformities, and low bone mineral density (BMD) within the post-cranial skeleton. OI type III often co-occurs with craniofacial defects, such as dentinogenesis imperfecta (DI). The goals of this study are: (1) to examine whether type I collagen defects, as seen in OI type III, affect BMD within the craniofacial skeleton; (2) to determine whether BMD varies among specific region of the craniofacial skeleton; (3) to examine whether diet-related variation in biomechanical loading is related to higher craniofacial BMD.

The homozygous recessive murine mouse ($OIM^{-/-}$) is a model for OI Type III. Similar to human OI patients, $OIM^{-/-}$ mice exhibit low post-cranial BMD, smaller body size, and DI. $OIM^{-/-}$ mice and WT littermates were weaned at 21 days and raised on either hard (high loading) or soft (low loading) diets. This resulted in four genotype x diet treatment groups: OIM-soft (n=3), OIM-hard (n=6), WT-soft (n=3), and WT-hard (n=9). Micro-CT scans were collected at 16 weeks (skeletal maturity). BMD was measured using Bruker CTAnalyzer software for eight regions of interest (ROIs) within the mandible (TMJ, corpus at the second molar, and symphysis), facial skeleton (nasal bone, maxilla at the second molar, premaxilla at the incisor), and cranial vault (frontal and parietal bones). Pairwise Mann-Whitney U tests were used to statistically compare BMD between genotypes ($\alpha = 0.100$).

When controlling for diet, WT mice had significantly greater BMD values than OIM mice at each ROI except at the maxilla at M2. Although variation between treatment groups, a

general trend for increased BMD in “high” strain regions, such as the mandibular symphysis or the maxillary incisor, existed. Lastly, WT mice raised on a hard diet were observed to have the highest BMD measurements across each region the craniofacial skeleton, however no significant differences were observed between $OIM^{-/-}$ mice raised on hard versus soft diets.

These results suggest that craniofacial BMD is generally lower in individuals with type I collagen defects, consistent with the post-cranial presentation. Additionally, regions associated with high strain during routine masticatory loading exhibited increased BMD as compared to regions of the skull that experience relatively “low” strain during chewing. While diet-associated loading may influence craniofacial BMD, in this study type I collagen status appears to be the primary determinant of BMD.

CRANIOFACIAL BONE MINERAL DENSITY IN MICE

WITH OSTEOGENESIS IMPERFECTA (OI)

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CRANIOFACIAL BONE MINERAL DENSITY IN MICE WITH OSTEOGENESIS IMPERFECTA (OI)

INTERNSHIP PRACTICUM REPORT

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For the Degree of

MASTER OF SCIENCE

By

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CHAPTER I

INTRODUCTION TO THE STUDY

The following practicum report was performed as a requirement for the Master of Science-Anatomy Track program, from May 2018-May 2019, at the University of North Texas Health Science Center (UNTHSC). The study was conducted under the direct supervision of Rachel Menegaz, PhD, in the Center for Anatomical Sciences and Department of Physiology and Anatomy at UNTHSC.

Osteogenesis imperfecta (OI)[*] is a rare genetic disorder that leads to the formation of brittle or weak bones that are prone to fracture (Phillips et al., 2000). With an incidence between 1/10,000 and 1/25,000 cases worldwide, OI presents itself in four novel forms, ranging from mild (Type I), mild-moderate (Type IV), severe (Type III), and perinatal lethal (Type II). In humans, the severe type III OI is characterized by a dominant null mutation in genes COL1A1 and COL1A2 (Phillips et al., 2000). Such mutations lead to abnormal synthesis and assembly of type I collagen, a major organic component of bone (Phillips et al., 2000). Clinical manifestations include small body size, decreased bone mineral density (BMD) in the post-cranial skeleton, limb deformities, and osteopenia (Phillips et al., 2000). Craniofacial abnormalities include a characteristic “triangular face”, short basicrania, and both true (absolute) and relative macrocephaly (O’Connell and Marini; Harrington et al., 2014). Dental abnormalities such as dentinogenesis imperfecta and class III malocclusions (underbite) can also be present in these patients (Eimar et al., 2016). Given these clinical manifestations, patients with OI experience a poor quality of life. Current treatments include anti-resorptive medications and surgical bracing (Oestrich et al., 2016). Non-invasive behavioral and exercise treatments

[*] Abbreviations used in this report are listed in Appendix A

represent an underutilized area for pediatric OI, due to current gaps in our understanding of the relationship between craniofacial biomechanics and bone integrity in this disorder.

In addition to aberrant collagen formation, numerous studies have suggested that alterations in the mineral component of bone, such as reduced crystallinity and abnormal apatite structure, have contributed to the compromised structural biomechanical properties of bone in patients with OI (Camacho et al., 1999; Phillips et al., 2000). Indeed, there is a known relationship between the organization of the organic (collagen) and inorganic (calcium hydroxyapatite crystals) components of bone that produce the tissue's resultant mechanical properties (Landis et al., 1995). In this study, we assess the quality of inorganic bone tissue by measuring bone mineral density (BMD). Per the Bruker micro-CT analyzer protocol, BMD is defined as "the volumetric density of calcium hydroxyapatite (CaHA)" measured in $g.cm^{-3}$ (Bruker, 2016). A study by Davie et al. (1994) noted that patients with OI have as great as three standard deviations decreased BMD than those with healthy bone tissue. Mouse models of osteogenesis imperfecta have been found to largely replicate the type III OI phenotype seen in humans: small body size, increased fracture incidence, dentinogenesis imperfecta, craniofacial phenotype, etc. (Phillips et al., 2000; Eimar et al., 2016; Menegaz & Organ, 2018). Additionally, decreased BMD in post-cranial elements such as the femur (Phillips et al., 2000) has been observed. In this study, we use the osteogenesis imperfecta murine ($OIM^{-/-}$) model in order to investigate the relationship between abnormal type I collagen formation and bone mineralization in the craniofacial skeleton.

Bone modeling and remodeling is an important biological function for maintaining healthy bone mass and BMD in active individuals (Lanyon & Rubin, 1985; Iura et al., 2015). Bone deformation, or strain, produced by biomechanical loading has been shown to stimulate

signaling pathways involved in the mineralization process during remodeling and thus increases BMD (Iura et al., 2015). Consequently, regions of the skeleton that experience high strains during loading associated with activities such as weight bearing and muscle contractions show increased BMD as compared to those that experience low strains or low frequencies of loading (Lanyon & Rubin, 1985; Phillips et al., 2000). Post-cranial skeletal elements such as the femur experience routine loading during locomotion, while regions of the craniofacial skeleton experience routine loading during mastication and other feeding activities (Lanyon & Rubin, 1985; Ravosa et al., 2013). Regions proximal to the bite point, such as the temporomandibular joint (TMJ), and sites of masticatory muscle attachment experience high strains during chewing. Conversely, more distant regions in the skull, such as the cranial vault, experience comparatively low strain (Hylander et al., 1992; Ravosa et al., 2010, 2013; Herring & Teng, 2000). These strain gradients play an important role in maintaining bone mass in the craniofacial skeleton by directing bone remodeling.

Activities such as swimming and running that increase biomechanical loading in the post-cranial skeleton are known to improve BMD outcomes in those regions (Huddelston et al., 1980; Judex et al., 2003). Our goal is to test the hypothesis that similar increases in biomechanical loading in the skull, created by changes in diet and feeding behaviors, will result in increased craniofacial BMD. In this study, $OIM^{-/-}$ mice were raised on either a “hard’ diet that produced high levels of biomechanical loading during feeding or a “soft” diet that produced relatively low loading. We then measured BMD in both “high” and “low” strain regions of the craniofacial skeleton to assess the combined effects of type I collagen integrity and biomechanical loading on BMD.

This project uses a mouse model of type III osteogenesis imperfecta (the $OIM^{-/-}$ mouse) to address three specific aims. The first aim is to determine whether mutations of type I collagen correspond to decreased BMD in the craniofacial skeleton. We hypothesize that $OIM^{-/-}$ mice will exhibit decreased BMD within the skull as compared to their wild type ($OIM^{+/+}$ or WT) littermates. Our second aim is to assess whether BMD varies among specific regions of the craniofacial skeleton with the hypothesis that regions that experience high loading during mastication, such as the TMJ, will exhibit higher BMD than regions that experience low loading, such as the parietal bone. Our third and final aim is to discuss whether increased masticatory loading can help recover the craniofacial phenotype and increase BMD in OI mice. We hypothesize that $OIM^{-/-}$ mice raised on a "hard" or mechanically challenging diet, which produces higher strains during mastication (Williams et al., 2005; Menegaz & Ravosa, 2017), will exhibit craniofacial BMD values more similar to WT mice than to $OIM^{-/-}$ mice raised on a soft, non-challenging diet.

CHAPTER II
INTERNSHIP SUBJECT

BACKGROUND AND LITERATURE

Section 1: Osteogenesis Imperfecta (OI)

Osteogenesis imperfecta (OI) is a rare genetic disorder that is characterized by a spontaneous dominant mutation in type I collagen genes, such as COL1A1 and COL1A2 (Phillips et al., 2000). Type I collagen is synthesized by fibroblasts as procollagen and provides tissues such as tendon, bone, skin, and cartilage with structure and tensile strength (Phillips et al., 2000). Procollagen is composed of three α polypeptide chains, typically two α1 and one α2, that fold together to form a heterotrimeric triple-helix (Viguet-Carrin et al., 2006). Such α chains are dominated by repetitive Gly-X-Y triplet sequences that are responsible for collagen's tight helical configuration (Viguet-Carrin et al., 2006). Most mutations in COL1A1 and COL1A2 genes involve glycine deletions or substitutions for larger amino acids, which disrupts regular synthesis and assembly of mature type I collagen fibrils (Gajko-Galicka, 2002). The severity and clinical manifestations of OI depends upon the nature and location of such mutations within COL1A1/COL1A2 genes (Gajko-Galicka, 2002).

OI is a phenotypically heterogeneous disease and presents in four novel forms ranging from mild to lethal. Type I is considered the mild type and is clinically characterized by moderate bone fragility, blue sclera (the whites of the eye are blue), and premature deafness (Sillence et al., 1979). Patients with type I OI typically produce *normal* collagen fibers, but at about 50% quantity as compared to healthy individuals (Van Dijk & Sillence, 2014). Type II typically results from internal deletions in COL1A1/COL1A2 genes and is specified as perinatal lethal (Van Dijk & Sillence, 2014). Infants with type II experience low bone mass and

intrauterine fractures and often die shortly after birth (Valadares et al., 2014). Type III OI is characterized by poor quality and quantity of type I collagen due to mutations in either COL1A1 or COL1A2 genes (Phillips et al., 2000). Clinical manifestations include small body size, limb deformities, spontaneous fractures, and dental abnormalities (Phillips et al., 2000). Similar to type I, type IV OI is classified as mild-moderate and is compatible with survival (Sillence et al., 1979).

Type III OI is classified as a severe presentation of the disease among those that survive infancy (Phillips et al., 2000). At birth, infants often present with blue sclera, long bone deformities, and fractures (Cheung & Glorieux, 2008). Type III OI is commonly referred to as the progressively deforming type since bending and bowing of post-cranial skeletal elements, particularly the femur, worsens with age (Sillence et al., 1986). As adults, individuals retain a short stature with an average height ranging from 90 to 120 centimeters (cm) and are commonly confined to wheelchairs (Cheung & Glorieux, 2008). Most patients experience severe bone fragility and are therefore at high risk for fracture (Cheung & Glorieux, 2008). Such bone fragility often leads to long bone deformities that can negatively affect locomotive and respiratory function resulting in compromised quality of life (Cheung & Glorieux, 2008). Similar phenotypic presentations have been documented in mouse models of osteogenesis imperfecta. The $OIM^{-/-}$ mouse used in this study is particularly known to replicate the human phenotype of type III OI, such as small body size, decreased BMD, high fracture risk, and dentinogenesis imperfecta (Phillips et al., 2000).

In addition to post-cranial element deformities, patients with type III OI experience craniofacial skeletal abnormalities such as a characteristic “triangular” shaped face, cranial vault deformities, macrocephaly, and dentinogenesis imperfecta (Phillips et al., 2000; Chang et al.,

2007). A study assessing cephalometric characteristics, such as facial height, facial divergence, and posterior angle, in the skulls of 16 children ranging from 8 to 15 years of age found similar craniofacial abnormalities (Chang et al., 2007). For example, facial divergence in these patients was found to be greater than 10 degrees larger than in controls (Chang et al., 2007). Facial divergence is defined as the shift of the lower jaw (anterior or posterior) relative to the forehead (Goldberg et al., 2013). Additionally, anterior and posterior cranial base lengths were significantly shorter in the OI patients as compared to their control counterparts (Chang et al., 2007). Early studies of mouse models suggest that similar facial and basicranial changes are seen in mouse models with type I collagen defects (Eimar et al., 2016; Menegaz and Organ, 2018).

Numerous dental abnormalities are also reported in human patients with type III OI, particularly in juveniles. Common clinical manifestations affecting the dentition include dentinogenesis imperfecta, class III malocclusions, tooth discoloration, and abnormal dentin mineralization (Eimar et al., 2016; Phillips et al., 2000). With an incidence rate of 1 in 8000, dentinogenesis imperfecta is a heritable disorder that often accompanies OI and is characterized by brittle or weak teeth (Schwartz & Tsipouras, 1984). Numerous studies have also identified class III malocclusions as common dental manifestations of OI (Schwartz & Tsipouras, 1984; Eimar et al., 2016). A class III malocclusion, commonly called an “underbite”, is characterized by the first mandibular molar positioned anteriorly relative to the first maxillary molar (Schwartz & Tsipouras, 1984). Previous studies of mouse models of OI have assessed craniofacial and dental defects and discovered aberrant dentin mineralization (Phillips et al., 2000; Eimar et al., 2016). Phillips et al. found that $OIM^{-/-}$ mice have altered mineral content in the enamel of the maxillary incisors (Phillips et al., 2000). Similarly, $Col1a1^{Jrt/+}$ mice, a combined OI and Ehlers-Danlos Syndrome model, have lower mineralized dentin volume/tooth volume ratios as

compared to their WT littermates (Eimar et al., 2016). These results suggest that OI affects normal dental development and mineralization.

There is currently no cure for OI, therefore current treatments and interventions are often aimed at disease management rather than prevention. As with any disease, early detection of OI is key to the success rate of treatments and interventions. Management of OI is multidisciplinary, which includes but is not limited to medication, orthopedic surgery, dietary adjustments, and physical therapy (Cheung & Glorieux, 2008). Bisphosphonate therapy, which serves anti-resorptive functions, is the most widely used treatment for OI (Cheung & Glorieux, 2008). Bisphosphonates, such as pamidronate and zoledronic acid, increase bone mass by inhibiting osteoclast activity and numerous studies have sighted its efficacy (Cheung & Glorieux, 2008). Among several large studies, a majority of patients experienced increased mobility and increased stature height after being treated with pamidronate over a period of several years (Glorieux et al., 1998; Zeitlin et al., 2003). Similarly, other studies have sighted increased BMD within the lumbar vertebrae of OI children and decreased incidence of fractures (Glorieux et al., 1998; Land et al., 2007). While bisphosphonate has demonstrated the ability to improve aspects of bone health in patients with OI, other methods of intervention are important for long-term treatment as extended use of bisphosphonates has been associated with BP-induced osteonecrosis of the jaw (Hewitt & Farah, 2007).

Non-medicinal treatments have also shown to be advantageous in improving the quality of life for patients living with OI. Physical therapy is often recommended to patients with OI to improve motor skills. Approaches vary depending on the severity of the disease (Harrington et al., 2014). For example, hydrotherapy has been a useful weight-bearing activity that gradually increases strength while walking braces have helped improve balance (Harrington et al., 2014).

Invasive surgical treatments, commonly used to repair fractures, are often necessary for more severe phenotypes. Deformities in long bones, such as the femur or tibia, are often treated via osteotomy, a procedure in which bones are cut and reshaped in order to reduce pressure between opposing skeletal elements (Harrington et al., 2014). Additionally, the insertion of intramedullary rods helps maintain mechanical strength and provides the bone with support to endure future weight-bearing activities such as locomotion (Harrington et al., 2014).

Section 2: Bone Composition

The composition of bone is defined by an inorganic mineral phase consisting of calcium hydroxyapatite and an organic phase consisting of type I collagen, other non-collagenous proteins, and water (Phillips et al., 2000). The relative quality and quantity of such components varies depending on age, gender, site, and health status and determines the mechanical properties of bone such as elasticity and plasticity (see Section 3: Biomechanical Properties of Bone) (Boskey, 2013). Consequently, a mutation in type I collagen leads to abnormal synthesis and assembly of collagen fibrils in conjunction with decreased bone tissue quality and strength (Boskey, 2013). During bone development, type I collagen forms an intimate relationship with the inorganic mineral component through the deposition of calcium hydroxyapatite (CaHA) crystals within spaces between parallel collagen fibrils (Landis et al., 1995). Using high-voltage electron microscopic tomography, Landis et al. (1995) compared the microstructure of hydroxyapatite crystals and its interaction with collagen within tendons of healthy mice to tendons of mice with osteogenesis imperfecta ($OIM^{-/-}$). They observed CaHA as bulky deposits inconsistently arranged along collagen fibrils in the $OIM^{-/-}$ mouse as compared to fibril arrangement in the WT mice. For this reason, bone fragility and liability to fracture are common

clinical manifestations of abnormal type I collagen disorders, such as OI (Van Dijk & Sillence, 2014).

Other studies have sighted the interaction between organic and inorganic components as critical for regular biomechanical functioning of bone tissue. Using the $OIM^{-/-}$ mouse as their model and bone mineral density (BMD) as their parameter for mineral content, Phillips et al. performed a longitudinal study to assess the effect that type I collagen mutations have on the mineralization process (2000). Mice with OI exhibited abnormal mineral content and decreased BMD within the femur as compared to their WT littermates, thus suggesting a co-dependent relationship between organic and inorganic components of bone to biomechanical function. Both experimentally and clinically, BMD serves as an important non-invasive parameter for assessing mineral quality and content of bone (Phillips et al., 2000). Additionally, in their study Phillips et al. assessed ion content in the femur and incisors of OI mice and WT littermates. Magnesium, an ion thought to negatively affect the mineral matrix and inhibit normal hydroxyapatite crystal formation, was significantly increased in femurs of OI mice as compared to their WT littermates (Phillips et al., 2000). These findings by Phillips et al. illustrate the intimate relationship between organic and inorganic components of bone and how the deterioration of that relationship can affect bone quality (2000).

Section 3: Biomechanical Properties of Bone

During routine activities such as locomotion or mastication, bones are subjected to deformation and tension as a result of external loading. Deformation is quantified by the amount of strain a tissue undergoes, which is defined as the change in length per unit of length (van Eijden, 2000). When bone deforms during loading, tension occurs in the tissue, which is defined

as stress (van Eijden, 2000). Stress is quantified as force applied to a tissue per unit area and is measured in Pascals (Pa) (van Eijden, 2000). Depending on the direction and force to which a load is applied, stress can be compressive, tensile, or shear (van Eijden, 2000). Compression occurs if the tissue decreases in length, tensile stress occurs if the tissue increases in length, and shear stress occurs if one region moves parallel relative to the other region (van Eijden, 2000). The range and distribution of such stresses and strains that a particular tissue is able to tolerate depends in large part on its biological characteristics (i.e. chemical composition, size, geometry, etc.) and on the nature of loading (Bouvier & Hylander, 1981; van Eijden, 2000). If bone is able to recoil back to its original shape after the applied force has ceased, it is said to be elastic (Smith & Walmsley, 1959). On the other hand, if the tissue is not able to recoil and the deformation persists after forces have ceased, it is termed plastic (Smith & Walmsley, 1959).

The elasticity and plasticity of a particular substance can be described by its stress-strain curve. Along the curve exists an elastic region and a plastic region, which are separated by a yield point (van Eijden, 2000). Stress-strain curves are unique for a given component of the skeleton depending on its architecture and composition. For example, the elastic modulus (E), which is represented by the slope of the elastic region, in cancellous bone ranges from 0.76 to 20 GPa while the elastic modulus in compact bone ranges from 10 to 20 GPa (Turner et al., 1990; van Eijden, 2000). Furthermore, elastic properties within a given component of the skeleton, such as the mandible, can vary. For example, previous studies have found that the elastic modulus is lower in the molar regions of the mandible and increases anteriorly towards the symphysis (Dechow et al., 1993; Schwartz-Dabney & Dechow, 1997). These findings are consistent with strain gauge studies on macaque zygomatic arches performed during feeding (both mastication and incision). When strain gauges were placed at the anterior, middle, and

posterior portion of the zygomatic arch of macaques, strain was found to be highest anteriorly and lowest posteriorly (Hylander and Johnson, 1997). Although the macaque facial skeleton morphology differs from that of humans, it is likely that biomechanical properties vary within the human craniofacial skeleton in a similar fashion.

The loading conditions experienced by bone during locomotion or mastication are thought to play a critical role in bone homeostasis (van Eijden, 2000). As a mechanosensing organ, bone has the ability to respond to the external environment and alter in morphology over the course of development (Ravosa et al., 2008). This dynamic response of bone to its loading environment is termed functional adaptation (Lanyon and Rubin, 1985). Functional adaptation refers to the response by bone tissue to load bearing to achieve optimal shape and size through a process called remodeling (Lanyon and Rubin, 1985; Ravosa et al., 2008). Remodeling occurs throughout life and involves the reabsorption of bone by osteoclasts followed by the deposition of new bone matrix by osteoblasts (Hadjidakis & Androuslakis, 2006). Signaling pathways involved in bone remodeling have been shown to be influenced by the presence of weight-bearing activities, such as exercise (Iura et al., 2015). Bone morphometric protein (BMP) is an important signaling factor involved in bone development and in controlling bone mass (Iura et al., 2015). In order to understand the relationship between load bearing and bone remodeling, Iura et al. used mice that were null for *Bmpr1a*, a gene that encodes for a BMP receptor (2015). Both WT and mutant mice ranging from 11 to 16 weeks of age were placed on a treadmill for 5 days a week and trabecular volume of the tibia was measured. Trabecular tibial volume in the knockout mice had increased as a result of exercise, whereas exercise in the WT mice did not appear to have an effect (Iura et al., 2015). These findings illustrate the role that functional

adaptation and the subsequent response (modeling and remodeling) by osteogenic cells play in bone development and homeostasis.

In addition to external forces, loading conditions and bone remodeling pathways are influenced by muscle mass. Skeletal muscle serves as an important natural loading source on bone. Additionally, bone strength has been cited as being proportional to muscle mass (Oestreich et al., 2015; Daly et al., 2004). To test this point, Oestreich et al., bred mice that were deficient for myostatin ($MSTN^{+/-}$) with heterozygous $OIM^{+/-}$ mice (2015). Myostatin is a protein that serves as a negative regulator for muscle growth (Oestreich et al., 2015). Body mass, bone physiochemical microarchitecture, and biomechanical integrity were assessed in the resulting adult offspring ($MSTN^{+/-}$ / $OIM^{+/-}$). Their findings demonstrated that increased muscle mass as a result of myostatin deficiency was correlated with increased body mass in the OI phenotype, but did not alter mid-shaft geometry (Oestriech et al., 2015). The fact that mid-shaft geometry was not greatly influenced by myostatin deficiency reinforces prior knowledge that geometrical changes in bone are primarily localized to the sites of muscle insertion (Oestreich et al., 2015). Femoral torsional ultimate strength (T_{max}) had also increased, suggesting that increased muscle mass indirectly improves biomechanical function (Oestreich et al., 2015). Results from this study illustrate how loading by muscle influences bone quality and function.

BMD currently serves as the most common parameter for clinically assessing bone strength and determining potential fracture risk (Morseth et al., 2011). Physical activity and subsequent bone remodeling is important for maintaining bone mass and BMD in healthy, active individuals. As previously mentioned, an increase in BMD is one of several indicators of increased osteoblastic activity and bone remodeling (Morseth et al., 2011). Numerous studies involving a wide array of individuals have supported this notion. For example, BMD within the

femoral neck of young girls engaging in high-impact physical activity over a 10-month period increased by 12.0 % while BMD only increased 1.7% in controls (Judex et al., 2003). Similarly, a human study reported a 22% increase in BMD within the dominant arm of professional tennis players (Huddleson et al., 1980).

Like the post-cranial skeleton, the craniofacial skeleton undergoes morphological and biochemical changes via remodeling mechanisms in response to stress and strain experienced during load bearing activities. Animal studies involving the manipulation of dietary consistency as a means for generating force on the masticatory system have demonstrated differences in biomineralization and BMD (Bouvier & Hylander, 1981; Ravosa et al., 2008). A study conducted by Ravosa et al. involved 20 rabbits obtained as weanlings (4 weeks old) that were fed either a soft-pellet diet to stimulate under-use (U) of masticatory elements or a hard-pellet diet to stimulate over-use (O) (2008). Micro-CT was used to visualize and quantify differences in biomineralization of the articular surface, subarticular bone, and cortical bone along the symphysis and the condylar head of the TMJ (Ravosa et al., 2008). Consistent with previous studies involving post-cranial skeletal elements, the group exposed to the “high” strain condition (hard-pellet diet) displayed elevated BMD as compared to the “low” strain (soft-pellet diet) group (Ravosa et al., 2008). Furthermore, jaw adductor muscle mass (masseter muscle) was significantly greater in the O-diet than the U-diet thus demonstrating the load-bearing role that muscle plays in increasing BMD (Ravosa et al., 2008).

Experimental and clinical studies (Harrington et al., 2014) have validated the use of biomechanical loading through exercise as a therapeutic way to address post-cranial bone fragility in OI. In this study, we investigate whether biomechanical loading through feeding can similarly be used to recover the craniofacial phenotype of mice with type III OI. Our

experimental design consisted of two experimental diets ("hard" pellets and "soft" meal) which created variation in the biomechanical loading ("high" and "low" loading, respectively) experienced by the craniofacial skeleton during feeding. We examined the effects of each of these diets on craniofacial BMD in both $OIM^{-/-}$ and WT mice. In doing so, we hope to gain a greater understanding of the relationship between type I collagen and BMD, and use this knowledge to improve treatment options and quality of life for patients with OI.

SPECIFIC AIMS

In this study, we use a mouse model of the severe type III osteogenesis imperfecta (the $OIM^{-/-}$ mouse) to investigate the individual and combined effects of type I collagen integrity and biomechanical loading on BMD in the face skeleton. Our three specific aims are:

Aim 1: To determine whether mutations of type I collagen synthesis correspond to decreased BMD in the craniofacial skeleton.

Hypothesis 1: We hypothesize that mice with severe OI ($OIM^{-/-}$) will exhibit decreased BMD within the skull as compared to their wild type ($OIM^{+/+}$ or WT) littermates.

Aim 2: To assess whether BMD varies within specific regions of the craniofacial skeleton.

Hypothesis 2: Regions that experience high loading, such as the TMJ, during mastication will have higher BMD than regions that experience low loading, such as the parietal bone.

Aim 3: To investigate whether increased masticatory loading can help recover the craniofacial phenotype and increase BMD in OI mice.

Hypothesis 3A: Mice raised on a "hard" or mechanically challenging diet, which produces higher strains during mastication (Williams et al., 2005; Menegaz, 2013), will exhibit craniofacial BMD values that are significantly greater than mice of the same genotype that are raised on a "soft", non-challenging diet.

Hypothesis 3B: $OIM^{-/-}$ mice raised on a "hard" or mechanically challenging diet will exhibit craniofacial BMD values that are more similar to WT mice than to $OIM^{-/-}$ mice raised on a "soft" diet.

SIGNIFICANCE

Describing the craniofacial phenotype in OI mice will provide insight into possible treatment and management interventions for humans living with OI. Specifically, analyzing the cellular processes and the biomechanical properties in the OI mouse model will allow us to understand how OI presents in humans and how to approach treatment. In OI, it is important to understand the cellular processes as the disease is a result of mutations in type I collagen genes (Phillips et al., 2000). Type I collagen is the most abundant structural protein in tissues such as skin, tendons, cartilage, and bone, all of which could negatively affect the quality of life for an individual if these tissues' regular form and function is compromised (Phillips et al., 2000). It is equally important to describe the biomechanical properties of the craniofacial skeleton and how these properties change throughout ontogeny. With this knowledge, we can elucidate the nature of the human facial skeleton and how a defect in type I collagen affects masticatory systems. A greater understanding of type I collagen mutations and the implication on load-bearing and biomechanical function will allow us to discover new treatments and improve the quality of life for those living with OI.

MATERIALS AND METHODS

Section 1: Experimental Model and Diets

All procedures and animal care were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC protocol 11133). The osteogenesis imperfecta murine (OIM) is a mouse strain with a nonlethal recessive inherited mutation of the COL1A2 gene. Homozygous $OIM^{-/-}$ mice (B6C3FE a/a-Col1a2OIM/J) are a model for the human presentation of type III OI. The wild type (WT) control in this experiment are $OIM^{+/+}$ (B6C3FeF1/J) $mice^{-/-}$.

WT littermates were raised from weaning (21 days) to adulthood (16 weeks). At weaning, individuals from both genotypes were randomly sorted into dietary treatment groups (Table 1). The “hard” diet consisted of standard LabDiet mouse diet pellets, and the “soft” diet consisted of the same dietary formula presented in a powdered meal form. Both the “hard” and “soft” diets had identical nutritional profiles. All animals were fed *at libitum*. Body weights were collected twice weekly. Visual inspections were performed regularly by veterinary staff to monitor for incisal malocclusions, and minimal trimming was used when necessary to ensure normal feeding behaviors.

Table 1 : Treatment Groups

Treatment Group	Genotype	Diet	n
OIM-soft	$OIM^{-/-}$	Meal	3
OIM-hard	$OIM^{-/-}$	Pellets	6
WT-soft	WT or $OIM^{+/+}$	Meal	3
WT-hard	WT or $OIM^{+/+}$	Pellets	9
			Total n = 21

Section 2: Micro-CT imaging

Mice were anesthetized with inhalation isoflurane at 3-5% and maintained at 1.5% in preparation for in-vivo micro-CT scans. Scans were then performed at 4 weeks (post-weaning juveniles), 10 weeks (adolescents), and 16 weeks (adults). This study discussed only the BMD analyses for the week 16 mice. Scans were collected using a Skyscan 1176 micro-CT machine at a resolution of 8 or 16 μm^3 voxels followed by reconstruction of CT images using NRecon software.

Section 3: BMD data collection

To assess whether BMD varies within the craniofacial skeleton, eight regions of interest (ROI) were selected from the craniomandibular skeleton (Table 2; Figure C1). ROIs were selected based on their relative amount of strain ("high" vs. "low") experienced during routine mastication. Regions proximal to the bite point, temporomandibular joint (TMJ), and sites of masticatory muscle attachment experience high strains during chewing, while more distant regions in the skull (such as the cranial vault) experience comparatively low strain (Hylander et al., 1992; Ravosa et al., 2010, 2013; Herring & Teng, 2000).

Using Skyscan CT-analyser software, we measured mean BMD at selected ROIs in the cortical bone of mouse specimens. Phantom rods of a known density of CaHA were used to calibrate measurements (Bruker, 2016). Two mouse-sized (2mm) phantom rods (0.25 and 0.75 $g.cm^{-3}$ CaHA embedded in epoxy resin) were scanned in the Skyscan 1176 micro-CT machine at similar settings (kV, mA, voxel size, etc.) as the in-vivo scans. The phantoms were scanned in water to approximate the x-ray absorption of soft tissues. CTan software (Bruker) was used to calculate the attenuation coefficients from the two rods, and these coefficients were used to calibrate the BMD measurements of the in-vivo scans.

A repeatability trial (n=3; trial=3) was conducted at the TMJ, mandibular corpus at the second molar (M2), and the mandibular symphysis to ensure precision of ROI selection with resulting standard deviation less than 3% for each ROI. Measurements at remaining ROIs were then conducted on all subjects on the left side of the skull assuming symmetry.

Table 2 : Regions of Interest (ROI)

Region of Interest (ROI)	ROI Classification	Relative Strain
TMJ	Mandible	High
Mandibular corpus near the second molar (**M2**)	Mandible	High
Mandibular symphysis	Mandible	High
Nasal bone	Face	Moderate
Maxilla at the second molar (**M2**)	Face	High
Maxillary incisor	Face	High
Parietal bone	Cranial Vault	Low
Frontal bone	Cranial Vault	Low

Section 4: Statistical Analysis

Due to the relatively small sample sizes in this study (Table 1), BMD measurements were analyzed using non-parametric ANOVAs (Mann Whitney U-Tests). Male and female mice were grouped together by treatments for the statistical analysis, as no significant sex differences were observed in BMD within the treatment groups. While $\alpha = 0.050$ is most commonly used in the biological sciences, we chose to use a α set to 0.100 in our non-parametric analyses due to our relatively small sample size.

To compare BMD between genotypes (Aim 1), we performed two Mann-Whitney U-tests ($\alpha = 0.100$). Our goal in this analysis was to compare $OIM^{-/-}$ to WT while controlling for diet, so

two analyses were performed for all ROIs: OIM-soft versus WT-soft; and OIM-hard versus WT-hard.

To compare BMD among specific regions of the craniofacial skeleton (Aim 2), we used a Kruskal-Wallis test ($\alpha = 0.100$) to first assess if any significant difference existed among the 8 ROIs within a single treatment group. The Kruskal-Wallis tests produced significant *p* values for all four treatment groups, so pair-wise Mann-Whitney U-tests ($\alpha = 0.100$) were then used to identify which ROIs significantly differed in the BMD values. Variation in BMD among craniofacial regions was also visually illustrated by classifying the ROIs into 3 groups (face, mandible, and cranial vault). Face ROIs include the nasal bone, maxilla at M2, and root of maxillary incisors. Mandibular ROIs include the mandibular symphysis and corpus and TMJ. The parietal bone and frontal bone are classified as cranial vault ROIs.

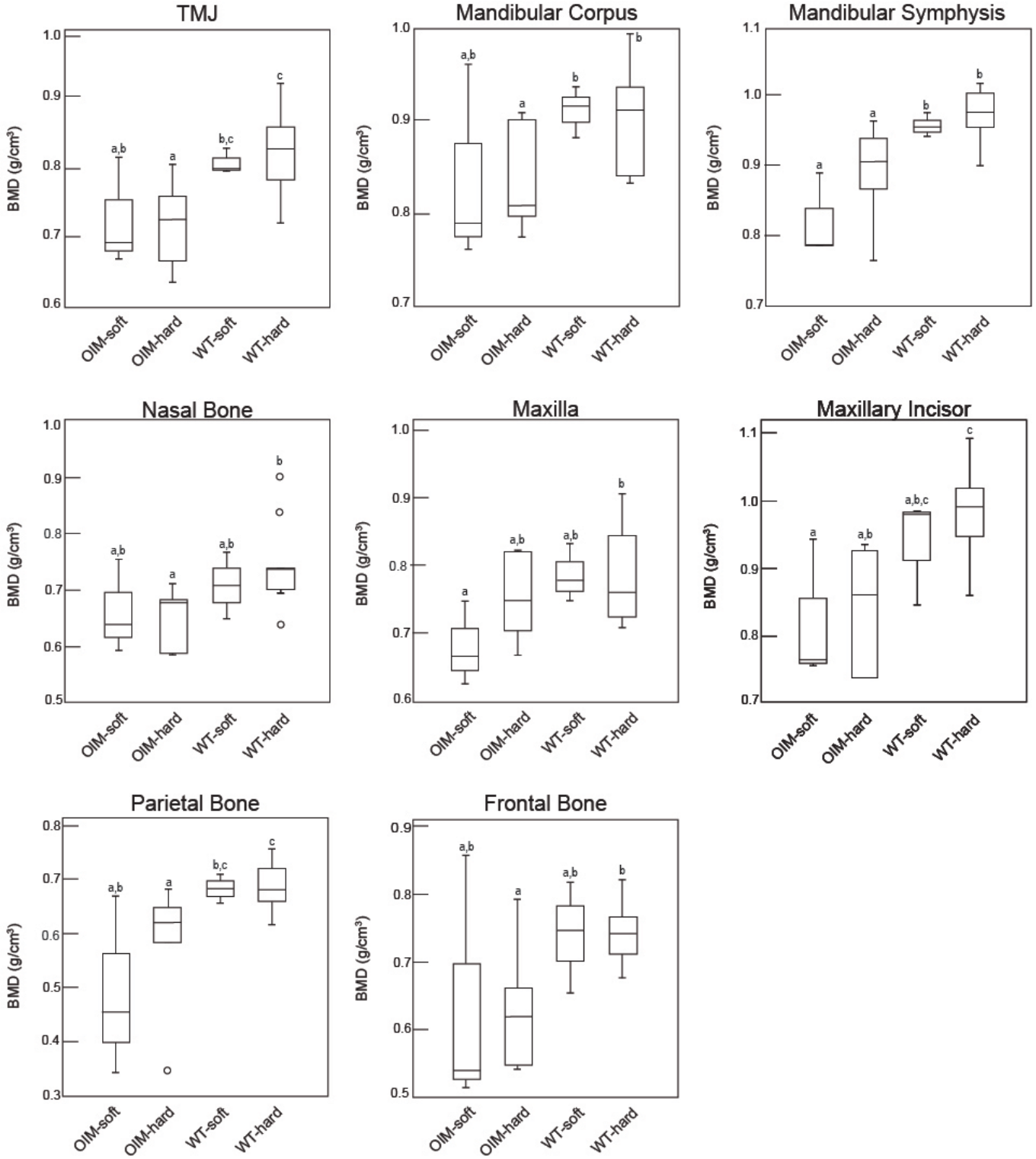
To assess whether manipulating diet can help recover the craniofacial phenotype and increase BMD (Aim 3), we performed pairwise Mann-Whitney U-tests ($\alpha = 0.100$) to compare dietary treatments within the same genotype. Two analyses were performed to test Aim 3 for all ROIs: OIM-soft versus OIM-hard; and WT-soft versus WT-hard.

RESULTS

Section 1: (Aim 1) BMD differences between genotypes (OIM vs. WT)

With an α value set to 0.100, the results of this study show that BMD is statistically greater in WT-hard mice as compared to OIM-hard mice. OI-hard mice displayed significantly less BMD compared to WT-hard mice across all eight ROIs, except at the buccal corpus of the maxilla at M2 (p-value= 0.313) (Table B1, Figure 1). For soft-diet mice, differences in mean BMD between genotypes were only significant at the mandibular symphysis (Table B1; Figure 1).

Figure 1: BMD differences between genotypes (OIM vs. WT) (Aim 1 & 3 results)



Section 2: (Aim 2) BMD differences among ROIs (within genotypes)

BMD in OIM-soft mice

BMD from mandibular ROIs was generally higher than BMD from ROIs in the face and cranial vault (Figure 2). Cranial vault ROIs were found to have BMD values with a wide distribution, but tended to be lower than BMD in the mandible and the face.

Pair-wise tests revealed that BMD at the mandibular corpus was significantly greater than BMD in the parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$); BMD at the mandibular symphysis was significantly greater than BMD in the parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$); BMD at the maxillary incisor was significantly greater than BMD in the parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$) (Table B2; Figure 3).

BMD in OIM-hard mice

BMD from mandibular ROIs was generally higher than BMD from ROIs in the face and cranial vault (Figure 2). Cranial vault ROIs were found to have BMD values with a wide distribution, but tended to be lower than BMD in the mandible and the face.

Pair-wise tests revealed that BMD at the TMJ was significantly greater than BMD in the mandibular corpus ($p = 0.015$), mandibular symphysis ($p = 0.004$), and the parietal bone ($p = 0.015$); BMD at the mandibular corpus was significantly greater than BMD in the TMJ ($p = 0.015$) parietal bone ($p = 0.002$), nasal bone ($p = 0.002$), and the frontal bone ($p = 0.004$); BMD at the mandibular symphysis was significantly greater than BMD in the TMJ ($p = 0.004$), parietal bone ($p = 0.002$), nasal bone ($p = 0.002$), frontal bone ($p = 0.004$), and maxilla at M2 ($p = 0.015$); BMD at the maxillary incisor was significantly greater than BMD in the parietal bone ($p = 0.002$), nasal bone ($p = 0.002$), and frontal bone ($p = 0.009$); BMD in the maxilla at M2 was

significantly greater than BMD at the parietal bone ($p = 0.004$), nasal bone ($p = 0.002$), and frontal bone ($p = 0.009$) (Table B3; Figure 3).

BMD in WT-soft mice

BMD from mandibular ROIs was generally higher than BMD from ROIs in the face and cranial vault (Figure 2). Cranial vault ROIs were found to have BMD values with a wide distribution, but tended to be lower than BMD in the mandible and the face.

Pair-wise tests revealed that BMD at the TMJ was significantly greater than BMD in the parietal ($p = 0.100$) and nasal bones ($p = 0.100$); BMD at the mandibular corpus was significantly greater than BMD in the TMJ ($p = 0.100$), parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), frontal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$); BMD at the mandibular symphysis was significantly greater than BMD in the TMJ ($p = 0.100$), mandibular corpus ($p = 0.100$), parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), frontal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$); BMD in the maxilla at M2 was significantly greater than BMD in the parietal bone only ($p = 0.100$); BMD in the maxillary incisor was significantly greater than BMD at the TMJ ($p = 0.100$), parietal bone ($p = 0.100$), nasal bone ($p = 0.100$), frontal bone ($p = 0.100$), and maxilla at M2 ($p = 0.100$) (Table B4; Figure 3).

BMD in WT-hard mice

BMD from mandibular ROIs was generally higher than BMD from ROIs in the face and cranial vault (Figure 2). Cranial vault ROIs were found to have BMD values with a wide distribution, but tended to be lower than BMD in the mandible and the face.

Pair-wise tests revealed that BMD at the TMJ was significantly greater than BMD in the parietal bone ($p = 0.000$), nasal bone ($p = 0.031$), frontal bone ($p = 0.008$), and maxilla at M2

($p = 0.000$); BMD at the mandibular corpus was significantly greater than BMD in the TMJ ($p = 0.024$), parietal bone ($p = 0.000$), nasal bone ($p = 0.001$), frontal bone ($p = 0.000$), and maxilla at M2 ($p = 0.006$); BMD at the mandibular symphysis was significantly greater than BMD in the TMJ ($p = 0.000$), mandibular corpus at M2 ($p = 0.004$), parietal bone ($p = 0.000$), nasal bone ($p = 0.000$), frontal bone ($p = 0.000$), and maxilla at M2 ($p = 0.000$); BMD in the maxilla at M2 was significantly greater than BMD in the parietal bone only ($p = 0.004$); BMD in the maxillary incisor was significantly greater than BMD at the TMJ ($p = 0.000$), mandibular corpus ($p = 0.019$), parietal bone ($p = 0.000$), nasal bone ($p = 0.000$), and frontal bone ($p = 0.000$) (Table B5; Figure 3).

Figure 2 : BMD differences among ROI categories (within genotypes) (Aim 2 results)

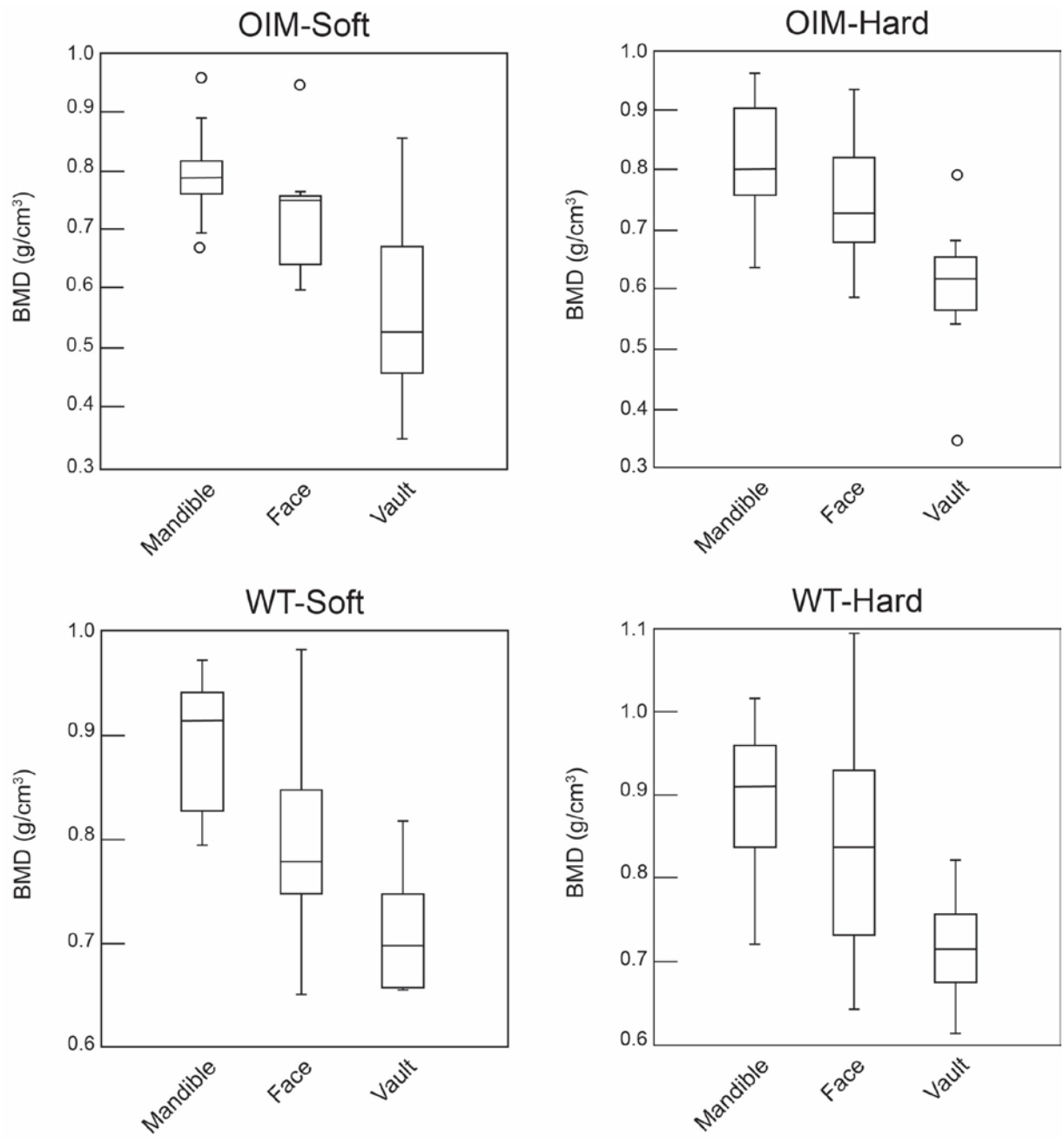
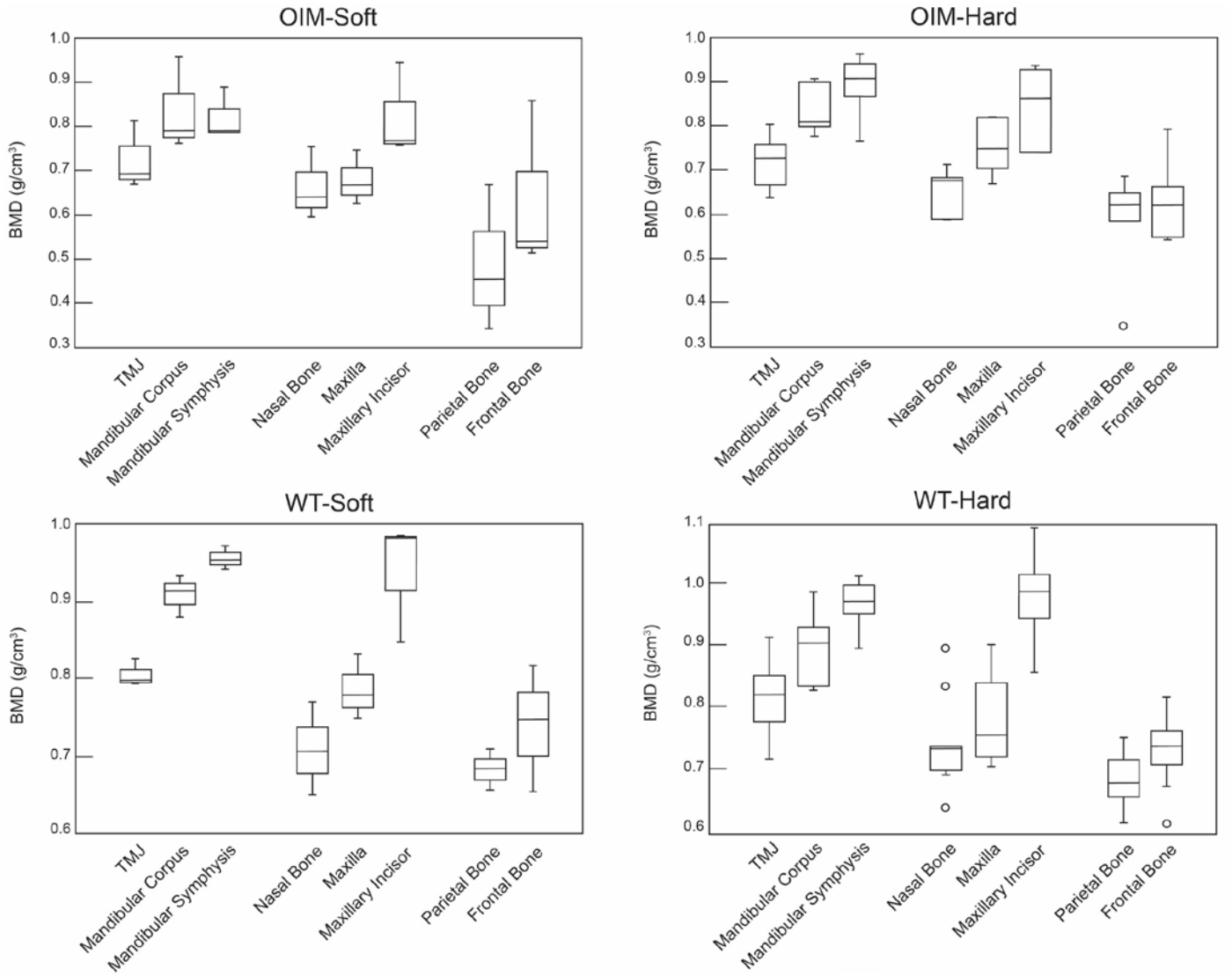


Figure 3: BMD differences among individual ROIs (within genotypes) (Aim 2 results)



Section 3: (Aim 3) BMD differences between diets

Aim 3A: BMD in Hard vs Soft Diet Mice

BMD among "hard" diet mice, regardless of genotype, tended to be higher than BMD in "soft" diet mice at each ROI, with the exception of the frontal and nasal bones (Table B6). However, contrary to our hypothesis, pair-wise comparisons revealed that differences between diet (hard vs. soft) among mice of the same genotype were not statistically significant ($p > 0.100$) given the sample size.

Aim 3B: BMD recovery in OIM-hard mice versus WT mice

Pairwise comparisons reveal that BMD values in OIM-hard mice are significantly smaller than those in WT mice (both WT-soft *and* WT-hard) at the TMJ ($p = 0.095$ and 0.049), mandibular symphysis ($p = 0.095$ and 0.007), and parietal bone ($p = 0.095$ and 0.049) (Table B7). OIM-hard mice were also observed to have significantly lower BMD values than WT-soft mice at the mandibular corpus near M2 ($p = 0.095$), and significantly lower BMD values than WT-hard mice at the maxilla near M2 ($p = 0.049$) and maxillary incisor ($p = 0.028$).

DISCUSSION

In this study, we measured BMD within specific regions of the craniofacial skeleton of OIM and WT mice raised on diets differing in consistency. The three specific aims of this study were as follows: (Aim 1) to determine whether a significant difference in BMD of the craniofacial skeleton between $OIM^{-/-}$ mice and their WT littermates exists; (Aim 2) to identify where BMD varies at specific regions within the facial skeleton; and (Aim 3) to determine if a

mechanically challenging diet can help recover the OI craniofacial phenotype and increase BMD.

Our results show that, controlling for diet, craniofacial BMD is generally higher in WT mice than in $OIM^{-/-}$ mice, thus providing support our hypothesis for Aim 1. Among mice raised on a mechanically challenging diet, BMD was significantly greater in WT mice than in $OIM^{-/-}$ mice at each ROI except the frontal bone and maxilla at M2. These results are generally consistent with previous studies of BMD in the post-cranial skeleton of mice and humans with OI (Davie & Haddaway, 1994; Phillips et al., 2000). Davie & Haddaway observed as great as three standard deviations decrease in BMD in OI patients as compared to healthy individuals (1994). Likewise, using a similar model that we use in this study, Phillips et al., noted that BMD in OI mice femurs was statistically decreased as compared to their WT counterparts (2000). We can attribute non-significant values at the maxilla at M2 as this ROI was difficult to trace while excluding tooth enamel on the micro-CT scans. Tooth enamel is considered to be the densest substance in the body, therefore it is possible that BMD for $OIM^{-/-}$ mice is elevated at this ROI for this reason (Brand & Isselhard, 2018, 16). Additionally, cortical bone in this location of the craniofacial skeleton is quite thin, thus increasing the difficulty of ROI tracing. It would be beneficial for future studies assessing BMD using micro-CT scans to employ a large voxel image size than this current study (16 μm^3). When analyzing small structures, such as cortical bone within mice skulls, a high voxel to structure size ratio is recommended (Bouxsein & Boyd, 2010). We mainly attribute the non-significant value in the frontal bone to our relatively small sample size. Likewise, we believe that similar trends will materialize in future studies with larger sample sizes.

The hypothesis for Aim 2 remains more ambiguous. As expected, we observe similar trends when ROIs were classified into location-based groups across treatment groups. Specifically, average BMD within the mandibular ROIs were greater than average BMD within facial ROIs, which was in turn greater than average BMD within cranial vault ROIs (Figure 2). These results are consistent with our hypothesis that regions of the skull that are subjected to "high" strain during loading caused by incision and mastication will exhibit higher BMD than regions that are exposed to "low" strain or "low" frequency of loading during chewing. As previously mentioned, to determine whether BMD varies within the craniofacial skeleton we performed pairwise Mann-Whitney U-Tests ($\alpha = 0.100$) for each treatment group. While results between treatment groups were not entirely consistent across all treatment groups (i.e. BMD in the TMJ was found to be significantly greater than the frontal bone in WT-hard mice, but not in OIM-hard mice), we do observe reoccurring trends (Figure 3). We attribute these non-significant values to our small sample size. Out of the 8 ROIs selected in this study, the mandibular symphysis and the region near the maxillary incisors had the highest average BMD for each treatment group, excluding OIM-soft mice. Conversely, the parietal bone exhibited the lowest average BMD across all treatment groups.

Numerous studies have shown that regions proximal to the bite point experience relatively higher strain as compared to regions that are more distal, such as the cranial vault (Hylander et al., 1992; Ravosa et al., 2010 and 2013; Herring & Teng, 2000). As previously discussed, increased biomechanical loading locally signals bone remodeling and increases BMD (Lanyon and Rubin, 1985; Ravosa et al., 2008; Iura et al., 2015). Although a few exceptions exist, our results are consistent with previous knowledge that regions subjected to high strain

during loading (such as the femur or the TMJ) generally have higher average BMD (Phillips et al., 2000; Ravosa et al., 2008).

Contrary to what we would expect, differences in BMD means between the TMJ and the frontal bone were not significant in OIM-soft mice. BMD within the frontal bone is likely increased due to the proximal attachment of the temporalis muscle in mice, which is classified as a muscle of mastication (Hiiemae & Houston, 1971). However, this does not explain why we do not see increased BMD in the parietal bone as compared to the frontal bone, as both of these bones are generally classified as "low" strain. (see Table 2). As noted by Herring & Teng, biomechanical strain varies within a given bone of the craniofacial skeleton (2000). More specifically, biomechanical strain is relatively low in the center of bones of the cranial vault, but with higher strain gradients near the cranial sutures (Herring & Teng, 2000). Therefore, it is likely that BMD within the frontal bone significantly differs from that of the parietal bone due to differences in strain at the relative location within the bone that data was collected (1 cm from suture). In short, we mainly attribute such exceptions to differences in strain at the specific location of data collection.

In Aim 3, we investigated whether an altered diet can increase BMD among mice of the same genotype. Differences in BMD values between WT-hard vs. WT-soft and OIM-hard vs. OIM-soft were not statistically significant. However, we did observe non-significant *trends* for higher BMD values in mice raised on hard diets than in mice of the same genotype raised on meal. Interestingly, BMD values at the mandibular symphysis and maxillary incisor appeared to be most affected by diet (Table B6; Figure 1). These results are not entirely surprising considering that mice chew or "gnaw" primarily using their proximal dentition. While genotype and collagen I status appears to be the primary determinant of BMD in this study, more research

and larger sample sizes are needed to definitively evaluate our hypothesis that animals raised on a hard diet will have higher BMD values than those raised on a soft diet (hypothesis 3A).

Similarly, our results do not provide conclusive support for hypothesis 3B, that $OIM^{-/-}$ mice raised on hard diets would have BMD values more similar to WT mice than to OIM-soft mice. Therefore, we cannot conclude that diet fully recovers the craniofacial BMD phenotype in OI mice. However, it is apparent from the trends observed in the Aim 3A analyses that diet plays *some* role in increasing BMD. Based on our small sample sizes and the aforementioned observed trends, we do believe further research is warranted to examine the role of biomechanical loading in the development and management of the OI phenotype.

SUMMARY AND CONCLUSIONS

Osteogenesis imperfecta is a rare genetic disease that results from mutations in type I collagen genes such as COL1A1 and COL1A2. This study compared mean BMD between $OIM^{-/-}$ mice, a rodent model for type III OI, and their WT littermates (Aim 1). Next, we compared BMD across different regions of the skull associated with “high” and “low” strain during routine masticatory function (Aim 2). Lastly, we tested whether increased biomechanical loading through altered diet can recover the craniofacial BMD phenotype in the OI model (Aim #3).

For Aim 1, we hypothesized that the craniofacial region of the OIM mouse model, compared to their diet-matched WT littermates, would have decreased mean BMD at each selected ROI. As expected, BMD was significantly higher in WT mice in all ROIs with the exception of the maxilla at M2. However, this may be related to technical issues involved in collecting data at this ROI. We observed a general correlation between type I collagen mutations and a decrease in BMD across the craniofacial skeleton in mice.

For Aim 2, we hypothesized that BMD varies within specific regions of the craniofacial skeleton. We expected that regions proximal to the bite point, which experience relatively "high" strain during mastication, would exhibit increased BMD. Although variation existed among the treatment groups, we observed a general trend for increased BMD in high strain regions (i.e. mandibular symphysis) and lower mean BMD in low strain regions (i.e. parietal bone).

For Aim 3, we hypothesized that increased masticatory loading in the $OIM^{-/-}$ model would affect biomineralization of the craniofacial region and help to recover the phenotype. Given our sample sizes, we cannot conclusively say that a mechanically challenging (pellet) diet resulted in increased BMD. However, at several ROIs (i.e. mandibular symphysis and maxillary incisor) we did observe trends for an increase in BMD in $OIM^{-/-}$ mice raised on a mechanically challenging diet compared to $OIM^{-/-}$ mice raised on soft, non-challenging diets. While genotype and type I collagen status appears to be the primary determinant of craniofacial BMD, our results suggest that biomechanical loading can influence bone development and homeostasis through genotype-environment interactions.

In conclusion, our findings show that a quantitative abnormality in type I collagen leads to decreased BMD within the craniofacial skeleton, thus decreasing mechanical integrity and masticatory function. In the future, studies should be completed with larger sample sizes in order to decipher significant differences in specific regions of the skull and whether increased masticatory loading can help to recover the OI phenotype. Furthermore, a continuation of the present study will longitudinally assess differences in craniofacial BMD throughout ontogeny in order to understand the development of the craniofacial phenotype in both pre- and post-weaning individuals.

CHAPTER III

INTERNSHIP EXPERIENCE

DESCRIPTION OF INTERNSHIP SITE AND EXPERIENCE

This internship practicum was performed at the University of North Texas Health Science Center in Fort Worth, TX under the direct supervision of Rachel A. Menegaz, PhD over the course of a year as a partial requirement for the degree of Master of Science. When I began the program in May 2018, I was first introduced to the project and previous studies performed by Dr. Menegaz. During this time, a general schedule was created for the upcoming year, which was maintained and adjusted throughout the project by both Dr. Menegaz and myself. From May 2018-August 2018, I met with Dr. Menegaz once a week to discuss previous studies pertaining to our current project. In September 2018, I presented my practicum background and research proposal as a "Work-in-Progress" (WIP) seminar in the Center for Anatomical Sciences.

The mice in our study were housed at Indiana University School of Medicine where micro-CT scans were performed and sent to us for further evaluation. Dr. Menegaz then trained me on the software (NRecon) that I would be using to reconstruct the CT images. Over the course of the Fall 2018 semester, I performed reconstructions for week 16 mice. During a few weeks in September and October 2018, I temporarily halted research to focus on classes such as Head & Neck Anatomy and Structural Neuroscience.

After my classes ended, Dr. Menegaz trained me on the software (Bruker CT-Analyser) we would use for measuring BMD. To measure BMD in our sample population, I first calibrated the software by using BMD phantoms, which were of a known density. Over the course of several months, I measured BMD at eight different regions of the skull in a total of 24 mice.

Once data acquisition was complete, I analyzed the data with Dr. Menegaz and discussed the implications of our results. These results have been reported in this practicum report. I also presented these results at the 2019 UNTHSC Research Appreciate Day (March 29, 2019) and at the 2019 annual meeting of the American Association of Anatomists at Experimental Biology in Orlando, Florida (April 9, 2019).

Throughout my experience, I was exposed to the ins and outs of conducting and presenting research. I was given the opportunity to learn from my PI and committee members, which has allowed me to develop critical thinking skills and to improve as a student.

JOURNAL SUMMARY

8/20/18

- Became familiar with reconstruction software, NRecon
- Practiced performing reconstructions

8/21/18

- Performed reconstructions for week 16 mice
    - Individuals 66, 80, 115, 116, 107, and 192
        - No issues

8/23/18

- Performed reconstructions for week 16 mice
    - Individuals 193, 86, 81, and 194
        - May need to repeat recon for individual 194; No other issues

8/27/18

- Performed reconstructions for week 16 mice
    - Individuals 152, 153, 154, 156, and 159
        - No issues

8/30/18

- Performed reconstructions for BMD phantoms
    - Phantom B (0.25 and 0.75 g. cm^{-3})
    - Phantom A (0.25 and 0.75 g. cm^{-3})

9/3/18

- Performed reconstructions for BMD phantoms

- Phantom E (0.25 and 0.75 g. cm^{-3})
- Phantom D (0.25 and 0.75 g. cm^{-3})
- Phantom C (0.25 and 0.75 g. cm^{-3})

9/5/18

- Practice WIP with Summer Ladd

9/6/18

- Practiced WIP presentation with Dr. Menegaz

9/7/18

- Gave WIP presentation to CAS department
- Lab meeting with PI
  - Discussed progress in project thus far
  - Discussed upcoming courses (Head & neck anatomy; Structural neuroscience)

10/23/18

- Performed calibrations for
  - Phantom A (18 μm^3 41 kV)
  - Phantom B (18 μm^3 55 kV; 445 μA)
  - Phantom C (18 μm^3 55kv; 455 μA)
  - Phantom D (18 μm^3 65 kV)
  - Phantom E (9 μm^3 50 kV)

- No issues

10/25/18

- Lab meeting with Dr. Menegaz
    - Discussed upcoming deadlines and goals for the Spring semester
    - Discussed Experimental Biology (EB) conference in Orlando, FL in April 2019

10/29/18

- Practiced forming ROIs (TMJ, M2 corpus, and mandibular symphysis) with Dr. Menegaz on Individual 67
- Began BMD precision study (n=3; trials=3). Started at left TMJ
    - Individual 67: 63195.185
    - Individual 80: 151232. 065
    - Individual 107: 89311.269
- Issues: Noticed BMD measurements were much higher than expected. Made note to repeat these measurements and check if calibrations were conducted correctly for next day

10/30/18

- Repeat trial #1 for individuals 67, 80, and 107 at left TMJ
    - No issues, numbers are more consistent with what we would expect
- Perform trial #1 for individuals 67, 80, and 107 at left M2 corpus and mandibular symphysis

- Made note that parsing out M2 corpus on CT images is difficult; no other issues

11/5/18

- Perform trial #2 for individuals 67, 80, and 107 at left TMJ, left M2 corpus, and mandibular symphysis
  - M2 corpus continues to be a difficult ROI to select; no other issues
- Begin writing abstract for EB Conference

11/6/18

- Perform trial #3 for individuals 67, 80, and 107 at left TMJ, left M2 corpus, and mandibular symphysis
  - M2 corpus continues to be a difficult ROI to select; no other issues

11/7/18

- PI ran stats for repeatability trial
  - All 3 ROIs had less than 3% standard deviation between trials
  - Will continue to collect data on remaining individuals

11/8/18

- Performed reconstructions using NRecon software for the following individuals
  - 73, 75, 76, 115, 192
    - No issues

- Perform BMD measurements for individual 66 on left TMJ, left M2 corpus, and mandibular symphysis
    - No issues

11/9/18

- Performed reconstructions for individual 79
    - No issues
- Performed BMD measurements for the following individuals at the left TMJ, left M2 corpus, and mandibular symphysis
    - 73, 75, 76, 79, 81, 86, and 194
        - M2 corpus continues to be difficult to delineate; no other issues

11/13/18

- Ran data on week 16 mice (n=12) for EB abstract with PI
    - Performed Kruskal-Wallis test (α=0.10) to compare BMD between genotypes
        - M2 corpus was only ROI that was statistically different between genotypes
        - Results may change once more measurements are added to sample

11/16/18

- Performed BMD measurements for individual 220 at left TMJ, left M2 corpus, and mandibular symphysis
    - No issues

11/19/18

- Performed BMD measurements for individuals 193 and 221 at left TMJ, left M2 corpus, and mandibular symphysis
    - No issues
- Filed intent to graduate with GSBS

11/20/18

- Performed BMD measurements for individuals 192 and 116 on left TMJ, left M2 corpus, and mandibular symphysis
    - No issues

11/26/18

- Meeting with PI
    - Discussed registering for Spring 2019 classes
    - Chose remaining 5 ROIs
    - PI helped identify these ROIs in mice CT scans
    - Set January 7th goal to complete introduction of practicum paper
- Performed BMD measurements on individual 152 on left TMJ, left M2 corpus, and mandibular symphysis
    - No issues

11/27/18

- Performed BMD measurements on the following individuals at the left TMJ, left M2 corpus, and mandibular symphysis
    - Individual 153, 154, 156, and 159
        - No issues

11/30/18

- Performed BMD measurements on the following individuals at the left TMJ, left M2 corpus, and mandibular symphysis
    - Individuals 71 and 72
        - No issues
- Began repeatability study (trial #1) for remaining neurocranium ROIs
    - ROIs: Parietal bone, frontal bone, and nasal bone
    - Individuals: 67, 80, and 107
        - No issues

12/4/18

- Performed trial #2 on the following individuals at the parietal bone, frontal bone, and nasal bone
    - Individuals 67, 80, and 107
        - No issues

12/6/18

- Performed trial #3 on the following individuals at the parietal bone, frontal bone, and nasal bone
    - Individuals 67, 80, and 107
        - No issues

12/10/18

- Initiated repeatability study (trial #1) on maxillary ROIs (M2 maxilla, Maxillary incisors)
    - Individuals 67, 80, and 107
        - M2 Maxilla on individual 67 and 107 was difficult to select; no other issues

12/11/18

- Performed trial #2 on M2 maxilla and maxillary incisor
    - Individuals 67, 80, and 107
        - M2 maxilla on individual 67 and 107 were difficult to select; no other issues
- Performed trial #3 on M2 maxilla and maxillary incisor
    - Individuals 67, 80, and 107
        - M2 maxilla on individual 80 was difficult to select; no other issues

12/18/18

- Ran statistics on repeatability trials using Excel

    - Of all 5 remaining ROIs, only the M2 maxilla on individual 80 had greater than 5% standard deviation
    - Sent this information to PI
- Repeated two trials on individual 80 at M2 maxilla
    - Results from repeated trials did not change results; will keep original results from trial #3

12/20/18

- Performed BMD measurements at remaining 5 ROIs
    - Individuals 67, 80, and 107
        - These measurements were re-done so that measurements were taken 1mm away from sutures (this was not done before)

12/22/18

- Performed BMD measurements at remaining 5 ROIs
    - Individual 66
        - No issues
- Performed BMD measurements at parietal and frontal bones
    - Individual 71
        - No issues

1/7/19

- Lab meeting with PI
    - Discussed strategy for RAD and EB conference
- Performed BMD measurements at nasal bone, M2 maxilla, and maxillary incisor
    - Individual 71
        - No issues
- Performed BMD measurements at parietal bone
    - Individuals 72, 73, 75, 76, 79, 81, 86, 115, 116, 152, 153, 154, 156, 159, 192, 193, 194, 220, and 221
        - Scans for individual 73 and 75 were tilted which made ROI selection difficult
        - Scans for individuals 192, 193, 194, 220, and 221 may need to be repeated; scans are very faint
        - No other issues

1/8/19

- Performed BMD measurements at frontal bone
    - Individuals 72, 73, 75, 76, 79, 81, 86, 115, 116, 152, 153, 154, 156, 159, 192, 193, 194, 220, and 221
        - Scans for individual 73 and 75 were tilted which made ROI selection difficult
        - Scans for individuals 192, 193, 194, 220, and 221 may need to be repeated; scans are very faint

        - No other issues

1/11/19

- Performed BMD measurements at nasal bone
    - Individuals 72, 73, 75, 76, 79, 81, 86, 115, 116, 152, 153, 154, 156, 159, 192, 193, 194, 220, and 221
        - Scans for individual 73 and 75 were tilted which made ROI selection difficult
        - Scans for individuals 192, 193, 194, 220, and 221 may need to be repeated; scans are very faint
        - No other issues
- Performed BMD measurements at M2 maxilla
    - Individuals 72, 73, 75, 76, 79, 81, 86, and 115
        - Scans for individual 73 and 75 were tilted which made ROI selection difficult
        - No other issues

1/14/19

- Performed BMD measurements at M2 maxilla
    - Individuals 116, 152, 153, 154, 156, 159, and 192
        - Scans for individuals 192 may need to be repeated; scan is faint
        - No other issues

1/15/19

- Performed BMD measurements at M2 maxilla
    - Individuals 193, 194, 220, and 221
        - Scans for individuals 193, 194, 220, and 221 may need to be repeated; scans are very faint
        - No other issues
- Performed BMD measurements at Maxillary incisor
    - Individuals 72, 73, 75, 76, 79, 81, 86, 115, 116, 152, 153, 154, 156, 159, 192, 193, 194, 220, and 221
        - Scans for individual 73 and 75 were tilted which made ROI selection difficult
        - Scans for individuals 192, 193, 194, 220, and 221 may need to be repeated; scans are very faint
        - No other issues
- Ran Mann-Whitney U-test with PI to compare genotypes
    - At α=0.05, reject null at mandibular symphysis and maxillary incisor
    - At α=0.10, reject null at mandibular symphysis, maxillary incisor, TMJ, and parietal
    - Outliers are all (except 1) from phantom E
        - Phantom E was used to calibrate individuals 80, 81, and 86

2/7/19

- Discussed week 16 data with PI

    - Individuals 80 and 81 were clearly outliers for TMJ, parietal bone, maxillary incisors, and mandibular symphysis → going to repeat measurements for these individuals

- Repeated BMD measurements for individuals 80 and 81 at TMJ, parietal bone, maxillary incisors, and mandibular symphysis
    - Repeated measurements did not change overall data trends
    - Will use original data
- Ran statistics for Aim #1
    - WT BMD > OIM BMD
- Ran statistics for Aim #3
    - Mixed results but trends are apparent

2/20/19

- Met with PI to run statistics for Aim #2
    - Mixed results but trends are apparent

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APPENDIX A

Table A1: Abbreviations used in this practicum report.

Abbreviation	Definition
BMD	Bone mineral density, or the volumetric density of calcium hydroxyapatite (CaHA) measured in g.cm-3
BMP	Bone morphometric protein
CaHa	Calcium hydroxyapatite
cm	Centimeters
COL1A1	Collagen, type 1, alpha-1(I) chain precursor, the genes that encode type I (α1) collagen.
COL1A2	Collagen, type 1, alpha-2(II) chain precursor, the genes that encode type I (α2) collagen.
Col1a1$^{Jrt/+}$	Combined osteogenesis imperfecta and Ehlers-Danlos syndrome mouse model
M2	Second molar
OI	Osteogenesis Imperfecta
OIM	Osteogenesis imperfecta murine; OIM$^{-/-}$ mice display severe (type III) osteogenesis imperfecta
OIM-soft	OIM$^{-/-}$ mice fed soft meal diet
OIM-hard	OIM$^{-/-}$ mice fed hard pellet diet
Pa	Pascals
WT	Wild-type or OIM$^{+/+}$
WT-soft	Wild-type mice fed soft meal diet
WT-hard	Wild-type mice fed hard pellet diet

APPENDIX B

Table B1 : WT vs. OIM Mann-Whitney p-values (Aim 1 results)

ROI	Mann-Whitney *p*-value (WT-soft vs. OIM-soft)	Mann-Whitney *p*-value (WT-hard vs. OIM-hard)
TMJ	0.400	0.005*
Mandibular corpus	0.700	0.016*
Mandibular symphysis	0.100*	0.011*
Nasal bone	0.400	0.011*
Maxilla at M2	0.200	0.313
Maxillary incisor	0.200	0.005*
Parietal bone	0.200	0.016*
Frontal bone	0.700	0.052*

* *Indicates significant difference (α=0.100)*

Table B2 : Mann-Whitney p-values between ROIs amongst OIM-soft mice (Aim 2 Results)

	TMJ	Mandibular M2	Mandibular Symphysis	Parietal Bone	Nasal Bone	Frontal Bone	M2 Maxilla	Maxillary Incisor
TMJ								
M2 Mandible	0.400							
Mandibular Symphysis	0.400	1.000						
Parietal Bone	0.200	0.100*	0.100*					
Nasal Bone	0.400	0.100*	0.100*	0.400				
Frontal Bone	0.700	0.400	0.400	0.400	0.700			
M2 Maxilla	0.400	0.100*	0.100*	0.400	1.000	0.700		
Maxillary Incisor	0.400	0.700	0.700	0.100*	0.100*	0.400	0.100*	

* *Indicates significant difference (α= 0.100)*

Table B3 : Mann-Whitney p-values between ROIs amongst OIM-hard mice (Aim 2 Results)

	TMJ	Mandibular M2	Mandibular Symphysis	Parietal Bone	Nasal Bone	Frontal Bone	M2 Maxilla	Maxillary Incisor
TMJ								
M2 Mandible	0.015*							
Mandibular Symphysis	0.004*	0.240						
Parietal Bone	0.015*	0.002*	0.002*					
Nasal Bone	0.180	0.002*	0.002*	0.310				
Frontal Bone	0.065	0.004*	0.004*	0.937	0.394			
M2 Maxilla	0.310	0.180	0.015*	0.004*	0.041*	0.026*		
Maxillary Incisor	0.065	0.818	0.310	0.002*	0.002*	0.009*	0.065	

** Indicates significant difference (α= 0.100)*

Table B4 : Mann-Whitney p-values between ROIs amongst WT-soft mice (Aim 2 Results)

	TMJ	Mandibular M2	Mandibular Symphysis	Parietal Bone	Nasal Bone	Frontal Bone	M2 Maxilla	Maxillary Incisor
TMJ								
M2 Mandible	0.100*							
Mandibular Symphysis	0.100*	0.100*						
Parietal Bone	0.100*	0.100*	0.100*					
Nasal Bone	0.100*	0.100*	0.100*	1.000				
Frontal Bone	0.400*	0.100*	0.100*	0.700	0.700			
M2 Maxilla	0.700*	0.100*	0.100*	0.100*	0.200	0.400		
Maxillary Incisor	0.100*	0.700	0.700	0.100*	0.100*	0.100*	0.100*	

** Indicates significant difference (α= 0.100)*

Table B5 : Mann-Whitney p-values between ROIs amongst WT-hard mice (Aim 2 Results)

	TMJ	**Mandibular M2**	**Mandibular Symphysis**	**Parietal Bone**	**Nasal Bone**	**Frontal Bone**	**Maxilla M2**	**Maxillary Incisor**
TMJ								
M2 Mandible	0.024*							
Mandibular Symphysis	0.000*	0.004*						
Parietal Bone	0.000*	0.000*	0.000*					
Nasal Bone	0.031*	0.001*	0.000*	0.136				
Frontal Bone	0.008*	0.000*	0.000*	0.113	0.796			
M2 Maxilla	0.000*	0.006*	0.000*	0.004*	0.161	0.222		
Maxillary Incisor	0.000*	0.019*	0.796	0.000*	0.000*	0.000*	0.000	

** Indicates significant difference (α= 0.100)*

Table B68 : Hard vs Soft Diet Mann-Whitney p-values (Aim 3A Results)

ROI	**Mann-Whitney *p*-value** **OIM-hard vs OIM-soft**	**Mann-Whitney *p*-value** **WT-hard vs WT-soft**
TMJ	0.905	0.573
Mandibular corpus	0.714	0.937
Mandibular symphysis	0.262	0.217
Nasal bone	0.905	0.573
Maxilla at M2	0.167	0.937
Maxillary incisor	0.905	0.287
Parietal bone	0.548	0.811
Frontal bone	0.548	0.937

Table B7 : BMD in OIM-Hard Mice versus WT Mice (Aim 3B Results)

ROI	**Mann-Whitney *p-value* OIM-hard vs. WT-soft**	**Mann-Whitney *p-value* OIM-soft vs. WT-hard**
TMJ	0.095*	0.049*
Mandibular corpus	0.095*	0.287
Mandibular symphysis	0.095*	0.007*
Nasal bone	0.381	0.217
Maxilla at M2	0.548	0.049*
Maxillary incisor	0.167	0.028*
Parietal bone	0.095*	0.049*
Frontal bone	0.167	0.371

* *Indicates significant difference (α= 0.100)*

APPENDIX C

Figure C1: Mouse Skull with ROIs