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Bone is much more than a simple, static organ. While its classical functions of mineral storage, organ protection, and locomotion still hold true, further studies into the skeleton have revealed a dynamic system capable of signaling, communication, remodeling, and responding to various stimuli. Overall skeletal health is critically important to maintaining high quality of life. Unfortunately, the skeleton is susceptible to various ailments such as fracture, cancer, and deterioration. Decreased bone mass and structural deterioration may lead to the silent disease of osteoporosis. With limited treatment options, focus has been shifted to prevention. The best method of osteoporosis prevention is to maximize peak bone mass during development. Nutrition plays an important role in developmental bone health and one nutrient is synonymous with skeletal health: milk. Supplying biologically relevant levels of bone specific nutrients, along with various bioactive compounds, milk should be nature's perfect food for increasing bone health. However, little evidence exists regarding milk's direct effect on bone development and strength. To fill this gap, we used pre-pubertal pigs as a model for children to test the effect of 750 mL of milk supplementation (n=6) vs. isocaloric maltodextrin solution (n=6) on the growing skeleton. Two experiments were conducted for 13 and 11 weeks and bones of the appendicular skeleton and mandible were collected and analyzed, for a total of 12 piglets per group. Short-term milk supplementation had no effect on bone growth, mineral density, or strength. However, piglets receiving milk had lower BMD of the mandible. The detected effect can be explained by the known anxiolytic effect of milk, that may have exerted behavioral effects, leading to lower mandibular activity and lower bone mineral density of the mandible. The major limitation of the present study was the short duration of the

treatment. Further research is still needed to determine long-term direct effects of milk consumption on the pre-pubertal and pubertal skeleton.

Effect of Milk Supplementation on Bone Development in Pre-Pubertal Pigs

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Brandon Batty, Author

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CHAPTER 1

LITERATURE REVIEW

1.1 Bone Formation

Bone formation occurs in two major modes, both of which require rapidly proliferating cells that reside in the marrow tissue and periosteal tissue. The two modes are intramembranous ossification and endochondral ossification. ***Intramembranous ossification*** is responsible for the formation of the flat bones of the skeleton. Neural crest-derived mesenchymal cells migrate to sites of future bones. This form of ossification does not use an intervening cartilage model and the stem cells differentiate directly into bone-committed osteoblasts [1]. The osteoblasts will secrete a collagen/proteoglycan matrix known as osteoid that is capable of binding to calcium salts and phosphorus to construct the mineralized bone matrix known as hydroxyapatite [2,3]. While osteoblasts are usually separated from the layer of calcification, some become trapped in the osteoid matrix and terminally differentiate into osteocytes, which are mature bone cells [4]. The transition to an osteocyte involves a dramatic shift from a cuboidal cell capable of matrix secretion and differentiation to a small bodied cell with projecting processes for cell to cell communication [4]. As the calcification process continues, bone spicules emanate from the osteocytes forming a network of connected cells that are visible as lacunae and canaliculi [4]. Directly adjacent to the developing bone is the periosteum. A fibrous sheet of connective tissue, the periosteum is tightly bound to the bone by thick fibers knows as Sharpeys' fibers [3]. The inner surface layer of cells will differentiate into osteoblasts and deposit osteoid. By this mechanism, many layers of bone are formed.

Endochondral ossification utilizes a cartilage intermediate that is subsequently replaced by bone. This process is responsible for the development of the long bones and the appendicular skeleton. First, mesenchymal stem cells become committed to the cartilage cell lineage [2]. These committed cells then condense to form compact nodules and differentiate into chondrocytes. Cells near the middle of the condensations will secrete a cartilage matrix consisting of type II, IX, and XI collagen, as well as proteoglycans [2,5]. Cells near the outer edge of the developing bone form the perichondrium and secrete type I collagen [5]. Next, the chondrocytes undergo a period of rapid proliferation that engages the linear growth of the developing bones. After rapid proliferation, the cells stop dividing, undergo hypertrophy, then apoptosis, and calcify [1]. In mammals, this type of ossification begins in the center of the bone and moves outward in both directions. This results in the formation of ossification centers located at both ends of the developing bone. The chondrocytes follow a pattern of hypertrophy, death, and subsequent mineralization that results in longitudinal bone growth [1,2]. Vascular incursion results in a source of perivascular osteoprogenitor cells and osteoclasts, both of which ensure correct skeletal development and aid in modeling and remodeling of bone throughout life [1,2].

1.2 Bone Microstructure

Bone structure can be divided into two categories: ***macrostructure***, which refers to the whole bone and its extrinsic properties such as shape, size, and mechanical strength [6,7], and ***microstructure,*** which refers to the individual elements that make up bone. Immediately

surrounding the outside of the bone is the periosteum. With the exception of articular surfaces and sesamoid bones, nearly every bony surface is covered in periosteum [1]. The ***periosteum*** contains two distinct layers: a fibrous outer layer and an inner layer with osteoblastic potential [1]. The outer layer can further be divided into two layers with the outer layer representing an inelastic, cell poor portion that is dense with collagenous matrix, and the inner layer representing a fibro-elastic layer with significant elasticity [1]. This inner layer of the periosteum is dense with osteogenic progenitor cells, osteoblasts, and fibroblasts that lie directly above the bone. [1,2]. Located under the periosteum is the ***cortical or compact bone***. Cortical bone is the dense, solid layer that surrounds the marrow cavity and is assembled by osteons which serve as its functional units [3,8]. Together, osteons and their vasculature make up the Haversian canals and system (large animals only). The walls of these canals are formed of lamellae, which are concentric layers of compact bone, and lacunae, which are small pores within the bone matrix eventually populated by an osteocyte [3,9]. Connecting the lacunae are microscopic channels called canaliculi. These fluid-filled channels serve as a transport system for canicular fluid and may serve to sense changes in load [10]. Sensing load releases anti-apoptotic factors and may result in increased bone formation [3,4,9,11]. These canals run parallel to each other and are connected perpendicularly by Volkmann's canals [12]. Lamellar bone is a highly-organized structure formed by remodeling woven bone, a haphazard arrangement of collagen fibers [2,13]. Lamellar bone is stronger and provides resistance to torsion forces on the bone. Further inside the bone, particularly toward the ends of long bone is a matrix called ***trabeculae or spongy bone***. This is the inner layer of bone and has a honeycomb-like structure [13] affording great elasticity and flexibility. Trabecular bone is the main load-bearing bone and its microarchitecture significantly influences bone strength [14].

1.3 Mesenchymal Stem Cell Features and Their Involvement in Bone Repair

Mesenchymal stem cells (**MSCs**) are fibroblast-like cells capable of adhering to plastic under normal culture conditions and having differentiation potential into osteoblasts, adipocytes, and chondroblasts *in vitro* while expressing in humans CD 105, CD90, CD73, and CD44 and lacking expression of CD 45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR [15,16]. Originally classified as marrow stromal cells, MSCs have been located throughout the body in bone marrow, adipose tissue and the umbilical cord [16]. Mesenchymal stem cells are multipotent cells with the capability of regenerating damaged tissue through different differentiation patterns stimulated by damages or other factors associated with growth and repair [16,17]. Different lineages of MSCs offer flexibility for stem cell therapy research as they each have different potentials for tissue regeneration [15,18,19]. Bone regeneration, fracture healing, and osteoporosis treatment with MSCs has been vigorously researched in recent decades [20]. When a tissue injury occurs, endogenous MSCs relocate to the injured area for participation in immunomodulation and tissue repair [17]. Granero-Molto and coworkers tracked MSCs by a bioluminescence technique expressing luciferase to a fracture site and showed that MSC improve fracture healing and biomechanical properties [21]. ***Bone morphogenic protein*** (**BMP**) has also been identified to be critical for normal fracture healing. BMP is produced by osteoprogenitor cells, MSCs, osteoblasts, and chondrocytes and reside in the extracellular matrix of bone. BMPs efficiently regulate the differentiation and proliferation of MSC to bone-forming osteoblasts. During fracture healing, BMP-2 is highly expressed and triggers a cascade of osteoblastic differentiation, chemotaxis, angiogenesis and controlled synthesis of extracellular matrix which

is critical for normal collagen deposition [21,22]. Different signaling molecules enhance MSC differentiation [23].

1.4 Role of the RANK/RANKL/OPG Cytokine System in Bone Physiology

Osteoclasts and their precursors express a receptor from the ***tumor necrosis factor*** (**TNF**) family known as ***receptor activator of nuclear factor-κB*** (**RANK**). RANK interacts with its ligand (**RANKL**), and activates ***TNF receptor associated factors*** (**TRAFs**) to stimulate osteoclastogenesis [23,24]. Together, RANK and TRAF regulate osteoclast activity through many signaling pathways that can modulate the rate and frequency of osteoclast maturation and bone resorption [25]. RANKL is a type II homotrimeric transmembrane protein that may be either secreted by osteoblasts to regulate bone resorption or is derived from the membrane as a result of alternative splicing or proteolytic cleavage [26]. By binding to its receptor, RANKL controls the differentiation, proliferation, and functionality of osteoclasts. In clinical trials where denosumab (AMG 162), a bisphosphonate antibody with high affinity for RANKL, is prescribed there is a significant reduction in RANKL binding to RANK with a consequent decrease in urinary markers for bone turnover due to up to 80 percent suppression of bone turnover [27]. In a clinical trial testing the efficacy of AMG 162 on post-menopausal women with low bone mass, an increase in lumbar spine and total hip bone mineral density (frequent location of fracture) and decrease in bone turnover was observed [28]. These results detail the importance of RANKL in bone resorption and potential mechanisms for treating osteoporosis therapeutically [28]. In contrast to therapeutic treatment of low bone mass, ***osteoprotegerin*** (**OPG**) levels could hold the

answer to inhibiting increased bone loss. Also known as osteoclastogenesis inhibitory factor, OPG is produced not only by osteoblasts, but also the heart, liver and spleen [23]. OPG is a natural inhibitor of osteoclastogenesis that acts as a decoy receptor of RANKL. When OPG and RANKL bind, RANKL is unable to bind to RANK on the immature osteoclast and stimulate the signaling cascade for the fusion of monocytes into mature osteoclasts [24–26]. Simonet and collaborators found that mice overexpressing OPG lacked osteoclasts, resulting in increased bone mass that led to osteopetrosis (higher than normal bone mineral density) [29,30]. In contrast, Bucay and colleagues used osteoprotegerin deficient mice and found they developed early onset osteoporosis. This data confirmed that OPG is an important regulator of bone development and accrual [81]. The RANKL/RANK/OPG cytokine system was one of the most important discoveries regarding bone health and metabolism that has been made in recent decades [106]. Inhibition of RANKL by OPG, or other synthetic factors, could be key is slowing the progression and diagnosis of osteoporosis.

1.5 Hormonal Regulation of Bone Metabolism

One of the most researched areas regarding bone health is post-menopausal women and estrogen deficiency. Osteoclasts express receptors for estrogen and inhibition of osteoclastogenesis is considered estrogens main function in protecting against bone loss [32–34]. When ***osteoclast-specific estrogen receptor alpha*** (**ERα**) was removed, there was a substantial decrease in trabecular bone mass. Moreover, estrogen blocks RANKL/M-CSF-induced activator protein-1-dependent transcription, and suppresses osteoclastic differentiation in response to RANKL

binding [34,35]. It has also been shown that estrogen suppresses the production of RANKL by osteoblasts and increases synthesis of its inhibitor, OPG [32]. Menopausal loss of estrogen is the main cause of imbalance in bone remodeling and increased bone turnover in women, resulting in osteoporosis [36]. ***Parathyroid hormone*** (**PTH**) is another hormone directly related to bone metabolism. Secreted by the parathyroid gland in response to low blood calcium, the 84-amino acid polypeptide regulates serum calcium homeostasis, renal phosphate and calcium reabsorption, and modulates bone turnover [37]. PTH receptors are located on osteoblasts and stimulate osteoclastogenesis by increasing secretion of RANKL and decreasing OPG release, thus increasing bone resorption and serum calcium levels [36–38]. As the body ages, renal function declines and calcium absorption across the intestinal lining slows. This increases PTH levels, inducing remodeling and resorption events, and eventually an imbalance in bone homeostasis, resulting in decreased bone mass and potentially osteoporosis [36]. In opposition of PTH is ***calcitonin*** (**CT**). It is well established that CT's effects on bone metabolism are through the inhibition of bone resorption [39,40]. CT binds to its receptor on osteoclasts and disrupts the ruffled border of osteoclasts and decreases osteoclast number, therefore limiting bone resorption [38]. CT also protects against bone resorption by decreasing the secretory activity of osteoclasts by altering Na^+-K^+-ATPase activity and reducing acid secretion [41]. ***Insulin like growth factor 1*** (**IGF-1**) has various effects on the skeleton. IGF-1 stimulates longitudinal bone growth, favors MSC differentiation into osteoblasts, and increases periosteal bone expansion [36,42]. Yakar and others found that IGF-1 increases bone strength, regulates size and shape, and plays a key role in adaptation to increased mechanical stress from total body growth [43]. In addition, it was confirmed by Zhang and coworkers that IGF-1 increases the rate of mineralization, which is of critical importance during the rapid bone growth of puberty [44]. Furthermore, IGF-1 has been

shown to stimulate RANKL secretion and osteoclastogenesis and maturation, suggesting IGF-1 as a critical regulator in bone turnover and remodeling [45,46].

1.6 Osteoporosis and Bone Mineral Density

Bone is often regarded as a simple, static tissue that supports our weight and stops growing when we reach our genetic potential for height. But in reality, bone is a dynamic tissue undergoing constant remodeling (concurrent action of bone formation and bone resorption) to adjust for physical pressures, physiological stress, and overall aging [11]. In addition to growing in length and volume, bone increases in overall density until the ***peak bone mass*** (PBM) is achieved. It is essential to reach the highest level of PBM as determined by our genetics to reduce risk of osteoporotic fracture later in life [47–49]. After PBM has been achieved, the rate of bone formation by osteoblasts is overtaken by bone resorption by osteoclasts. When this occurs in excess, bones become weak, brittle, and there is an increased risk of fracture due to a fall [11,48,50]. The clinical term for this decrease in bone mass and integrity is osteoporosis. Defined quantitively, osteoporosis is diagnosed when body bone mineral density is at least 2.5 standard deviations below a reference range of healthy individuals [51,52]. Considered a silent disease, osteoporosis affects approximately 35 million Americans and annually results in 17-20 billion U.S. dollars of healthcare costs due to osteoporosis related injuries [53,54]. Diagnosis of osteoporosis can be achieved through ***dual-energy x-ray absorptiometry*** (**DEXA**). DEXA is the gold standard for measuring bone mineral density and provides a simple, non-invasive method of pinpointing individuals that may be at an increased risk for fracture [53,55]. DEXA uses high and low-energy photons to differentiate between hard and soft tissue. Photons are absorbed by

the bone and remaining photons not absorbed are used to determine ***bone mineral density*** (**BMD**), higher absorption means greater BMD [55,56]. While DEXA is a very powerful tool in osteoporosis diagnosis, it is only a two-dimensional analysis of a three-dimensional problem [55]. Bone has a diverse role in the body that is somewhat paradoxical. It must provide support, protection, and serve as a reserve for calcium and other minerals. If bone is too dense, it will not have the flexibility to absorb mechanical load and will fail; if bone is too soft, it will surely break under stress and have no physical capacity to aid in locomotion. This creates a need to assess not only bone mineral density for prevention and diagnosis of osteoporosis, but also to evaluate bone quality and its microarchitecture [50,53]. Though requiring biopsy or excised bones, ***Micro-computed tomography*** (**μ-CT**) is a high-resolution imaging technology capable of building a three-dimensional model of bone microstructure [57]. Along with decreased BMD, deterioration of trabecular bone thickness and number will compromise bone integrity [11,58].

1.7 Bone Biomechanics

Bone composition and structure will determine the capability of a bone to resist fracture due to extreme mechanical stress that occurs during a fall [53]. Bone strength is defined as the maximum amount of force that can be tolerated by the skeleton before fracture or structural failure occurs [14]. The properties of the bone, in addition to its structure and mineral content, allow it to absorb load by elastic deformation while resisting permanent changes in shape [12,14,58]. Critical measurements of bone are used to determine its capacity to resist fracture: BMD, total bone volume, cortical width or thickness, and trabecular thickness, separation, and

number, are all variables considered when estimating load bearing [58]. Trabecular bone presents two levels of organization that allow for load transfer. At the macroscale, trabecular bone is composed of a lattice that forms the framework for a soft, cell-dense marrow that fills the intertrabecular area [59]. The microscale is organized in a way that allows for optimal weight distribution and forms new bone in response to increased stress [59]. A key attribute of bone is the ability to adapt to external stimuli by adding or removing bone. Mechanosensation by osteocytes is not fully understood, but it is accepted that the displacement of canicular fluid, deformed bone matrix, and graded electrical potentials are all identified by osteocytes and subsequent information is transferred to osteoblasts and/or osteoclasts, leading to adjustments or increases in bone mass and architecture [10–12,59–61].

Cortical bone mass is critical to overall bone strength and health as it comprises roughly 80 percent of the skeleton [58]. Increases in cortical porosity from excessive bone resorption and thinning of diaphyseal cortices are responsible for increased fracture risk and compromised bone integrity [11,14,58]. Bone geometry also plays an important role in the determination of bone strength. Cross-sectional area, shape, and size are frequently used to evaluate potential fracture risk [14]. Taes and coworkers found that men reaching peak bone mass with lower cortical area and thickness and lower trabecular density were more susceptible to fractures [62]. A method of directly determining strength is a three-point bending test; which uses a constant force applied to the bone until failure occurs [14,58]. From this, elasticity, stress, strain, and overall strength can easily be calculated. Unfortunately, this method is only suitable *post-mortem* and with animal models, making it impossible to be used as a means for diagnosis. Overall, the entire composition of bone mineral density, bone geometry, and bone microarchitecture illustrates the critical variables for evaluating the strength and fracture resistance of bone.

1.8 Effect of Milk on Bone Health

The consumption of milk has provided mankind with nutrients for thousands of years and the diverse nature and utilization of milk has given it extreme flexibility to yield hundreds of different dairy products [63]. Several key nutrients critical for bone health are available in one serving of milk (**Table 1**). The thought that consumption of dairy products improves bone health is longstanding and many studies have sought to prove this correlation [64]. Simply put, an increased amount of bioavailable calcium in the diet has been shown to improve bone development and health [63,65,66]. Calcium is often seen as the most important component of milk for bone health and adequate intake is recommended during adolescence to not only increase peak bone mass, but also help prevent osteoporosis later in life [67]. When mice were treated with low, adequate, and high levels of dietary calcium, it was confirmed that low dietary calcium negatively impacted bone strength, architecture, and overall peak bone mass [47]. However, the diverse blend of nutrients in milk have shown to increase bone health and development. Besides calcium, milk contains biologically relevant amounts of phosphorus, magnesium, potassium, proteins, and vitamin D (due to fortification) that all contribute to bone growth and mineral accrual [65,66,68,69]. Vitamin D is critical for maintaining calcium homeostasis and assists in the active transport of calcium across the intestinal lumen [67]. High levels of dietary vitamin D have resulted in greater tibial strength and greater trabecular bone volume [70]. It is theorized that high vitamin D levels achieved with fortified milk intake are suitable for higher bone mass and osteoporosis prevention [70]. Deficiency in vitamin D, both dietary and environmental, increases risk of bone disease and reduces bone strength and health

[67,68,71]. Dietary protein intake has also been associated with bone health. Bone health can be improved when three servings of *dairy* protein are included in the diet [66,72]. Dairy proteins are high in aromatic amino acids which increases synthesis of IGF-1**.** IGF-1 indirectly stimulates calcium absorption across the intestinal lumen by enhancing production of circulating calcitriol [48]. An increase in serum IGF-1 exerts growth effects on bone mass during adulthood [73]. Phosphorus, magnesium, and zinc are all critical to maintaining a healthy skeleton [69]. Adequate phosphorus is critical to form the matrix of hydroxyapatite, the mineralized portion of bone. Magnesium serves to regulate calcium homeostasis through its interaction with parathyroid hormone. Zinc, while found in small amounts, is an important cofactor for enzymes while forming part of the apatite portion of bone [67]. Interactions between these different milk components reduce bone resorption and increase bone formation, decreasing the rate of bone loss and risk of osteoporosis. However, it is important to consider that bone health is the results of multiple factors including a healthy lifestyle, as well as adequate nutrition to be considered optimal [68,69,74].

1.9 Bioactive Protein-Derived Compounds in Milk and Their Potential Effects on Bone

Milk is the first source of nutrients for mammals and contains critical amounts of nutrients vital for growth and development [65]. Uniquely for humans, milk consumption continues past the weaning stage and may be consumed throughout one's entire life in some cultures. Considered an important factor of a healthy diet, milk contains a diverse source of nutrients and various bioactive compounds [75,76]. The protein fraction of milk is receiving further attention

regarding its application for improving bone health [77]. The protein fraction of milk is comprised of two main portions: approximately 80 percent being casein proteins and 20 percent being whey proteins. Further classification of casein proteins have split the group into α-, β-, and k-caseins, while the whey portion contains the major constituents β-lactoglobulin, α-lactoalbumin, and various other minor proteins [75,78]. When ingested, milk proteins undergo enzymatic digestion that yield milk peptides. These milk-derived peptides have bioactive properties [78]. Such peptides have antimicrobial, immunomodulatory, antioxidant, bone forming, and various other properties that suggest ingestion of milk is not only beneficial nutritionally, but could also serve as a nutraceutical [75,76,78]. A study presented by Toba and coworkers (2001) followed 30 adult men receiving a beverage containing 300 mg of milk basic protein (MBP) daily for 16 days. Serum osteocalcin, a product of osteoblast activity, significantly increased in the treatment group, suggesting the increased activity of bone formation. In the same study, urinary cross-linked ***N-telopeptides of type-1 collagen*** (**NTX**), a measure of bone resorption, was significantly decreased. Thus, supplementation of milk basic protein increased bone formation and decreased bone resorption [79]. An *in vitro* study evaluating ***concentrated bovine milk whey active proteins*** (**CBP**) determined that CBPs can promote osteoblastic differentiation and mineralization under normal human culture conditions [80]. Mohanty and colleagues summarized the functional roles of milk-derived bioactive peptides [78]. Physiological digestion of the casein protein portion of milk yields various peptides with effects on bone metabolism [81]. ***Casein phosphopeptides*** (**CPPs**) may enhance calcium solubility and bone calcification. Overall, CPPs have the potential to directly affect osteoblast-like cell growth and result in greater deposition of calcium in bone extracellular matrix [81]. Using a rodent model for oral administration of lactoferrin, it was concluded that

lactoferrin can act as a preserving agent for bone mass and microarchitecture in ovariectomized mice. Lactoferrin can offset estrogen-dependent bone loss and deterioration [82]. Contributing to the research of milk as a functional food, bioactive peptides may be another critical piece in the puzzle against disease. Specifically, bioactive peptides from milk can protect against bone loss both *in vitro* and *in vivo* and their effect on the expression of genes regarding bone formation may confirm their role as a nutraceutical [78].

1.10 MicroRNA and Bone Development

Micro RNAs (**miRNA**) are small, non-coding segments of nucleotides that function in post-transcriptional gene silencing and target messenger RNA degradation, resulting in inhibition of expression of their target genes [83–85]. After transcription occurs by RNA polymerase II, the pri-miRNA are cleaved into precursor miRNA which are transported to the cytoplasm and processed into mature, single-stranded miRNA [85]. *In vitro* studies have tracked various miRNA and evaluated their effect on osteogenic differentiation and osteoblastic activity. In 2015, Li and colleagues investigated miR-26a. This specific miRNA has been negatively correlated with bone loss in osteoporotic mice with induced estrogen deficiency, conditions that simulate menopause [86]. Through cell transfection, it was discovered that overexpression of miR-26a rescued osteogenic function of ovariectomized-MSC and increased expression of ***alkaline phosphatase*** (***Alp***), an early osteogenic marker, and ***osteocalcin*** (***Ocn***), a marker of mineralization[86]. miRNA also regulate osteoblastic differentiation by targeting specific signaling pathways critical for bone development. For example, the Wnt pathway induces MSC

proliferation and drives differentiation toward the osteoblastic lineage [87,88]. Concurrent activation of the Wnt pathway and inhibition of ***glycogen synthase kinase 3*** (**GSK3**) increase osteoblastic differentiation. miR-346 directly binds to the 3' untranslated region of GSK3 β-mRNA and, in turn, activates the Wnt signaling pathway [85,88]. Numerous other miRNA can stimulate or inhibit osteogenic pathways; miR-378 enhances osteoblast differentiation in the presence of bone morphogenic protein-2 [85], miR-335-5p is highly expressed in osteoblasts and promotes osteogenesis through downregulation of Wnt antagonist, Dickkopf-related protein 1 (DKK1) [89], and high levels of miR-17-5p and miR106a inhibit mineralization and ALP activity [90]. Thus, miRNA can be a powerful regulator of various pathways that either inhibit or stimulate bone formation [85]. miRNA can target genes and transcription factors directly related to osteogenic differentiation and may regulate bone formation. ***Runt-related transcription factor 2*** (**Runx2**) is required for commitment and differentiation of osteoblasts [91]. This protein alone is regulated by at least 11 miRNA [92]. Precise control of Runx2 is required for proper bone formation and overexpression does not result in increased bone formation, but rather osteopenia as increased levels of Runx2 augment the secretion of RANKL [92]. Therefore, miRNA serve as a control mechanism for proper development of bone. ***Osterix*** (***Osx***) is a transcription factor that is required to complete maturation of pre-osteoblasts to osteoblasts [93]. miR-637, miR-125b, and miR-138 inhibit Osx and can negatively affect bone formation [85,92].

In addition to endogenous synthesis, miRNA can be derived from the diet. Specifically, extracellular vesicles, also known as exosomes, are highly enriched with miRNA and virtually all the circulating miRNA are enclosed in exosomes [94] The exosome provide protection for their precious cargo of miRNA, proteins, and lipids [95]. Exosomes are absorbed by intestinal and endothelial cells by endocytosis, and can accumulate in the peripheral tissues [95]. However,

the biological activity of miRNA derived from dietary exosomes is still controversial [95]. Baier and colleagues (2014) found that miRNA from bovine milk exosomes are absorbed and affect gene expression in peripheral immune cells in mice and feeding a diet devoid of cow's milk derived exosome decreased plasma concentration of miR-29b by 61 percent [84]. Oral delivery of bovine derived extracellular vesicles in mice was shown to increase overall osteocyte number and woven bone formation [96]. Conversely, Snow and colleagues concluded that ingestion of miRNA through the diet was not a frequent mechanism for horizontal transfer of miRNA to different organisms [97]. Overall, miRNAs and exosomes could be potent sources to transfer genetic information between animals; however, further research regarding uptake and horizontal transfer of dietary miRNA is still needed.

1.11 Pigs as an Experimental Model

Choosing an animal model adequate to mimic human physiology is paramount for any health-related experiment. Rodents have long been used as models of human biology and disease as they are easy to maintain and offer various genetic strains suitable for evaluating different experimental conditions [98]. However, using rodents as a model for bone development may be difficult when extrapolating results to humans. Critical differences including rapid rate of growth, differences in bone structure (rodents lack Haversian systems), and that mice are roughly 1/2500 the size of humans makes the justification for rodent models problematic [98,99]. Pigs are anatomically and physiologically more similar to humans and their comparable size is more reasonable for orthopedic studies [100]. In terms of relevance and application to human bone,

pigs have similar bone mineral density, morphology, remodeling rate, and have a rapid-remodeling (growth) phase and adult skeletal phase [99]. In addition, further knowledge is continually obtained regarding the gene expression of bone-related genes in porcine, making pigs not only relevant on the physical level but also on the molecular level [100]. Nutrient digestion and metabolism are similar between humans and swine which may prove useful for optimizing dietary strategies in the fight against obesity and other metabolically related diseases [101,102].

1.12 Conflicting Literature

Although milk has been proven beneficial for bone health, there is literature that suggests otherwise. Michaelsson and colleagues (2014) evaluated the relationship between high milk consumption (3+ glasses/day), mortality, and fracture risk. High intake was associated with increased mortality in men and women and with an increased risk of fracture in women. As this study is observational with the possibility of confounding variables, results are recommended to be interpreted cautiously [103]. Even so, results of a 22-year prospective follow-up of the Nurses' Health Study and the Health Professionals Follow-up study were evaluated for the risk of hip fracture at 50 years or older associated with their milk intake during the ages of 13-18. An additional glass of milk per day was associated with significantly higher hip fracture risk in men while milk consumption had no effect of hip fracture risk in women [104]. The latest systematic review and meta-analysis revealed inconsistent and unreliable evidence for the association of milk consumption and risk of hip fracture [105]. Further evaluation into the direct effect of milk on fracture risk, short and long term, is needed.

1.13 References

[1] J.R. Dwek, The periosteum: what is it, where is it, and what mimics it in its absence?, Skeletal Radiol. 39 (2010) 319–323. doi:10.1007/s00256-009-0849-9.

[2] S.F. Gilbert, Osteogenesis: The development of bones, (2000). https://www.ncbi.nlm.nih.gov/books/NBK10056/ (accessed November 3, 2017).

[3] B. Clarke, Normal bone anatomy and physiology, Clin. J. Am. Soc. Nephrol. CJASN. 3 (2008) S131–S139. doi:10.2215/CJN.04151206.

[4] M.B. Schaffler, W.-Y. Cheung, R. Majeska, O. Kennedy, Osteocytes: Master orchestrators of bone, Calcif. Tissue Int. 94 (2014) 5–24. doi:10.1007/s00223-013-9790-y.

[5] F. Long, D.M. Ornitz, Development of the endochondral skeleton, Cold Spring Harb. Perspect. Biol. 5 (2013) a008334. doi:10.1101/cshperspect.a008334.

[6] L. Dalle Carbonare, M.T. Valenti, F. Bertoldo, M. Zanatta, S. Zenari, G. Realdi, V. Lo Cascio, S. Giannini, Bone microarchitecture evaluated by histomorphometry, Micron. 36 (2005) 609–616. doi:10.1016/j.micron.2005.07.007.

[7] E. Donnelly, Methods for assessing bone quality: A Review, Clin. Orthop. 469 (2011) 2128–2138. doi:10.1007/s11999-010-1702-0.

[8] E. Seeman, Age- and menopause-related bone loss compromise cortical and trabecular microstructure, J. Gerontol. Ser. A. 68 (2013) 1218–1225. doi:10.1093/gerona/glt071.

[9] R.P. Main, Osteocytes and the bone lacunar-canalicular system: Insights into bone biology and skeletal function using bone tissue microstructure, Int. J. Paleopathol. 18 (2017) 44–46. doi:10.1016/j.ijpp.2017.05.002.

[10] U.T. Iwaniec, R.T. Turner, Influence of body weight on bone mass, architecture and turnover, J. Endocrinol. 230 (2016) R115–R130. doi:10.1530/JOE-16-0089.

[11] A.L. Boskey, R. Coleman, Aging and bone, J. Dent. Res. 89 (2010) 1333–1348. doi:10.1177/0022034510377791.

[12] G. Osterhoff, E.F. Morgan, S.J. Shefelbine, L. Karim, L.M. McNamara, P. Augat, Bone mechanical properties and changes with osteoporosis, Injury. 47 (2016) S11–S20. doi:10.1016/S0020-1383(16)47003-8.

[13] N. Little, B. Rogers, M. Flannery, Bone formation, remodelling and healing, Surg. Oxf. 29 (2011) 141–145. doi:10.1016/j.mpsur.2011.01.002.

[14] H. Fonseca, D. Moreira-Gonçalves, H.-J.A. Coriolano, J.A. Duarte, Bone quality: The determinants of bone strength and fragility, Sports Med. 44 (2014) 37–53. doi:10.1007/s40279-013-0100-7.

[15] L. Kong, L.-Z. Zheng, L. Qin, K.K.W. Ho, Role of mesenchymal stem cells in osteoarthritis treatment, J. Orthop. Transl. 9 (2017) 89–103. doi:10.1016/j.jot.2017.03.006.

[16] J. Kobolak, A. Dinnyes, A. Memic, A. Khademhosseini, A. Mobasheri, Mesenchymal stem cells: Identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche, Methods. 99 (2016) 62–68. doi:10.1016/j.ymeth.2015.09.016.

[17] W. Lin, L. Xu, S. Zwingenberger, E. Gibon, S.B. Goodman, G. Li, Mesenchymal stem cells homing to improve bone healing, J. Orthop. Transl. 9 (2017) 19–27. doi:10.1016/j.jot.2017.03.002.

[18] E. Yamachika, S. Iida, Bone regeneration from mesenchymal stem cells (MSCs) and compact bone-derived MSCs as an animal model, Jpn. Dent. Sci. Rev. 49 (2013) 35–44. doi:10.1016/j.jdsr.2012.11.003.

[19] M. Riminucci, C. Remoli, P.G. Robey, P. Bianco, Stem cells and bone diseases: New tools, new perspective, Bone. 70 (2015) 55–61. doi:10.1016/j.bone.2014.09.009.

[20] J. Phetfong, T. Sanvoranart, K. Nartprayut, N. Nimsanor, K. Seenprachawong, V. Prachayasittikul, A. Supokawej, Osteoporosis: the current status of mesenchymal stem cell-based therapy, Cell. Mol. Biol. Lett. 21 (2016) 12. doi:10.1186/s11658-016-0013-1.

[21] F. Granero-Moltó, J.A. Weis, M.I. Miga, B. Landis, T.J. Myers, L. O'Rear, L. Longobardi, E.D. Jansen, D.P. Mortlock, A. Spagnoli, Regenerative effects of transplanted mesenchymal stem cells in fracture healing, Stem Cells. 27 (2009) 1887–1898. doi:10.1002/stem.103.

[22] P. Garg, M.M. Mazur, A.C. Buck, M.E. Wandtke, J. Liu, N.A. Ebraheim, Prospective review of mesenchymal stem cells differentiation into osteoblasts, Orthop. Surg. 9 (2017) 13–19. doi:10.1111/os.12304.

[23] X. Chen, Z. Wang, N. Duan, G. Zhu, E.M. Schwarz, C. Xie, Osteoblast-osteoclast interactions, Connect. Tissue Res. 59 (2018) 99–107. doi:10.1080/03008207.2017.1290085.

[24] M. McClung, Role of RANKL inhibition in osteoporosis, Arthritis Res. Ther. 9 (2007) S3. doi:10.1186/ar2167.

[25] M.N. Weitzmann, The role of inflammatory cytokines, the RANKL/OPG Axis, and the immunoskeletal interface in physiological bone turnover and osteoporosis, Scientifica. (2013). doi:10.1155/2013/125705.

[26] B.F. Boyce, L. Xing, Biology of RANK, RANKL, and osteoprotegerin, Arthritis Res. Ther. 9 (2007) S1. doi:10.1186/ar2165.

[27] P.J. Bekker, D.L. Holloway, A.S. Rasmussen, R. Murphy, S.W. Martin, P.T. Leese, G.B. Holmes, C.R. Dunstan, A.M. DePaoli, A single-dose placebo-controlled study of AMG 162, a fully human monoclonal antibody to RANKL, in postmenopausal women, J. Bone Miner. Res. 19 (2004) 1059–1066. doi:10.1359/JBMR.040305.

[28] M.R. McClung, E.M. Lewiecki, S.B. Cohen, M.A. Bolognese, G.C. Woodson, A.H. Moffett, M. Peacock, P.D. Miller, S.N. Lederman, C.H. Chesnut, D. Lain, A.J. Kivitz, D.L. Holloway, C. Zhang, M.C. Peterson, P.J. Bekker, Denosumab in postmenopausal women with low bone mineral density, N. Engl. J. Med. 354 (2006) 821–831. doi:10.1056/NEJMoa044459.

[29] W.S. Simonet, D.L. Lacey, C.. Dunstan, M. Kelley, M.. Chang, R. Luthy, H.Q. Nguyen, Osteoprotegerin: A novel Secreted protein involved in the regulation of bone density - ScienceDirect, (1997). https://www.sciencedirect.com/science/article/pii/S0092867400802093?via%3Dihub (accessed March 13, 2018).

[30] Z. Stark, R. Savarirayan, Osteopetrosis, Orphanet J. Rare Dis. 4 (2009) 5. doi:10.1186/1750-1172-4-5.

[31] N. Bucay, I. Sarosi, C.R. Dunstan, S. Morony, J. Tarpley, C. Capparelli, S. Scully, H.L. Tan, W. Xu, D.L. Lacey, W.J. Boyle, W.S. Simonet, osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification, Genes Dev. 12 (1998) 1260–1268.

[32] S. Khosla, M.J. Oursler, D.G. Monroe, Estrogen and the skeleton, Trends Endocrinol. Metab. TEM. 23 (2012) 576–581. doi:10.1016/j.tem.2012.03.008.

[33] T. Nakamura, Y. Imai, T. Matsumoto, S. Sato, K. Takeuchi, K. Igarashi, Y. Harada, Y. Azuma, A. Krust, Y. Yamamoto, H. Nishina, S. Takeda, H. Takayanagi, D. Metzger, J. Kanno, K. Takaoka, T.J. Martin, P. Chambon, S. Kato, Estrogen Prevents Bone Loss via estrogen receptor α and induction of fas ligand in osteoclasts, Cell. 130 (2007) 811–823. doi:10.1016/j.cell.2007.07.025.

[34] S. Srivastava, G. Toraldo, M.N. Weitzmann, S. Cenci, F.P. Ross, R. Pacifici, Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-κB ligand (RANKL)-induced JNK activation, J. Biol. Chem. 276 (2001) 8836–8840. doi:10.1074/jbc.M010764200.

[35] N.K. Shevde, A.C. Bendixen, K.M. Dienger, J.W. Pike, Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7829–7834.

[36] J.S. Walsh, Normal bone physiology, remodelling and its hormonal regulation, Surg. Oxf. 36 (2018) 1–6. doi:10.1016/j.mpsur.2017.10.006.

[37] T. Bellido, V. Saini, P.D. Pajevic, Effects of PTH on osteocyte function, Bone. 54 (2013) 250–257. doi:10.1016/j.bone.2012.09.016.

[38] P.H.C. and E. Schipani, The roles of parathyroid hormone and calcitonin in bone remodeling: Prospects for novel therapeutics, Endocr. Metab. Immune Disord. - Drug Targets. (2006). http://www.eurekaselect.com/56669/article (accessed February 22, 2018).

[39] A.K. Huebner, J. Keller, P. Catala-Lehnen, S. Perkovic, T. Streichert, R.B. Emeson, M. Amling, T. Schinke, The role of calcitonin and α-calcitonin gene-related peptide in bone formation, Arch. Biochem. Biophys. 473 (2008) 210–217. doi:10.1016/j.abb.2008.02.013.

[40] A.G. Turner, F. Tjahyono, W.S.M. Chiu, J. Skinner, R. Sawyer, A.J. Moore, H.A. Morris, D.M. Findlay, J.D. Zajac, R.A. Davey, The role of the calcitonin receptor in protecting against induced hypercalcemia is mediated via its actions in osteoclasts to inhibit bone resorption, Bone. 48 (2011) 354–361. doi:10.1016/j.bone.2010.09.013.

[41] M. Zaidi, A.M. Inzerillo, B.S. Moonga, P.J.R. Bevis, C.L.-H. Huang, Forty years of calcitonin—where are we now? A tribute to the work of Iain Macintyre, FRS, Bone. 30 (2002) 655–663. doi:10.1016/S8756-3282(02)00688-9.

[42] E.M. Spencer, Modern Concepts of Insulin-Like Growth Factors, Elsevier Science Publishing Co., Inc., 1991.

[43] S. Yakar, E. Canalis, H. Sun, W. Mejia, Y. Kawashima, P. Nasser, H.-W. Courtland, V. Williams, M. Bouxsein, C. Rosen, K.J. Jepsen, Serum IGF-1 determines skeletal strength by regulating subperiosteal expansion and trait interactions, J. Bone Miner. Res. 24 (2009) 1481–1492. doi:10.1359/jbmr.090226.

[44] M. Zhang, S. Xuan, M.L. Bouxsein, D. von Stechow, N. Akeno, M.C. Faugere, H. Malluche, G. Zhao, C.J. Rosen, A. Efstratiadis, T.L. Clemens, Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization, J. Biol. Chem. 277 (2002) 44005–44012. doi:10.1074/jbc.M208265200.

[45] V. Locatelli, V.E. Bianchi, Effect of GH/IGF-1 on bone metabolism and osteoporsosis, Int. J. Endocrinol. (2014). doi:10.1155/2014/235060.

[46] S.F. Ahmed, C. Farquharson, The effect of GH and IGF1 on linear growth and skeletal development and their modulation by SOCS proteins, J. Endocrinol. 206 (2010) 249–259. doi:10.1677/JOE-10-0045.

[47] S. Viguet-Carrin, M. Hoppler, F. Membrez Scalfo, J. Vuichoud, M. Vigo, E.A. Offord, P. Ammann, Peak bone strength is influenced by calcium intake in growing rats, Bone. 68 (2014) 85–91. doi:10.1016/j.bone.2014.07.029.

[48] J.-P. Bonjour, T. Chevalley, S. Ferrari, R. Rizzoli, The importance and relevance of peak bone mass in the prevalence of osteoporosis, Salud Pública México. 51 (2009) s5–s17. doi:10.1590/S0036-36342009000700004.

[49] C.M. Weaver, C.M. Gordon, K.F. Janz, H.J. Kalkwarf, J.M. Lappe, R. Lewis, M. O'Karma, T.C. Wallace, B.S. Zemel, The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations, Osteoporos. Int. 27 (2016) 1281–1386. doi:10.1007/s00198-015-3440-3.

[50] Y. Yuan, X. Chen, L. Zhang, J. Wu, J. Guo, D. Zou, B. Chen, Z. Sun, C. Shen, J. Zou, The roles of exercise in bone remodeling and in prevention and treatment of osteoporosis, Prog. Biophys. Mol. Biol. 122 (2016) 122–130. doi:10.1016/j.pbiomolbio.2015.11.005.

[51] M.R. McClung, Prevention and management of osteoporosis, Best Pract. Res. Clin. Endocrinol. Metab. 17 (2003) 53–71. doi:10.1016/S1521-690X(02)00080-5.

[52] R. Eastell, Prevention and management of osteoporosis, Medicine (Baltimore). 45 (2017) 565–569. doi:10.1016/j.mpmed.2017.06.004.

[53] M.S. Nanes, C.B. Kallen, Osteoporosis, Semin. Nucl. Med. 44 (2014) 439–450. doi:10.1053/j.semnuclmed.2014.06.006.

[54] P. Sambrook, C. Cooper, Osteoporosis, The Lancet. 367 (2006) 2010–2018. doi:10.1016/S0140-6736(06)68891-0.

[55] Wilkinson J. Mark, Morris Richard M., Martin-Fernandez Miguel A., Pozo Jose M., Frangi Alejandro F., Maheson Marci, Yang Lang, Use of high resolution dual-energy x-ray absorptiometry-region free analysis (DXA-RFA) to detect local periprosthetic bone remodeling events, J. Orthop. Res. 33 (2015) 712–716. doi:10.1002/jor.22823.

[56] G.M. Blake, I. Fogelman, Technical principles of dual energy x-ray absorptiometry, Semin. Nucl. Med. 27 (1997) 210–228.

[57] A.J. Burghardt, T.M. Link, S. Majumdar, High-resolution computed tomography for clinical imaging of bone microarchitecture, Clin. Orthop. 469 (2011) 2179–2193. doi:10.1007/s11999-010-1766-x.

[58] M.L. Brandi, Microarchitecture, the key to bone quality, Rheumatology. 48 (2009) iv3–iv8. doi:10.1093/rheumatology/kep273.

[59] R. Oftadeh, M. Perez-Viloria, J.C. Villa-Camacho, A. Vaziri, A. Nazarian, Biomechanics and mechanobiology of trabecular bone: A Review, J. Biomech. Eng. 137 (2015) 0108021–01080215. doi:10.1115/1.4029176.

[60] C. Liu, Y. Zhao, W.-Y. Cheung, R. Gandhi, L. Wang, L. You, Effects of cyclic hydraulic pressure on osteocytes, Bone. 46 (2010) 1449–1456. doi:10.1016/j.bone.2010.02.006.

[61] J. Klein-Nulend, A.D. Bakker, R.G. Bacabac, A. Vatsa, S. Weinbaum, Mechanosensation and transduction in osteocytes, Bone. 54 (2013) 182–190. doi:10.1016/j.bone.2012.10.013.

[62] Taes Youri, Lapauw Bruno, Griet Vanbillemont, De Bacquer Dirk, Goemaere Stefan, Zmierczak Hans, Kaufman Jean-Marc, Prevalent fractures are related to cortical bone geometry in young healthy men at age of peak bone mass, J. Bone Miner. Res. 25 (2010) 1433–1440. doi:10.1002/jbmr.17.

[63] A. Caroli, A. Poli, D. Ricotta, G. Banfi, D. Cocchi, Invited review: Dairy intake and bone health: A viewpoint from the state of the art1, J. Dairy Sci. 94 (2011) 5249–5262. doi:10.3168/jds.2011-4578.

[64] H.J. Kalkwarf, Childhood and adolescent milk intake and adult bone health, Int. Congr. Ser. 1297 (2007) 39–49. doi:10.1016/j.ics.2006.08.008.

[65] P.C. Pereira, Milk nutritional composition and its role in human health, Nutrition. 30 (2014) 619–627. doi:10.1016/j.nut.2013.10.011.

[66] R. Rizzoli, Nutritional aspects of bone health, Best Pract. Res. Clin. Endocrinol. Metab. 28 (2014) 795–808. doi:10.1016/j.beem.2014.08.003.

[67] J.M. Hess, S.S. Jonnalagadda, J.L. Slavin, Dairy foods: Current evidence of their effects on bone, cardiometabolic, cognitive, and digestive health, Compr. Rev. Food Sci. Food Saf. 15 (2016) 251–268. doi:10.1111/1541-4337.12183.

[68] H.T. Viljakainen, Factors influencing bone mass accrual: focus on nutritional aspects, Proc. Nutr. Soc. 75 (2016) 415–419. doi:10.1017/S0029665116000252.

[69] T.K. Thorning, A. Raben, T. Tholstrup, S.S. Soedamah-Muthu, I. Givens, A. Astrup, Milk and dairy products: good or bad for human health? An assessment of the totality of scientific evidence, Food Nutr. Res. 60 (2016) 32527. doi:10.3402/fnr.v60.32527.

[70] L. Williamson, A. Hayes, E.D. Hanson, P. Pivonka, N.A. Sims, J.H. Gooi, High dose dietary vitamin D3 increases bone mass and strength in mice, Bone Rep. 6 (2017) 44–50. doi:10.1016/j.bonr.2017.02.001.

[71] H. McDevitt, S.F. Ahmed, Establishing good bone health in children, Paediatr. Child Health. 20 (2010) 83–87. doi:10.1016/j.paed.2009.09.012.

[72] D.F. Ma, W. Zheng, M. Ding, Y.M. Zhang, P.Y. Wang, Milk intake increases bone mineral content through inhibiting bone resorption: Meta-analysis of randomized controlled trials, E-SPEN J. 8 (2013) e1–e7. doi:10.1016/j.clnme.2012.10.005.

[73] R. Rizzoli, Dairy products, yogurts, and bone health, Am. J. Clin. Nutr. 99 (2014) 1256S-1262S. doi:10.3945/ajcn.113.073056.

[74] S. Sahni, K.M. Mangano, D.P. Kiel, K.L. Tucker, M.T. Hannan, Dairy intake is protective against bone loss in older vitamin d supplement users: The framingham study, J. Nutr. (2017) jn240390. doi:10.3945/jn.116.240390.

[75] S. Mills, R.P. Ross, C. Hill, G.F. Fitzgerald, C. Stanton, Milk intelligence: Mining milk for bioactive substances associated with human health, Int. Dairy J. 21 (2011) 377–401. doi:10.1016/j.idairyj.2010.12.011.

[76] C.-C. Hsieh, B. Hernández-Ledesma, S. Fernández-Tomé, V. Weinborn, D. Barile, J.M.L.N. de Moura Bell, Milk Proteins, Peptides, and Oligosaccharides: Effects against the 21st Century Disorders, BioMed Res. Int. 2015 (2015). doi:10.1155/2015/146840.

[77] Y. Morita, A. Ono-Ohmachi, S. Higurashi, H. Matsuyama, A. Serizawa, T. Takeya, The bone-strengthening activity of milk basic protein is not dependent on lactoferrin, Int. Dairy J. 27 (2012) 40–46. doi:10.1016/j.idairyj.2012.07.003.

[78] D.P. Mohanty, S. Mohapatra, S. Misra, P.S. Sahu, Milk derived bioactive peptides and their impact on human health – A review, Saudi J. Biol. Sci. 23 (2016) 577–583. doi:10.1016/j.sjbs.2015.06.005.

[79] Y. Toba, Y. Takada, Y. Matsuoka, Y. Morita, M. Motouri, T. Hirai, T. Suguri, S. Aoe, H. Kawakami, M. Kumegawa, A. Takeuchi, A. Itabashi, Milk basic protein promotes bone

formation and suppresses bone resorption in healthy adult men, Biosci. Biotechnol. Biochem. 65 (2001) 1353–1357. doi:10.1271/bbb.65.1353.

[80] K. Tsuji-naito, R.W. Jack, Concentrated bovine milk whey active proteins facilitate osteogenesis through activation of the JNK-ATF4 pathway, Biosci. Biotechnol. Biochem. 76 (2012) 1150–1154. doi:10.1271/bbb.110990.

[81] G. Tulipano, O. Bulgari, S. Chessa, A. Nardone, D. Cocchi, A. Caroli, Direct effects of casein phosphopeptides on growth and differentiation of in vitro cultured osteoblastic cells (MC3T3-E1), Regul. Pept. 160 (2010) 168–174. doi:10.1016/j.regpep.2009.11.018.

[82] H.Y. Guo, L. Jiang, S.A. Ibrahim, L. Zhang, H. Zhang, M. Zhang, F.Z. Ren, Orally administered lactoferrin preserves bone mass and microarchitecture in ovariectomized rats, J. Nutr. 139 (2009) 958–964. doi:10.3945/jn.108.100586.

[83] M. Nugent, MicroRNAs and Fracture Healing, Calcif. Tissue Int. 101 (2017) 355–361. doi:10.1007/s00223-017-0296-x.

[84] S.R. Baier, C. Nguyen, F. Xie, J.R. Wood, J. Zempleni, MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 Kidney Cell Cultures, and Mouse Livers, J. Nutr. 144 (2014) 1495–1500. doi:10.3945/jn.114.196436.

[85] C. Huang, J. Geng, S. Jiang, MicroRNAs in regulation of osteogenic differentiation of mesenchymal stem cells, Cell Tissue Res. 368 (2017) 229–238. doi:10.1007/s00441-016-2462-2.

[86] Y. Li, L. Fan, J. Hu, L. Zhang, L. Liao, S. Liu, D. Wu, P. Yang, L. Shen, J. Chen, Y. Jin, MiR-26a rescues bone regeneration deficiency of mesenchymal stem cells derived from osteoporotic mice, Mol. Ther. 23 (2015) 1349–1357. doi:10.1038/mt.2015.101.

[87] T. Kubota, T. Michigami, K. Ozono, Wnt signaling in bone, Clin. Pediatr. Endocrinol. 19 (2010) 49–56. doi:10.1297/cpe.19.49.

[88] V. Krishnan, H.U. Bryant, O.A. MacDougald, Regulation of bone mass by Wnt signaling, J. Clin. Invest. 116 (2006) 1202–1209. doi:10.1172/JCI28551.

[89] J. Zhang, Q. Tu, L.F. Bonewald, X. He, G. Stein, J. Lian, J. Chen, Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1, J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. 26 (2011). doi:10.1002/jbmr.377.

[90] H. Kang, A. Hata, The role of microRNAs in cell fate determination of mesenchymal stem cells : balancing adipogenesis and osteogenesis, BMB Rep. 48 (2015) 319–323. doi:10.5483/BMBRep.2015.48.6.206.

[91] L.D. Carbonare, G. Innamorati, M.T. Valenti, Transcription factor Runx2 and its application to bone tissue engineering, Stem Cell Rev. Rep. 8 (2012) 891–897. doi:10.1007/s12015-011-9337-4.

[92] J.B. Lian, G.S. Stein, A.J. van Wijnen, J.L. Stein, M.Q. Hassan, T. Gaur, Y. Zhang, MicroRNA control of bone formation and homeostasis, Nat. Rev. Endocrinol. 8 (2012) 212–227. doi:10.1038/nrendo.2011.234.

[93] K.M. Sinha, X. Zhou, Genetic and molecular control of Osterix in skeletal formation, J. Cell. Biochem. 114 (2013) 975–984. doi:10.1002/jcb.24439.

[94] A. Turchinovich, L. Weiz, A. Langheinz, B. Burwinkel, Characterization of extracellular circulating microRNA, Nucleic Acids Res. 39 (2011) 7223–7233. doi:10.1093/nar/gkr254.

[95] J. Zempleni, Milk exosomes: beyond dietary microRNAs, Genes Nutr. 12 (2017). doi:10.1186/s12263-017-0562-6.

[96] M.C. Oliveira, O.J. Arntz, E.N. Blaney Davidson, P.L.E.M. van Lent, M.I. Koenders, P.M. van der Kraan, W.B. van den Berg, A.V.M. Ferreira, F.A.J. van de Loo, Milk extracellular vesicles accelerate osteoblastogenesis but impair bone matrix formation, J. Nutr. Biochem. 30 (2016) 74–84. doi:10.1016/j.jnutbio.2015.11.017.

[97] J.W. Snow, A.E. Hale, S.K. Isaacs, A.L. Baggish, S.Y. Chan, Ineffective delivery of diet-derived microRNAs to recipient animal organisms, RNA Biol. 10 (2013) 1107–1116. doi:10.4161/rna.24909.

[98] R.L. Perlman, Mouse models of human disease, Evol. Med. Public Health. 2016 (2016) 170–176. doi:10.1093/emph/eow014.

[99] L.M. Wancket, Animal models for evaluation of bone implants and devices: Comparative bone structure and common model uses, Vet. Pathol. 52 (2015) 842–850. doi:10.1177/0300985815593124.

[100] F.L. Lüthje, K. Skovgaard, H.E. Jensen, L. Kruse Jensen, Pigs are useful for the molecular study of bone inflammation and regeneration in humans, Lab. Anim. (2018) 0023677218766391. doi:10.1177/0023677218766391.

[101] S.N. Heinritz, R. Mosenthin, E. Weiss, Use of pigs as a potential model for research into dietary modulation of the human gut microbiota, Nutr. Res. Rev. 26 (2013) 191–209. doi:10.1017/S0954422413000152.

[102] C. Perleberg, A. Kind, A. Schnieke, Genetically engineered pigs as models for human disease, Dis. Model. Mech. 11 (2018). doi:10.1242/dmm.030783.

[103] K. Michaëlsson, A. Wolk, S. Langenskiöld, S. Basu, E. Warensjö Lemming, H. Melhus, L. Byberg, Milk intake and risk of mortality and fractures in women and men: cohort studies, The BMJ. 349 (2014). doi:10.1136/bmj.g6015.

[104] D. Feskanich, H.A. Bischoff-Ferrari, L. Frazier, W.C. Willett, Milk consumption during teenage years and risk of hip fractures in older adults, JAMA Pediatr. 168 (2014) 54–60. doi:10.1001/jamapediatrics.2013.3821.

[105] S. Bian, J. Hu, K. Zhang, Y. Wang, M. Yu, J. Ma, Dairy product consumption and risk of hip fracture: a systematic review and meta-analysis, BMC Public Health. 18 (2018). doi:10.1186/s12889-018-5041-5.

[106] Food Composition Databases Show Foods -- Milk, reduced fat, fluid, 2% milkfat, with added vitamin A and vitamin D, (n.d.). https://ndb.nal.usda.gov/ndb/foods/show/72?fgcd=&manu=&lfacet=&format=&count=&max=50&offset=&sort=default&order=asc&qlookup=milk+2%25+fluid+cow&ds=Standard+Reference&qt=&qp=&qa=&qn=&q=&ing= (accessed March 13, 2018).

CHAPTER 2

EFFECT OF MILK SUPPLEMENTATION ON BONE DEVELOPMENT IN PRE-PUBERTAL PIGS

2.1 Introduction

An estimated 200 million people suffer from osteoporosis worldwide [1]. Each year in the U.S., approximately eight million people experience osteoporotic fractures, which results in 20 billion dollars in related healthcare costs [2,3]. Peak bone mass (**PBM**) can be described as the amount of bone mass attained during the growth period that peaks at the end of skeletal maturation [4]. During childhood and adolescence, a peak in bone mineral acquisition velocity (12 years old for girls, 14 years old for boys) results in accumulation of 39 percent of total body bone mineral and by 16-18 years of age, 95 percent of adult PBM has been accrued. Therefore, pre-puberty and puberty is critical for providing the necessary building blocks for proper bone development [4]. Achieving the largest relative PBM is pivotal for maintaining bone integrity. Higher PBM increases the amount of bone an individual can afford to lose without increasing risk of osteoporosis when bone resorption overtakes bone formation [5–7]. Therefore, preventing osteoporosis may be achieved by maximizing PBM and slowing the loss of bone after peak has been attained [4,8,9].

Non-modifiable factors such as age, gender, and genetics work in tandem with modifiable factors such as lifestyle, activity level, and nutrition to optimize bone health [10,11]. Among nutrients, milk has been considered the perfect food for bone development [12]. Despite this, the economic research service of the United States Department of Agriculture (USDA) has reported a decrease in milk consumption by almost 38% since 1975 [13].

Children (males and females age 5-14) are more susceptible to fractures than young or mature adults as peak in fracture risk occurs just before the onset of puberty [4]. Consumption of milk and other dairy products during childhood and adolescence is positively associated with total bone area, cortical area, and cortical content [14]. When children sustain a forearm injury, those with higher bone mineral density (**BMD**) have increased resistance to fracture [4,15]. In contrast, pre-pubertal children with bovine milk allergy displayed lower BMD despite normal growth, suggesting milk avoidance during growth is detrimental to mineral accrual and may affect PBM [8]. Unfortunately, these observational studies make it difficult to demonstrate the direct effect of milk consumption on bone health. According to the most recent review of the literature, dairy products improve total-body bone mineral content (**BMC**) in children only when baseline calcium intake is below 750 mg/day [16]. However, despite multiple randomized-controlled trials, there is still no direct evidence for an effect of dairy products in reducing fracture risk in children [16].

On any given day, 50% of adults and 60% of youth (ages 2-19) will consume a soda or sport drink, known as sugar-sweetened beverages (**SSBs**). After a spike in consumption from 1977 to 2001 [17], SSB consumption has seen a significant decline in consumption trends in the last 15 years [18]. Despite this decline, SSB consumption still contributes to roughly 7% of the total daily calorie intake among youth and adults [19]. Consuming SSBs may have deleterious effects on bone health [20]. Whiting and collaborators (2001) evaluated the average beverage type intake around peak bone mineral accrual and found that low nutrient-dense beverage choices exerted negative effects on total bone mass [21]. Rats provided with SSBs had significant decrease in femur BMD [22], while high-glucose beverages suppressed the differentiation and proliferation of osteoblasts, resulting in a decreased rate of bone formation [23]. Long-term

consumption of soft drinks appears to also have a catabolic effect on bone in both boys and girls [24]. These results suggest that consuming SSBs rather than nutrient-rich beverages may be detrimental to bone health during growth. However, to our knowledge, no work has been performed to evaluate the direct effects of milk consumption versus SSBs consumption on the growing skeleton.

Based on the above data, our hypothesis was that milk consumption increases BMC, BMD, and bone strength when compared to SSB consumption. Conventionally, rodents have been used to evaluate human disease due to ease of genetic manipulation and handling [25], including studies on bone growth [26–28]. However, their small size and absence of Harversian systems are weaknesses in rodents as an animal model to study effects of nutrients on bone growth in children [28]. The skeleton of growing piglets are more similar to humans than rodents, making them a more suitable animal model for use [29,30]. Therefore, to test our hypothesis, we used growing pre-pubertal piglets to determine the effect of supplementing milk or SSBs on 1) growth rates and bone mineral density, 2) biomechanical strength of bone, and 3) physical bone characteristics.

2.2 Materials and Methods

2.2.1 Study Design

Experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees of Oregon State University (protocol # 4691). Briefly, two experiments were performed. In Experiment 1, twelve 5-week-old Yorkshire piglets, 8 males and 4 females from

two litters, and in Experiment 2, twelve 8-9-week-old Duroc-Berkshire male piglets from 4 litters were used. Animals were blocked by litter and sex and randomly assigned to receive 750 mL of whole fresh cow milk collected from the bulk milk tank of the Oregon State University Dairy Center (4.8% fat, 3.6% protein, 4.8% lactose) or an isocaloric equivalent of maltodextrin solution (500 mL) (cat# 007-345-0341, Honeyville, USA). Piglets were treated for 13 or 11 weeks in Experiment 1 and 2, respectively, and euthanized using captive bolt (Experiment 1) and barbiturate injection (Experiment 2) at the end of the study.

2.2.2 *In-Vivo* Bone growth

Ultrasonography was used to track growth of the right femur and right last rib using an Mindray M5 Ultrasound (#MR-01001893, Shenzhen Mindray Bio-Medical Electronics C., LTD., Nanshan, Shenzhen, China) with a variable frequency 5-8 MHz micro-convex transducer. For Experiment 1 and 2, ultrasound measurements were taken at four (6, 10, 14, and 18 weeks of age) and three timepoints (8, 12, and 18 weeks of age), respectively. Ultrasounds were measured using ImageJ (National Institutes of Health, USA, v 1.51j8). First, the measurement scale was calibrated from the measuring depth of the image. Next, a straight line was drawn across the bone to measure bone diameter. Images were analyzed by an individual blinded to treatment.

2.2.3 Sample Collection and Preservation

Immediately after euthanasia, the following samples were collected. From Experiment 1: left femur, last left rib, and left and right tibia/fibula. From Experiment 2: left femur, last left rib, left

radius, left humerus, and left mandible. Bones to be used for DEXA analysis were stripped of all adherent tissue, fixed in 10% buffered formalin (cat# CA71007-348, VWR, Radnor, PA, USA) for 2 months and stored in 70% ethanol at room temperature until analysis. Bones for biomechanical evaluation had adherent tissue left on and were wrapped in gauze soaked with serological saline solution (lot#A130607-1, VEDCO, St. Joseph, MO, USA)*)* and stored at -20^{o} C until evaluation.

2.2.4 DEXA and Femur/Mandible Volume

Excised bones were scanned by DEXA (Hologic Discovery QDR series) at the Samaritan Athletic Medicine Center in Corvallis, Oregon. Calibration was performed on a lumbar spine phantom prior to scanning the samples. Bones were positioned parallel to scanner table and scanned using the Rat Whole Body setting at high definition. Parameters measured included BMC (g), bone area (cm^2), and BMD (g/cm^2). Regions of interest of the mandible (body, ramus, teeth), femur (proximal epiphysis, diaphysis, distal epiphysis), and humerus (proximal epiphysis, diaphysis, distal epiphysis) were further evaluated for the same parameters. All scans were performed by the same person to minimize operator variability. Femur and mandible volume were measured via water displacement. A 2000 milliliter (mL) graduated cylinder was filled with 1000-mL of distilled water and each femur was carefully placed inside and the new volume was recorded. Femur volume was then calculated by subtracting 1000-mL from the new volume reading and rounded to the nearest mL. To measure the volume of the mandible, a one-half inch hole was drilled into the side of a 1.5 L plastic container and a plastic tube was inserted and heated to create an airtight seal. The plastic tube drained into a 200-mL graduated cylinder.

Water was added to the container and allowed to reach equilibrium. Known volumes of water were added to create a standard curve for volume measurement. After this calibration, each mandible was carefully submerged and volume was recorded to the nearest mL (**Figure 1)**.

2.2.5 Three-Point Bending Evaluation

Prior to evaluation, frozen femurs were thawed at 4^{o} C for 24 hours. All adherent tissue was subsequently removed, and bones were re-wrapped in gauze soaked with saline to prevent drying. The femoral diameter was measured using a 0-150 mm range Swiss Precision Instruments (SPI) electronic caliper (cat#14-792-6, Garden Grove, CA, USA) with 0.01 mm display resolution and ± .03mm accuracy. An Instron 5900 series high-capacity testing system (100kN) dual-column floor model in the Instron Calibration Laboratory in the Department of Mechanical, Industrial, and Materials Engineering at Oregon State University was used for evaluation. Prior to testing, each femur was warmed to room temperature for one-hour and the midpoint of the diaphysis was determined. Each femur was supported by an adjustable fulcrum and adjusted to the active length of the bone and the load point was set in the middle of the diaphysis. The two supports and load applicator were rounded to avoid shearing. The anterior surface (flattest side) of the bone was facing downward and load was applied at 10 mm/min. Load was applied until bone fracture and corresponding force curves were generated and evaluated using Bluehill 3 Testing software (Instron, USA). Parameters measured were ultimate bending strength (N), experimental deformation at fracture (mm), and overall bone stiffness (N/mm).

2.2.6 Cortical Thickness Evaluation

After three-point bending evaluation, each femur was cut using a Dremel moto-tool model 395 type 5 variable speed saw equipped with cut-off wheels No. 409 at 30,000 rpm. Three locations were selected for evaluation: fracture point, proximal femur, and distal femur. Thickness measurements of the dorsal, ventral, lateral, and medial diaphysis were taken with the same SPI electronic caliper mentioned above. A picture was taken using an iPhone 7 with 16-megapixel camera of the midpoint and the cross-sectional area, medullary cavity area, cortical area, and average cortical thickness were measured using ImageJ. The measurement scale was calibrated from the ruler held against the bone during the picture. Next, using the tracing feature of ImageJ, the endosteal surface and periosteal surface were traced and measured to obtain medullary cavity area and cross-sectional area, respectively. Cortical area was obtained via subtraction (cross-sectional area – medullary cavity area = cortical area). A straight line was drawn to measure cortical thickness in 10 equidistant places along the cross-section to measure average cortical thickness (**Figure 2**).

2.2.7 Enamel Thickness

The fourth premolar was removed from the left-half of the mandible (using the Dremel mentioned above) and cut along a sagittal plane to expose the enamel. Pictures were taken of the tooth using an iPhone 7 with 16-megapixel camera and enamel thickness was evaluated using ImageJ. The measurement scale was calibrated from the ruler held against the tooth. Next, a straight line was drawn across the enamel in 10 equidistant places apart to measure average

enamel thickness (**Figure 3**). The thickness of the occlusal surface of each tooth was measured in the same manner.

2.2.8 Statistical Analysis

Statistical analysis was performed using R Studio (Version 3.4.3). Ultrasound measurements were analyzed with repeated measures ANOVA using the "stats" package in R. All other data were analyzed using a liner mixed effects model with fixed effects of treatment (and sex for experiment 1) and random effect of pig. Relationships between variables were made using Pearson correlations. This was accomplished with the "lme" function in R. Statistical significance was set at $p < 0.05$ and statistical tendencies at $p < 0.10$ and all data are expressed as mean ± SE.

2.3 Results and Discussion

2.3.1 *In-Vivo* Bone growth

The overall growth of the rib and femur had a steady but significant increase over time with no differences between treatments at any timepoint measured (**Figure 4**). The increases observed through time is consistent with growth during childhood in humans being relatively steady [31]. In the pre-adolescent years, human bone accrual occurs at a rate of approximately 5-6 cm per year (length) [7]. However, once the adolescent growth spurt occurs, peak growth velocity can reach approximately 8.5 and 10.4 cm per year for girls and boys, respectively [32].

The lack of differences observed in bone growth between piglets receiving milk or maltodextrin is consistent to data generated from the NHANES 1999-2002 survey that revealed no effect of milk consumption on height of 5-11-year-old children. In contrast, milk consumption was a predictor of height and bone health in adolescent children aged 12-18 years [33]. Similarly, Chevalley and colleagues (2005) found that bone size and standing height did not differ in children with baseline calcium consumption of ~750mg/d supplemented with milk-calcium enriched foods versus children receiving a placebo [34].

2.3.2 DEXA

We found no treatment effect on any measurement in Experiment 1 (**Table 2**). However, sex differences were evident in Experiment 1 (**Table 3**). Femur BMC was larger ($p = 0.025$) in males compared to females, as was femur BMD ($p = 0.016$), while femur area tended ($p = 0.10$) to be larger in males compared to females (**Figure 5)**. Further evaluation into specific regions of interest of the femur revealed that the proximal epiphyseal area, BMC, and BMD as well as the diaphyseal area, BMC, and BMD were significantly larger is males compared to females (**Table 3**). Due to high activity levels, pre-pubertal children are at the highest risk for fracture among population sub-groups [4]. Post-menopausal women, however, are ranked second for fracture risk and first for osteoporotic fracture risk [4]. Menopause is associated with a natural reduction in estrogen production, which leads to increased deterioration of bone mass and microarchitecture [11,35]. Similar to pubertal growth results reported by Yilmaz and collaborators (2005) in children [36], the data in the current study demonstrates that female pigs were predisposed to lower BMC and BMD before puberty..

We found no significant difference in the area, BMC, and BMD for the radius, femur, or humerus between the two treatment groups in male piglets in Experiment 2 (**Table 4)**. Unexpectedly, BMC and BMD of the mandible were significantly smaller ($p < 0.05$) in the group receiving milk when compared to the maltodextrin group. This warranted further evaluation of BMC and BMD and three points of the mandible (body, ramus, teeth) were determined to be regions of interest to understand what part of the mandible was responsible for the difference in BMC and BMD. The area, BMC, and BMD of the ramus had no difference between the two groups. The area of the mandibular body had no difference although the BMD was lower ($p < 0.05$) and the BMC tended to be lower ($p = 0.09$) in the group receiving milk compared to maltodextrin solution. Conversely, the area and the BMC of the teeth tended to be higher in the group receiving milk ($p = 0.08$ and $p = 0.09$, respectively), but not the BMD ($p = .79$), suggesting the increased mineral content of the teeth was due to the increased area.

The difference in bone mineral density of the mandible between the two groups suggests that there was a difference in overall mandibular activity. Several studies have examined masticatory hypofunction and mandible bone mineral density. In 2011, Tsai and collaborators injected botulinum toxin into the temporalis and masseter muscles of 30-day-old Long-Evans rats and found decreased mandibular bone mineral density as a result of disuse [37]. Kunii and colleagues also found that occlusal (contact between the upper and lower teeth during chewing or rest) hypofunction decreased total mandibular BMD of both trabecular and cortical bone, but was recovered when mandibular function was restored [38]. When considering the results of our experiment, the above data suggest that the group receiving milk likely had lower mandibular activity compared to the control group. To confirm this observation, the excised premolar was evaluated for wear patterns and occlusal thickness. The enamel thickness of the occlusal surface

was significantly thicker in the group receiving milk (**Table 4).** In addition, visual inspection of the teeth revealed increased wear and flattened areas of the tooth in the group receiving the maltodextrin solution (**Figure 6).** Milk is known to contain various bioactive peptides and one , α-casozepine, has anxiolytic effects similar to benzodiazepines [39–43]. Besides α-casozepine, milk contains other anxiolytic compounds that can reduce stress and anxiety [44–47] . Increased stress and anxiety can induce teeth-grinding (bruxism) that results in higher jaw activity [48]. Based on the above evidence, it is possible that the low BMD of the mandible of piglets receiving milk was due to a calming effect of the milk, resulting in lower mandibular activity.

2.3.3 Three-Point Bending Evaluation, Cortical Thickness, Cortical Area, Cross-Sectional Area, Medullary Cavity Area, and Femur Properties

Femurs were preserved for biomechanical evaluation for Experiment 2 only. There were no significant differences in ultimate bending strength, experimental deformation, or overall bone stiffness between the two groups (**Table 5**). Femur diameter, volume, and length, along with body weight, were also measured as these extrinsic properties affect bone strength and no significant differences were found between groups (**Table 5**). There were no significant correlations of ultimate ending strength with body weight, femur diameter, volume, area, BMC, or BMD. Experimental deformation at fracture was negatively correlated with femur diameter (r^2= -0.75), area (r^2= -0.69), and BMC (r^2= -0.62) and had tendencies to be negatively correlated with femur length (r^2= -0.56). In contrast, body weight was positively correlated with femur length in Experiment 1 (r^2= 0.77), while body weight was positively correlated with femur length (r^2= 0.58), area (r^2= 0.58), and BMC (r^2= 0.60) in Experiment 2 (**Table 6).** No significant

treatment differences in any of the measurements (average cortical thickness, cross-sectional area, cortical area, and medullary cavity area) were detected in Experiment 1 (**Table 5)**. Comparing the two sexes, the ventral cortical thickness of the proximal femur was significantly larger in males ($p < 0.05$) while the medullary cavity area tended to be larger in females ($p < 0.10$). In Experiment 2, the medial cortical thickness of the femur at the fracture point was smaller in milk compared to the control group ($p = 0.03$) while the lateral thickness at the fracture point was larger in the group receiving milk ($p = 0.05$). The medial cortical thickness of the distal femur tended to be higher in the group receiving milk ($p = 0.10$) **(Figure 7).** There were no differences in medullary cavity area, cross-sectional area, or cortical area between the two groups (**Table 5)**.

To our knowledge, this was the first study to evaluate the direct effect of whole and unfortified milk consumption on physical bone strength in pre-pubertal pigs. Bone strength is determined by various properties such as bone geometry, microarchitecture, and tissue-level properties [49]. Though we found no differences in mechanical strength or bone level properties between groups, an interesting observation was the greater area of the medullary cavity of the female pigs in Experiment 1. After menopause, females lose the bone-protective effect of estrogen and lose bone more rapidly than men [50]. Specifically, women experience an imbalance in bone turnover characterized by endosteal erosion, cortical thinning, and medullary cavity expansion which increases risk of fracture [50,51]. While we found no significant difference in average cortical thickness between treatments, males on average had thicker cortices than females (4.94 ± 0.11 mm vs. 4.60 ± 0.23 mm; $p = .05$). We found no significant correlations between femoral BMD and strength which contradicts work by Nielsen and colleagues (2007) who found that BMD and biomechanical strength in pigs were closely related [52]. However, it was not surprising that

bone strength was not significantly different between groups as BMC, BMD, and other femoral properties were not different. In our study, negative correlations of experimental deformation with femur diameter, area, BMC, and length suggests that the linear growth experienced during childhood and adolescence may increase risk of fracture from a fall or physical activity as the capability of bone to resist fracture by deforming is reduced [4]. The differences in cortical thickness measurements of the medial and lateral femur between treatment groups awaits an explanation. This could be due to the differences in age, breed, and duration of each trial. Final body weight was positively associated with length and mineral content of the femur. These data are in line with the *mechanostat hypothesis*, which states that increases in weight and force applied to bone can increase and adjust bone structure to adapt to the increase in mechanical loads [53–55]. While it is beyond the scope of our study, these data suggest that increases in mechanical forces, such as those experienced through physical activity, have a positive effect on bone mineral accrual and development

2.3.4 Enamel Thickness

The fourth premolar was only harvested for animals in Experiment 2. There were no differences in enamel thickness between the two treatment groups (1.15 ± 0.05 mm vs 1.16 ± 0.05; $p = 0.97$). Soft drinks have become the beverage of choice for most children [56]. Although we did not find any short-term effect of SSB consumption, enamel erosion from SSBs could have detrimental effects on children's teeth [57].

2.3.5 Calcium and Maltodextrin Intake

There were no differences in estimated calcium intake between treatment groups in Experiment 1 (10.52 ± 3.02 g/day vs. 9.00 ± 2.43 g/day, p = 0.28) or Experiment 2 (14.80 ± 2.85 g/day vs. 14.30 ± 1.07 g/day, p = 0.66). The piglets were provided an *ad libitum* grower ration containing 0.5% percent calcium and both groups achieved their National Research Council (NRC) recommended calcium requirements prior to any supplementation [58]. As the USDA recommended amount of milk for humans provides ~900 mg of calcium, this level of milk supplementation did not supply relevant levels of calcium to our growing piglets. In addition, the bioactive features of milk appear to have no effect on bone metabolism when supplemented short-term. Children who avoid dairy products or have low baseline calcium intakes have compromised skeletal health [8,59]. Milk supplementation and increased calcium intake has proven to increase bone mineral density and overall skeletal health when baseline calcium is below the daily recommended intake (DRI) [16,60,61]. However, as seen in the current study, milk supplementation when calcium intake is already adequate provides no further benefit. Our data is in agreement with Gibbons and colleagues (2004) [62]. Overall, when dietary calcium levels meet the DRI, milk supplementation has no effect on bone metabolism. SSBs have reported negative effects on skeletal development [63]. Cola consumption has been associated with decreased hip BMD and increased fracture risk [64], decreased serum calcium levels, and higher calcium excretion- all potential factors for developing osteoporosis [65]. No negative effects on bone development were seen in our experiment, suggesting no short-term effects of maltodextrin on skeletal growth and development.

2.3.6 Results from the combination of the two experiments

Statistical results of the combination of the same data (males only) from Experiment 1 and Experiment 2 can be found in **Table 7**. Despite the increased number of animals, there were no differences when comparing the two treatment groups (except for lateral cortical thickness). When the data were compared between the two experiments, we observed several significant differences, with almost all the bone being larger in animals in Experiment 2 vs. Experiment 1. This was not surprising as the piglets in Experiment 2 were older, heavier, and bigger when the trial was completed.

2.3.7 Limitations

Our study was not without limitations. As these were short-term experiments, it is difficult to make long-term inferences regarding the effects of milk supplementation on bone growth as pigs reach skeletal maturity around 25 months of age [66]. In Experiment 1, only the femur and tibia/fibula were collected whereas in Experiment 2, the femur, humerus, radius, and mandible were collected. This limits our *ex-vivo* comparison between studies to only the femur and its characteristics. Additionally, the current experiment may have missed the critical window of opportunity for evaluating the effect of milk on bone growth; specifically, when there is a peak in bone mass and mineral accrual during the growth spurt of puberty. In the present work we did not assess the microarchitecture of the trabecular bone using microcomputer tomography. This would have provided a better understanding of the long-term effect of milk on fracture resistance, since trabecular bone can be affected by milk in adolescents [14]. Another limitation of our study was the lack of measurement of any bone remodeling using, for instance, expression of genes related to bone turnover. Furthermore, long term effect on bone may be seeing on

mesenchymal and periosteal stem cells, as these cell lineages are responsible for bone formation [67,68].

2.4 Conclusion

Short-term milk supplementation does not affect bone growth, development, or strength in pre-pubertal growing piglets compared to maltodextrin supplementation. Milk may exert an indirect effect on bone formation by affecting behavior through the calming properties of casein-derived bioactive peptide. Adequate dietary calcium could have overridden any effect of milk on the skeleton in the short-term. Further research regarding milk's effect on pubertal mineral accrual in bone, longer experimental trials, evaluation of bone turnover, and assessment of the status of stem cells responsible for bone formation are needed to further understand the direct effect of milk on bone health.

2.5 References

[1] T. Sözen, L. Özışık, N.Ç. Başaran, An overview and management of osteoporosis, Eur. J. Rheumatol. 4 (2017) 46–56. doi:10.5152/eurjrheum.2016.048.

[2] M.S. Nanes, C.B. Kallen, Osteoporosis, Semin. Nucl. Med. 44 (2014) 439–450. doi:10.1053/j.semnuclmed.2014.06.006.

[3] J. Phetfong, T. Sanvoranart, K. Nartprayut, N. Nimsanor, K. Seenprachawong, V. Prachayasittikul, A. Supokawej, Osteoporosis: the current status of mesenchymal stem cell-based therapy, Cell. Mol. Biol. Lett. 21 (2016) 12. doi:10.1186/s11658-016-0013-1.

[4] C.M. Weaver, C.M. Gordon, K.F. Janz, H.J. Kalkwarf, J.M. Lappe, R. Lewis, M. O'Karma, T.C. Wallace, B.S. Zemel, The national osteoporosis foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations, Osteoporos. Int. 27 (2016) 1281–1386. doi:10.1007/s00198-015-3440-3.

[5] A. Caroli, A. Poli, D. Ricotta, G. Banfi, D. Cocchi, Invited review: Dairy intake and bone health: A viewpoint from the state of the art, J. Dairy Sci. 94 (2011) 5249–5262. doi:10.3168/jds.2011-4578.

[6] J.-P. Bonjour, T. Chevalley, S. Ferrari, R. Rizzoli, The importance and relevance of peak bone mass in the prevalence of osteoporosis, Salud Pública México. 51 (2009) s5–s17. doi:10.1590/S0036-36342009000700004.

[7] S. Stagi, L. Cavalli, C. Iurato, S. Seminara, M.L. Brandi, M. de Martino, Bone metabolism in children and adolescents: main characteristics of the determinants of peak bone mass, Clin. Cases Miner. Bone Metab. 10 (2013) 172–179.

[8] G. Mailhot, V. Perrone, N. Alos, J. Dubois, E. Delvin, L. Paradis, A.D. Roches, Cow's milk allergy and bone mineral density in prepubertal children, Pediatrics. 137 (2016) e20151742. doi:10.1542/peds.2015-1742.

[9] H.T. Viljakainen, Factors influencing bone mass accrual: focus on nutritional aspects, Proc. Nutr. Soc. 75 (2016) 415–419. doi:10.1017/S0029665116000252.

[10] J. Higgs, E. Derbyshire, K. Styles, Nutrition and osteoporosis prevention for the orthopaedic surgeon, EFORT Open Rev. 2 (2017) 300–308. doi:10.1302/2058-5241.2.160079.

[11] R. Rizzoli, Nutritional aspects of bone health, Best Pract. Res. Clin. Endocrinol. Metab. 28 (2014) 795–808. doi:10.1016/j.beem.2014.08.003.

[12] R. Rizzoli, Dairy products, yogurts, and bone health, Am. J. Clin. Nutr. 99 (2014) 1256S-1262S. doi:10.3945/ajcn.113.073056.

[13] USDA ERS - Dairy Data, (n.d.). https://www.ers.usda.gov/data-products/dairy-data/ (accessed March 14, 2018).

[14] E.Z. Movassagh, S. Kontulainen, A.D.G. Baxter-Jones, S. Whiting, M. Szafron, M. Papadimitropoulos, H. Vatanparast, Are milk and alternatives and fruit and vegetable intakes during adolescence associated with cortical and trabecular bone structure, density, and strength in adulthood?, Osteoporos. Int. 28 (2017) 609–619. doi:10.1007/s00198-016-3775-4.

[15] R. Rizzoli, M.L. Bianchi, M. Garabédian, H.A. McKay, L.A. Moreno, Maximizing bone mineral mass gain during growth for the prevention of fractures in the adolescents and the elderly, Bone. 46 (2010) 294–305. doi:10.1016/j.bone.2009.10.005.

[16] E.G.H.M. van den Heuvel, J.M.J.M. Steijns, Dairy products and bone health: how strong is the scientific evidence?, Nutr. Res. Rev. (2018) 1–15. doi:10.1017/S095442241800001X.

[17] E. Han, L.M. Powell, Consumption patterns of sugar sweetened beverages in the united states, J. Acad. Nutr. Diet. 113 (2013) 43–53. doi:10.1016/j.jand.2012.09.016.

[18] S.N. Bleich, K.A. Vercammen, J.W. Koma, Z. Li, Trends in Beverage Consumption Among Children and Adults, 2003-2014, Obesity. 26 (2018) 432–441. doi:10.1002/oby.22056.

[19] L.M. Powell, M.L. Maciejewski, Taxes and sugar-sweetened beverages, JAMA. 319 (2018) 229–230. doi:10.1001/jama.2017.19522.

[20] M.N. Händel, B.L. Heitmann, B. Abrahamsen, Nutrient and food intakes in early life and risk of childhood fractures: a systematic review and meta-analysis, Am. J. Clin. Nutr. 102 (2015) 1182–1195. doi:10.3945/ajcn.115.108456.

[21] S.J. Whiting, A. Healey, S. Psiuk, R. Mirwald, K. Kowalski, D.A. Bailey, Relationship between carbonated and other low nutrient dense beverages and bone mineral content of adolescents, Nutr. Res. 21 (2001) 1107–1115. doi:10.1016/S0271-5317(01)00324-4.

[22] L. Tian, X. Yu, Fat, Sugar, and bone health: A complex relationship, Nutrients. 9 (2017) 506. doi:10.3390/nu9050506.

[23] M. Terada, M. Inaba, Y. Yano, T. Hasuma, Y. Nishizawa, H. Morii, S. Otani, Growth-inhibitory effect of a high glucose concentration on osteoblast-like cells, Bone. 22 (1998) 17–23.

[24] L. Libuda, U. Alexy, T. Remer, P. Stehle, E. Schoenau, M. Kersting, Association between long-term consumption of soft drinks and variables of bone modeling and remodeling in a sample of healthy German children and adolescents, Am. J. Clin. Nutr. 88 (2008) 1670–1677. doi:10.3945/ajcn.2008.26414.

[25] R.L. Perlman, Mouse models of human disease, Evol. Med. Public Health. 2016 (2016) 170–176. doi:10.1093/emph/eow014.

[26] F. Elefteriou, X. Yang, Genetic mouse models for bone studies—Strengths and limitations, Bone. 49 (2011) 1242–1254. doi:10.1016/j.bone.2011.08.021.

[27] A.A. Abubakar, M.M. Noordin, T.I. Azmi, U. Kaka, M.Y. Loqman, The use of rats and mice as animal models in ex vivo bone growth and development studies, Bone Jt. Res. 5 (2017) 610–618. doi:10.1302/2046-3758.512.BJR-2016-0102.R2.

[28] R.L. Jilka, The relevance of mouse models for investigating age-related bone loss in humans, J. Gerontol. Ser. A. 68 (2013) 1209–1217. doi:10.1093/gerona/glt046.

[29] F.L. Lüthje, K. Skovgaard, H.E. Jensen, L. Kruse Jensen, Pigs are useful for the molecular study of bone inflammation and regeneration in humans, Lab. Anim. (2018) 0023677218766391. doi:10.1177/0023677218766391.

[30] L.M. Wancket, Animal models for evaluation of bone implants and devices: comparative bone structure and common model uses, Vet. Pathol. 52 (2015) 842–850. doi:10.1177/0300985815593124.

[31] P.M. Kelly, A. Diméglio, Lower-limb growth: how predictable are predictions?, J. Child. Orthop. 2 (2008) 407–415. doi:10.1007/s11832-008-0119-8.

[32] F. Rauch, D.A. Bailey, A. Baxter-Jones, R. Mirwald, R. Faulkner, The ‘muscle-bone unit’ during the pubertal growth spurt, Bone. 34 (2004) 771–775. doi:10.1016/j.bone.2004.01.022.

[33] A.S. Wiley, Does milk make children grow? relationships between milk consumption and height in NHANES 1999–2002, Am. J. Hum. Biol. 17 (2005) 425–441. doi:10.1002/ajhb.20411.

[34] T. Chevalley, J.-P. Bonjour, S. Ferrari, D. Hans, R. Rizzoli, Skeletal site selectivity in the effects of calcium supplementation on areal bone mineral density gain: A randomized,

double-blind, placebo-controlled trial in prepubertal boys, J. Clin. Endocrinol. Metab. 90 (2005) 3342–3349. doi:10.1210/jc.2004-1455.

[35] E. Seeman, Age- and Menopause-Related Bone Loss Compromise Cortical and Trabecular Microstructure, J. Gerontol. Ser. A. 68 (2013) 1218–1225. doi:10.1093/gerona/glt071.

[36] D. Yilmaz, B. Ersoy, E. Bilgin, G. Gümüşer, E. Onur, E.D. Pinar, Bone mineral density in girls and boys at different pubertal stages: relation with gonadal steroids, bone formation markers, and growth parameters, J. Bone Miner. Metab. 23 (2005) 476–482. doi:10.1007/s00774-005-0631-6.

[37] C.-Y. Tsai, Y.-M. Shyr, W.-C. Chiu, C.-M. Lee, Bone changes in the mandible following botulinum neurotoxin injections, Eur. J. Orthod. 33 (2011) 132–138. doi:10.1093/ejo/cjq029.

[38] R. Kunii, M. Yamaguchi, Y. Aoki, A. Watanabe, K. Kasai, Effects of experimental occlusal hypofunction, and its recovery, on mandibular bone mineral density in rats, Eur. J. Orthod. 30 (2008) 52–56. doi:10.1093/ejo/cjm057.

[39] C. Beata, E. Beaumont-Graff, V. Coll, J. Cordel, M. Marion, N. Massal, N. Marlois, J. Tauzin, Effect of alpha-casozepine (Zylkene) on anxiety in cats, J. Vet. Behav. Clin. Appl. Res. 2 (2007) 40–46. doi:10.1016/j.jveb.2007.02.002.

[40] S.M. McDonnell, J. Miller, W. Vaala, Calming benefit of short-term alpha-casozepine supplementation during acclimation to domestic environment and basic ground training of adult semi-feral ponies, J. Equine Vet. Sci. 33 (2013) 101–106. doi:10.1016/j.jevs.2012.05.060.

[41] C. Beata, E. Beaumont-Graff, C. Diaz, M. Marion, N. Massal, N. Marlois, G. Muller, C. Lefranc, Effects of alpha-casozepine (Zylkene) versus selegiline hydrochloride (Selgian,

Anipryl) on anxiety disorders in dogs, J. Vet. Behav. Clin. Appl. Res. 2 (2007) 175–183. doi:10.1016/j.jveb.2007.08.001.

[42] L. Miclo, E. Perrin, A. Driou, V. Papadopoulos, N. Boujrad, R. Vanderesse, J.-F. Boudier, D. Desor, G. Linden, J.-L. Gaillard, Characterization of α-casozepine, a tryptic peptide from bovine αs1-casein with benzodiazepine-like activity, FASEB J. 15 (2001) 1780–1782. doi:10.1096/fj.00-0685fje.

[43] G. Landsberg, B. Milgram, I. Mougeot, S. Kelly, C. de Rivera, Therapeutic effects of an alpha-casozepine and L-tryptophan supplemented diet on fear and anxiety in the cat, J. Feline Med. Surg. 19 (2017) 594–602. doi:10.1177/1098612X16669399.

[44] I.J.I. dela Peña, E. Hong, J.B. de la Peña, H.J. Kim, C.J. Botanas, Y.S. Hong, Y.S. Hwang, B.S. Moon, J.H. Cheong, Milk collected at night induces sedative and anxiolytic-like effects and augments pentobarbital-induced sleeping behavior in mice, J. Med. Food. 18 (2015) 1255–1261. doi:10.1089/jmf.2015.3448.

[45] I.-C. Hou, C. Suzuki, N. Kanegawa, A. Oda, A. Yamada, M. Yoshikawa, D. Yamada, M. Sekiguchi, E. Wada, K. Wada, K. Ohinata, β-Lactotensin derived from bovine β-lactoglobulin exhibits anxiolytic-like activity as an agonist for neurotensin NTS2 receptor via activation of dopamine D1 receptor in mice, J. Neurochem. 119 (n.d.) 785–790. doi:10.1111/j.1471-4159.2011.07472.x.

[46] A. Mika, M. Gaffney, R. Roller, A. Hills, C.A. Bouchet, K.A. Hulen, R.S. Thompson, M. Chichlowski, B.M. Berg, M. Fleshner, Feeding the developing brain: Juvenile rats fed diet rich in prebiotics and bioactive milk fractions exhibit reduced anxiety-related behavior and modified gene expression in emotion circuits, Neurosci. Lett. 677 (2018) 103–109. doi:10.1016/j.neulet.2018.01.052.

[47] J.K.B. Soares, A.P.R. de Melo, M.C. Medeiros, R.C.R.E. Queiroga, M.A.D. Bomfim, E.C.A. Santiago, R.C.A. Guedes, Anxiety behavior is reduced, and physical growth is improved in the progeny of rat dams that consumed lipids from goat milk: An elevated plus maze analysis, Neurosci. Lett. 552 (2013) 25–29. doi:10.1016/j.neulet.2013.07.028.

[48] P. Cavallo, L. Carpinelli, G. Savarese, Perceived stress and bruxism in university students, BMC Res. Notes. 9 (2016). doi:10.1186/s13104-016-2311-0.

[49] H. Fonseca, D. Moreira-Gonçalves, H.-J.A. Coriolano, J.A. Duarte, Bone Quality: The determinants of bone strength and fragility, Sports Med. 44 (2014) 37–53. doi:10.1007/s40279-013-0100-7.

[50] A.M. Cheung, H. Frame, M. Ho, E.S. Mackinnon, J.P. Brown, Bone strength and management of postmenopausal fracture risk with antiresorptive therapies: considerations for women's health practice, Int. J. Womens Health. 8 (2016) 537–547. doi:10.2147/IJWH.S112621.

[51] E. Seeman, Growth and age-related abnormalities in cortical structure and fracture risk, Endocrinol. Metab. 30 (2015) 419–428. doi:10.3803/EnM.2015.30.4.419.

[52] D.H. Nielsen, F.J. McEvoy, M.T. Madsen, J.B. Jensen, E. Svalastoga, Relationship between bone strength and dual-energy X-ray absorptiometry measurements in pigs, J. Anim. Sci. 85 (2007) 667–672. doi:10.2527/jas.2006-025.

[53] U.T. Iwaniec, R.T. Turner, Influence of body weight on bone mass, architecture and turnover, J. Endocrinol. 230 (2016) R115–R130. doi:10.1530/JOE-16-0089.

[54] H.M. Frost, The mechanostat: a proposed pathogenic mechanism of osteoporoses and the bone mass effects of mechanical and nonmechanical agents, Bone Miner. 2 (1987) 73–85.

[55] H.M. Frost, Bone's mechanostat: A 2003 update, Anat. Rec. A. Discov. Mol. Cell. Evol. Biol. 275A (2003) 1081–1101. doi:10.1002/ar.a.10119.

[56] J.F. Tahmassebi, M.S. Duggal, G. Malik-Kotru, M.E.J. Curzon, Soft drinks and dental health: A review of the current literature, J. Dent. 34 (2006) 2–11. doi:10.1016/j.jdent.2004.11.006.

[57] R. Barac, J. Gasic, N. Trutic, S. Sunaric, J. Popovic, P. Djekic, G. Radenkovic, A. Mitic, Erosive effect of different soft drinks on enamel surface in vitro: Application of stylus profilometry, Med. Princ. Pract. 24 (2015) 451–457. doi:10.1159/000433435.

[58] T.R. Edition, B.O. Agriculture, Nutrient requirements of swine, 1998.

[59] P.C. Pereira, Milk nutritional composition and its role in human health, Nutrition. 30 (2014) 619–627. doi:10.1016/j.nut.2013.10.011.

[60] J. Cadogan, R. Eastell, N. Jones, M.E. Barker, Milk intake and bone mineral acquisition in adolescent girls: randomised, controlled intervention trial., BMJ. 315 (1997) 1255–1260.

[61] S. Viguet-Carrin, M. Hoppler, F. Membrez Scalfo, J. Vuichoud, M. Vigo, E.A. Offord, P. Ammann, Peak bone strength is influenced by calcium intake in growing rats, Bone. 68 (2014) 85–91. doi:10.1016/j.bone.2014.07.029.

[62] M.J. Gibbons, N.L. Gilchrist, C. Frampton, P. Maguire, P.H. Reilly, R.L. March, C.R. Wall, The effects of a high calcium dairy food on bone health in pre-pubertal children in New Zealand, Asia Pac. J. Clin. Nutr. 13 (2004) 341–347.

[63] E. Tsanzi, H.R. Light, J.C. Tou, The effect of feeding different sugar-sweetened beverages to growing female Sprague–Dawley rats on bone mass and strength, Bone. 42 (2008) 960–968. doi:10.1016/j.bone.2008.01.020.

[64] K.L. Tucker, K. Morita, N. Qiao, M.T. Hannan, L.A. Cupples, D.P. Kiel, Colas, but not other carbonated beverages, are associated with low bone mineral density in older women: The Framingham Osteoporosis Study, Am. J. Clin. Nutr. 84 (2006) 936–942. doi:10.1093/ajcn/84.4.936.

[65] M. Mahmood, A. Saleh, F. Al-Alawi, F. Ahmed, Health effects of soda drinking in adolescent girls in the United Arab Emirates, J. Crit. Care. 23 (2008) 434–440. doi:10.1016/j.jcrc.2008.06.006.

[66] A.N. Mastrangelo, E.M. Magarian, M.P. Palmer, P. Vavken, M.M. Murray, The effect of skeletal maturity on the regenerative function of intrinsic ACL cells, J. Orthop. Res. Off. Publ. Orthop. Res. Soc. 28 (2010) 644–651. doi:10.1002/jor.21018.

[67] S.F. Gilbert, Osteogenesis: The Development of Bones, (2000). https://www.ncbi.nlm.nih.gov/books/NBK10056/ (accessed November 3, 2017).

[68] Y. Qin, J. Guan, C. Zhang, Mesenchymal stem cells: mechanisms and role in bone regeneration, Postgrad. Med. J. 90 (2014) 643–647. doi:10.1136/postgradmedj-2013-132387.

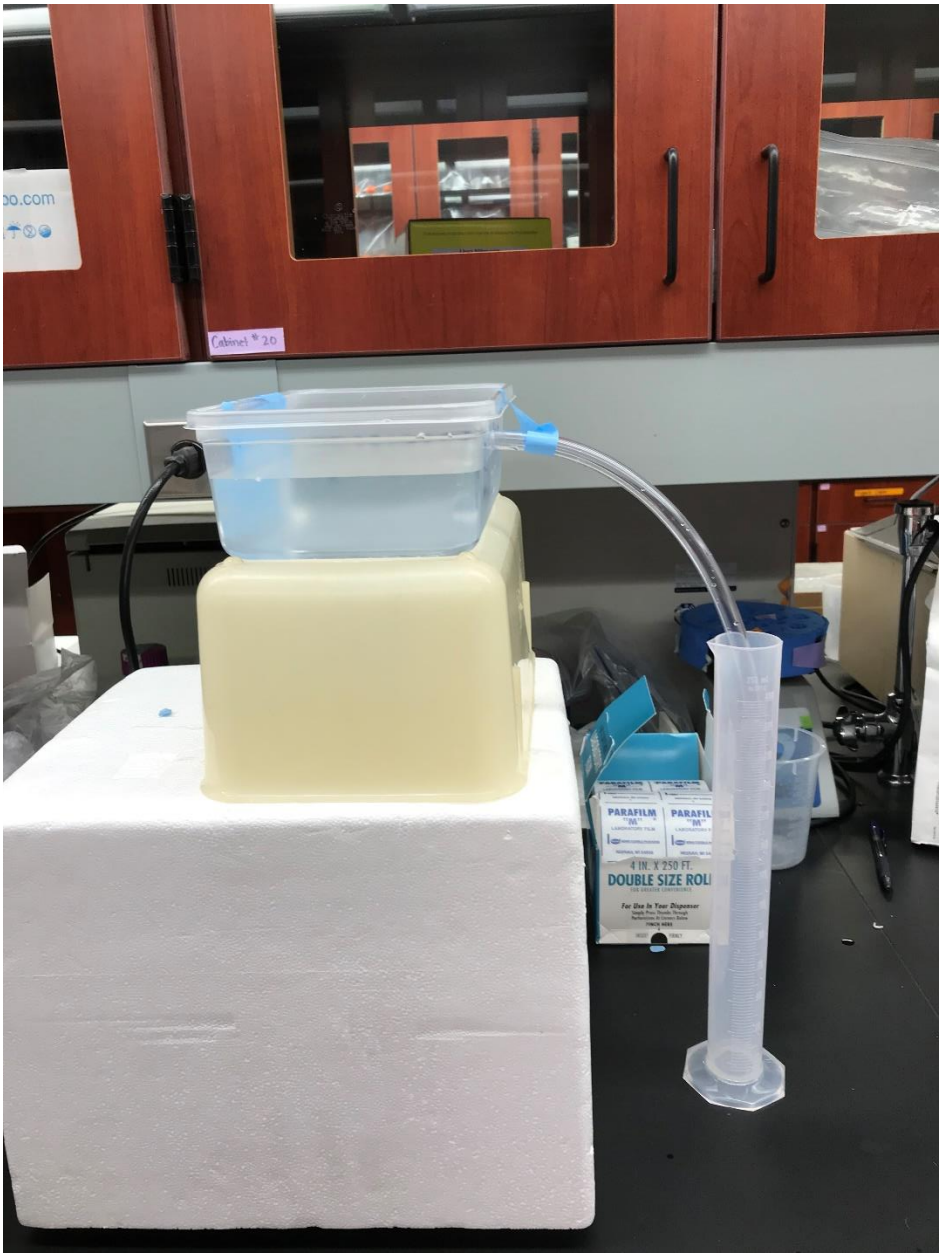

Figure 1- System used for mandibular volumetric analysis

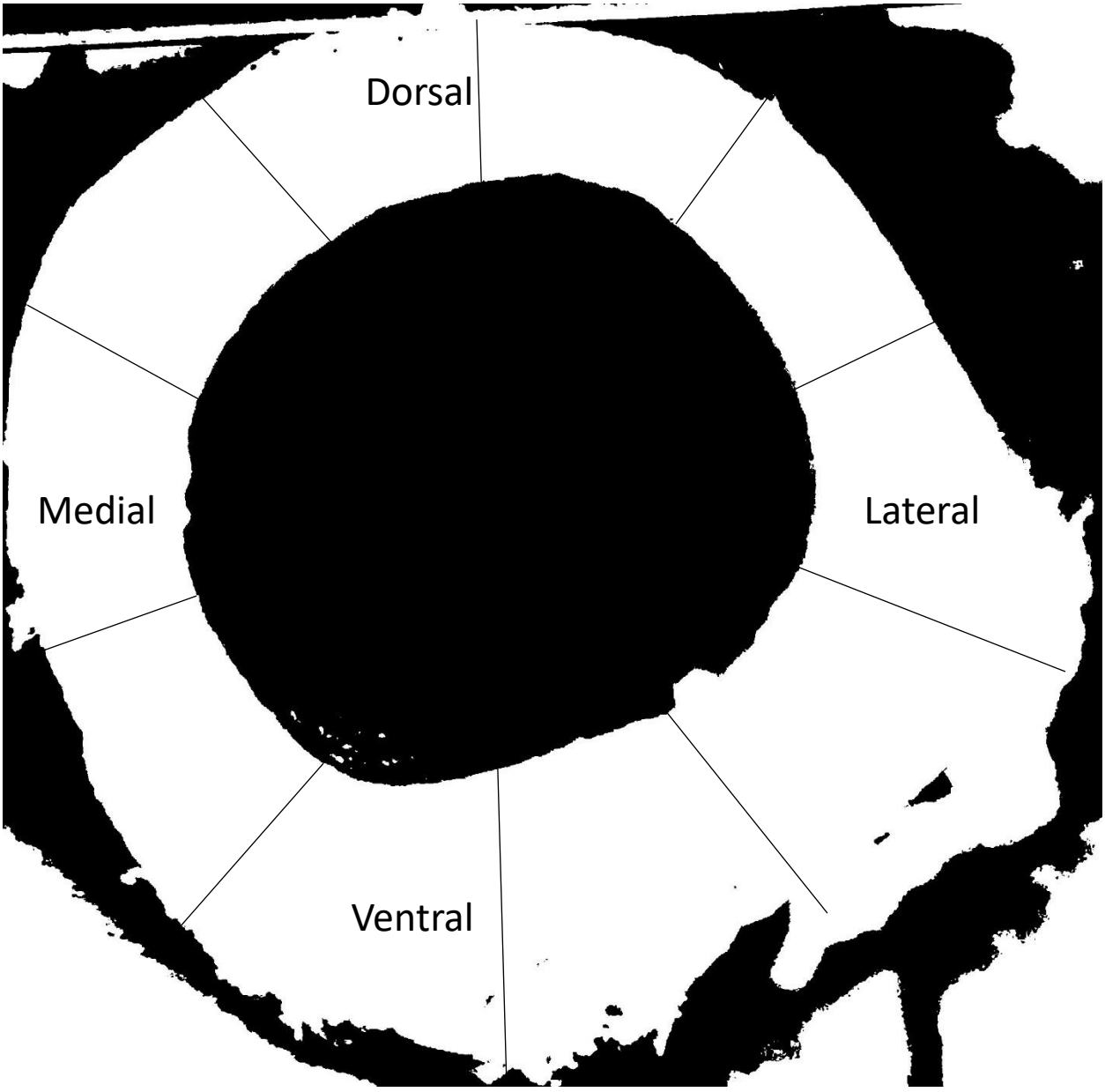

Figure 2: Binary picture of cross-section of femoral diaphysis. The prominent white ellipse is the cross-section of the midpoint of the femur. The black inner area is the medullary cavity. Measurements of the cross-sectional area, medullary cavity area, cortical area, and average cortical thickness were taken. 10 points were measured equidistant apart from each other for

average cortical thickness (spokes). Dorsal, Lateral, Ventral, and Medial labels indicate location of thickness measurement with electronic caliper.

Figure 3: Sagittal cross-section of the fourth premolar used for enamel thickness evaluation. Enamel and dentin layers are labelled for reference.

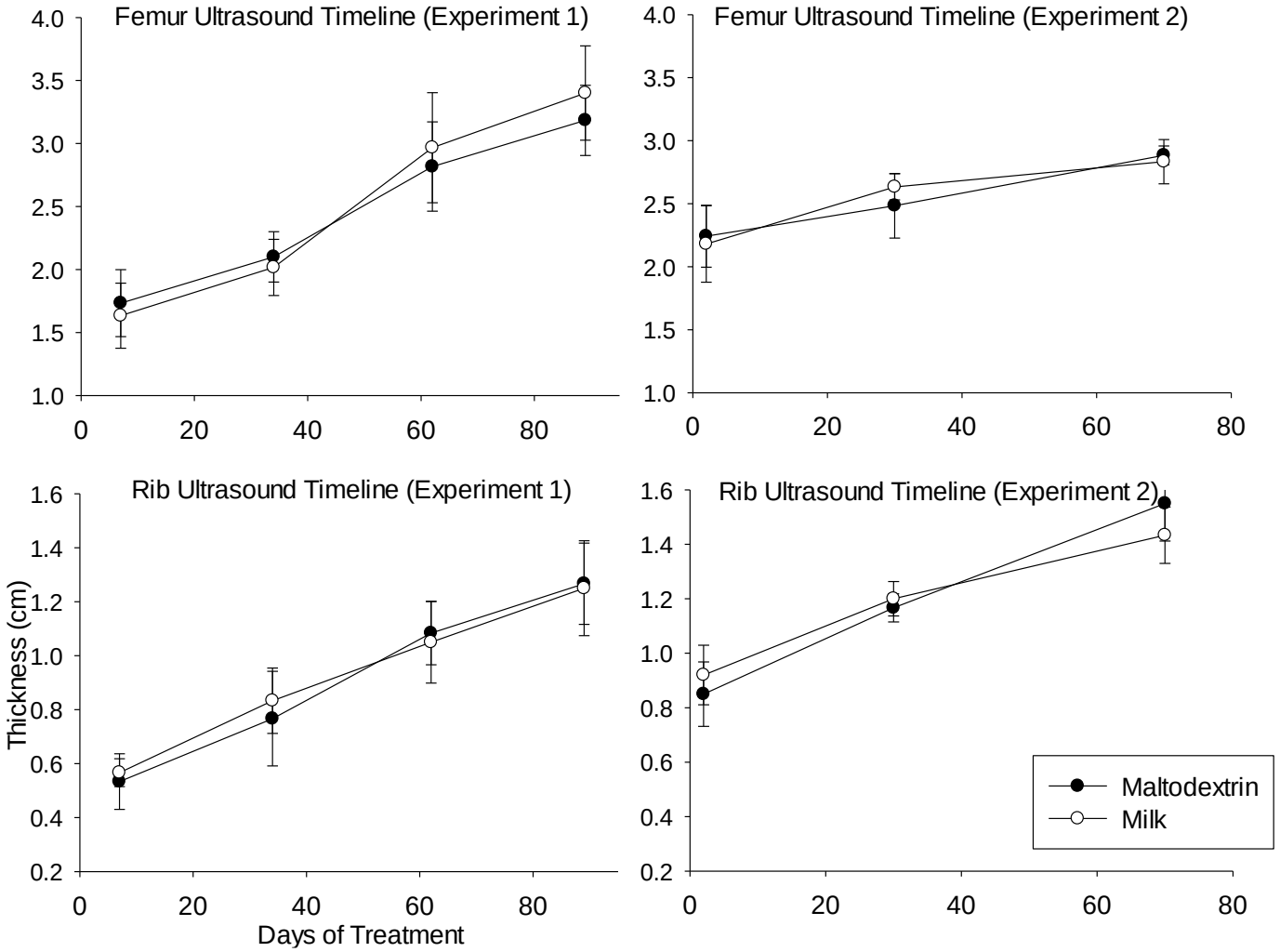

Figure 4. *In vivo* measurement of the thickness of the last right rib and right femur in 5 (Experiment 1) or 8 (Experiment 2) week old piglets receiving daily 750 mL of milk or isocaloric amount of maltodextrin in Experiment 1 (n=6/group) and Experiment 2 (n=6/group).

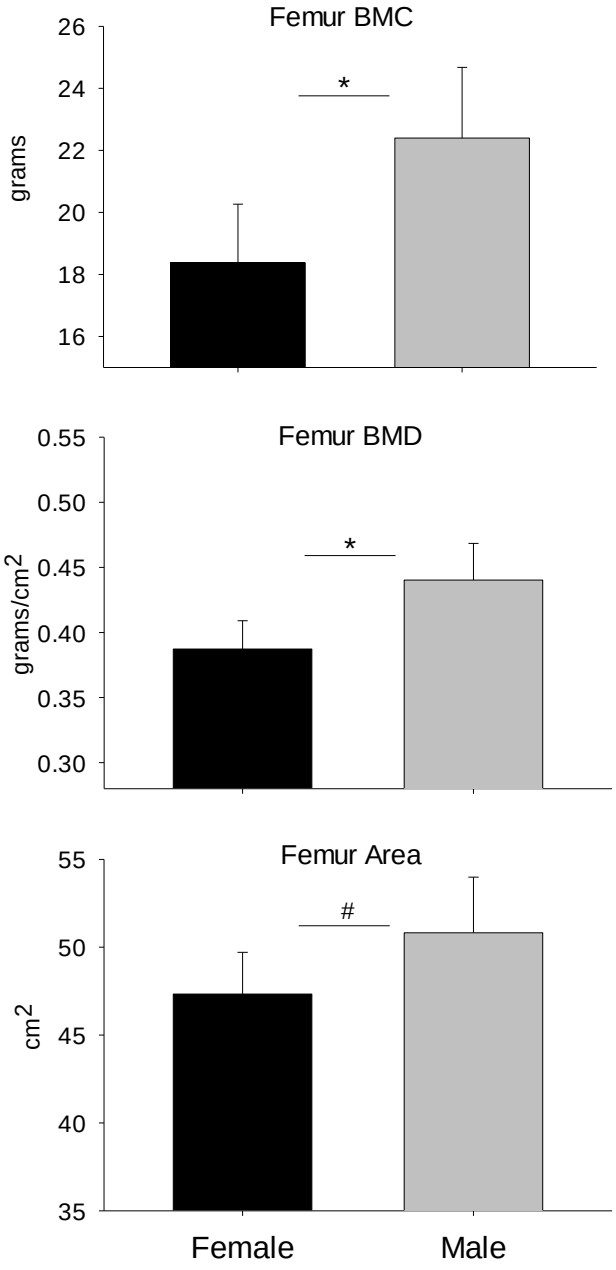

Figure 5. Femur bone mineral content (BMC), bone mineral density (BMD), and area between male (n=8) and female (n=4) piglets from Experiment 1. * $p < 0.05$; # $p < 0.10$.

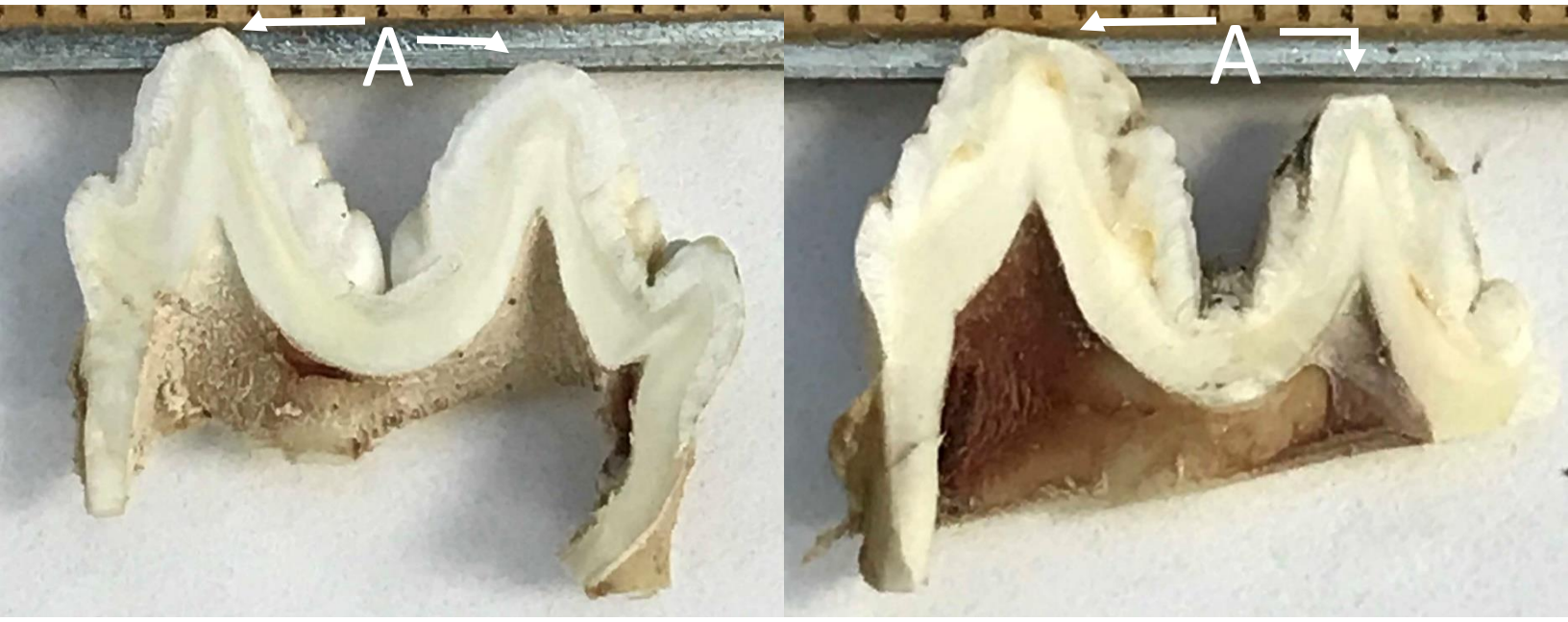

Figure 6. Comparison of occlusal surfaces in the fourth premolar in piglets receiving 750 mL/d of milk (Left) or an isocaloric maltodextrin solution (Right). The group receiving the maltodextrin solution showed increased wear and flattening on the occlusal surface (A) compared to the group receiving milk.

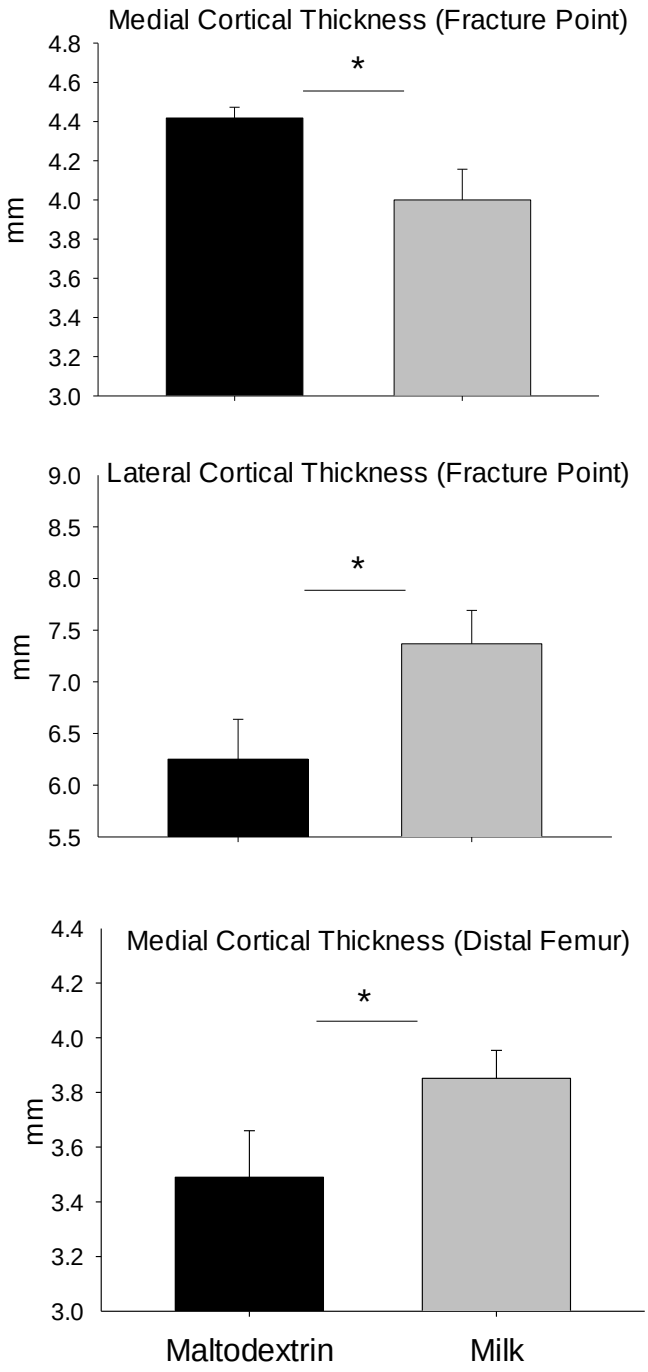

Figure 7. Differences in cortical thickness measurements for piglets receiving daily 750 mL of milk or isocaloric amount of maltodextrin in Experiment 2 (n=6/group). * $p < 0.05$; # $p < 0.10$

Table 1. Milk provides biologically relevant amounts of key nutrients essential for proper bone development- List of key nutrients critical for bone health available in one serving of milk

Nutrient	Daily recommended intake (DRI)[a] (Office of Dietary Supplements) [12]	Amount in 8 oz of 2% cow's milk[b]	Percent of DRI provided in 8 oz of 2% cow's milk
Calcium	1,300 mg/d	293 mg	22.5
Phosphorus	1,250 mg/d	224 mg	17.9
Magnesium	240 mg/d	27mg	11.2
Potassium	4.5 g/d	342 mg	7.6
Vitamin D	600 IU/d	120 IU	20.0
Zinc	8 mg/d	1.17 mg	14.6

[a] Daily recommended intake for children (both genders) ages 9-13, Office of Dietary Supplements [12] Section 1

[b] From National Nutrient Database for Standard Reference, USDA [106] section 1

Table 2. DEXA data from bones and regions of interest of piglets (n=6/group) receiving daily milk or isocaloric amount of maltodextrin in Experiment 1. Data are expressed as mean ± S.E.M.

Parameter	Maltodextrin	Milk	p-value
Femur BMC (g)	20.78 ± 1.26	21.33 ± 1.18	0.700
Femur BMD (g/cm^2)	0.42 ± 0.02	0.42 ± 0.01	0.961
Femur Area (cm^2)	48.96 ± 0.93	50.36 ± 1.71	0.465
Femoral Head BMC (g)	16.52 ± 0.40	16.93 ± 0.76	0.623
Femoral Head BMD (g/cm^2)	5.69 ± 0.41	6.04 ± 0.60	0.574
Femur Head Area (cm^2)	0.34 ± 0.02	0.36 ± 0.02	0.540
Femur Diaphysis BMC (g)	16.11 ± 0.85	15.98 ± 0.36	0.861
Femur Diaphysis BMD (g/cm^2)	8.52 ± 0.83	8.23 ± 0.29	0.616
Femur Diaphysis Area (cm^2)	0.53 ± 0.03	0.52 ± 0.01	0.749
Femur Distal Epiphysis BMC (g)	17.11 ± 0.71	18.2 ± 1.20	0.485
Femur Distal Epiphysis BMD (g/cm^2)	6.88 ± 0.36	7.3 ± 0.62	0.599
Femur Distal Epiphysis Area (cm^2)	0.40 ± 0.01	0.41 ±0.01	0.890
Left Tibia/Fibula BMC (g)	16.79 ± 1.34	18.45 ± 1.56	0.494
Left Tibia/Fibula BMD (g/cm^2)	0.36 ± 0.01	0.38 ± 0.01	0.430
Left Tibia/Fibula Area (cm^2)	46.04 ± 2.17	48.06 ± 2.2	0.578
Right Tibia/Fibula BMC (g)	18.11 ± 1.11	18.33 ± 1.25	0.904
Right Tibia/Fibula BMD (g/cm^2)	0.39 ± 0.01	0.39 ± 0.01	0.804
Right Tibia/Fibula Area (cm^2)	46.64 ±1.85	46.68 ±1.91	0.988
Total BMD (g/cm^2)	0.39±0.01	0.40 ± 0.01	0.777

Table 3. DEXA data for sex differences from bones and regions of interest of male (n=8) and female (n=4) piglets from Experiment 1. Data are expressed as mean ± S.E.M.

Parameter	Male	Female	p-value
Femur BMC (g)	22.4 ± 0.81	18.37 ± 0.95	**0.025***
Femur BMD (g/cm^2)	0.44 ± 0.01	0.39±0.01	**0.016***
Femur Area (cm^2)	50.82 ± 1.12	47.34±1.19	0.111
Femur Proximal Epiphysis BMC (g)	6.53±0.4	4.8±0.22	0**.037***
Femur Proximal Epiphysis BMD (g/cm^2)	0.38±0.01	0.31±0.01	**0.020***
Femur Proximal Epiphysis Area (cm^2)	17.26±0.45	15.66±0.62	*0.096#*
Femur Diaphysis BMC (g)	9.14±0.41	6.97±0.43	**0.007***
Femur Diaphysis BMD (g/cm^2)	0.54±0.02	0.47±0.03	*0.073#*
Femur Diaphysis Area (cm^2)	16.83±0.39	14.8±0.81	**0.041***
Femur Distal Epiphysis BMC (g)	7.06±0.47	6.83±0.54	0.656
Femur Distal Epiphysis BMD (g/cm^2)	0.40 ±0.01	0.39±0.02	0.284
Femur Distal Epiphysis Area (cm^2)	17.54±0.98	17.56±0.82	0.926
Left Tibia/Fibula BMC (g)	18.28 ± 1.41	16.67±1.11	0.549
Left Tibia/Fibula BMD (g/cm^2)	0.38 ± 0.01	0.36±0.01	0.586
Left Tibia/Fibula Area (cm^2)	47.95±1.87	45.74±2.39	0.580
Right Tibia/Fibula BMC (g)	18.18±1.09	18.25±0.99	0.977
Right Tibia/Fibula BMD (g/cm^2)	0.39±0.01	0.39±0.01	0.994
Right Tibia/Fibula Area (cm^2)	46.52±1.67	46.9±1.87	0.896
Total BMD (g/cm^2)	0.40±0.01	0.38±0.01	0.139

* $p < 0.05$; # $p < 0.10$)

Table 4. DEXA data from bones and regions of interest of piglets (n=6/group) receiving daily milk or isocaloric amount of maltodextrin in Experiment 2. Data are expressed as mean ± S.E.M.

Parameter	Maltodextrin	Milk	p-value
Radius BMC (g)	32.09 ± 2.77	31.8 ± 2.42	0.937
Radius BMD (g/cm^2)	0.51 ± 0.04	0.52 ± 0.04	0.810
Radius Area (cm^2)	63.12 ± 2.25	61.31 ± 1.55	0.521
Humerus BMC (g)	36.83 ± 1.97	34.94 ± 2.32	0.548
Humerus BMD (g/cm^2)	0.67 ± 0.03	0.66 ± 0.03	0.724
Humerus Area (cm^2)	54.72 ± 0.78	52.99 ± 1.14	0.238
Humeral Distal Epiphysis BMC (g)	12.51 ± 0.41	13.26 ± 1.01	0.505
Humeral Distal Epiphysis BMD (g/cm^2)	0.62 ± 0.02	0.68 ± 0.04	0.155
Humeral Distal Epiphysis Area (cm^2)	20.06 ± 0.70	19.49 ± 0.46	0.511
Humeral Diaphysis BMC (g)	13.27 ± 0.54	12.5 ± 1.09	0.541
Humeral Diaphysis BMD (g/cm^2)	1.02 ± 0.04	0.98 ± 0.04	0.492
Humeral Diaphysis Area (cm^2)	12.98 ± 0.47	12.64 ± 0.70	0.696
Humeral Head BMC (g)	11.48 ± 1.55	9.76 ± 0.58	0.325
Humeral Head BMD (g/cm^2)	0.52 ± 0.07	0.45 ± 0.02	0.397
Humeral Head Area (cm^2)	22.32 ± 0.76	21.64 ± 0.48	0.461
Femur BMC (g)	38.87 ± 1.02	37.67 ± 1.19	0.462
Femur BMD (g/cm^2)	0.58 ± 0.01	0.59 ± 0.01	0.560
Femur Area (cm^2)	67.14 ± 1.22	64.15 ± 1.09	*0.098#*
Total BMD (g/cm^2)	0.54 ± 0.01	0.53 ± 0.02	0.598
Mandible BMC (g)	29.68 ± 1.86	24.65 ± 1.22	**0.047***
Mandible BMD (g/cm^2)	0.41 ± 0.02	0.35 ± 0.01	**0.046***
Mandible Area (cm^2)	72.5 ± 1.24	69.59 ± 3.33	0.431
Ramus BMC (g)	8.17 ± 0.53	7.52 ± 0.76	0.468
Ramus BMD (g/cm^2)	0.23 ± 0.01	0.25 ± 0.02	0.291
Ramus Area (cm^2)	34.75 ± 1.75	31.41 ± 2.94	0.352
Mandibular Body BMC (g)	17.85 ± 1.53	13.88 ± 0.72	*0.092#*
Mandibular Body BMD (g/cm^2)	0.60 ± 0.05	0.46 ± 0.02	**0.027***
Mandibular Body Area (cm^2)	29.74 ± 0.93	30.41 ± 1.68	0.733
Teeth BMC (g)	3.06 ± 0.22	3.68 ± 0.25	*0.092#*
Teeth BMD (g/cm^2)	0.50 ± 0.02	0.49 ± 0.01	0.794
Teeth Area (cm^2)	6.17 ± 0.47	7.47 ± 0.50	*0.088#*
Occlusal Surface Thickness (mm)	0.792 ± 0.07	1.05 ± 0.08	**0.032***

*$p < 0.05$, # $p < 0.10$

Table 5. Femur characteristics and final body weight for 5 (Experiment 1) or 8 (Experiment 2) week old piglets receiving daily 750 mL of milk or isocaloric amount of maltodextrin in Experiment 1 (n=6/group) and Experiment 2 (n=6/group) and biomechanical data for piglets in Experiment 2. Data are expressed as mean ± S.E.M.

Parameter	Maltodextrin	Milk	p-value
Experiment 1			
Average Cortical Thickness (mm)	4.97 ± 0.13	4.69 ± 0.23	0.818
Cross-Sectional Area (mm^2)	453.11 ± 20.34	442.13 ± 9.91	0.639
Cortical Area (mm^2)	300.24 ± 11.88	284.22 ± 12.13	0.365
Medullary Cavity Area (mm^2)	154.27 ± 11.14	157.91 ± 12.87	0.309
Femur Diameter (mm)	22.73 ± 0.53	22.99 ± 0.44	0.724
Femur Length (mm)	157.67 ± 1.17	163.83 ± 2.23	*0.059#*
Body Weight (kg)	58.82 ± 3.37	63.05 ± 2.81	0.391
Experiment 2			
Average Cortical Thickness (mm)	4.78 ± 0.15	5.03 ± 0.11	0.213
Cross-Sectional Area (mm^2)	531.75 ± 20.25	521.23 ± 26.89	0.761
Cortical Area (mm^2)	327.71 ± 16.37	322.5 ± 6.38	0.773
Medullary Cavity Area (mm^2)	204.04 ± 6.38	198.73 ± 23.71	0.833
Ultimate Bending Strength (N)	3697.1 ± 95.76	3750.46 ± 199.03	0.814
Experimental Deformation (mm)	5.73 ± 0.22	5.96 ± 0.29	0.529
Stiffness (N/mm)	1151.65 ± 38.37	1149.88 ± 52.76	0.489
Femur Diameter (mm)	24.73 ± 0.40	24.26 ± 0.41	0.431
Femur Volume (mL)	234.0 ± 8.02	235.5 ± 6.13	0.880
Femur Length (mm)	177.83 ± 1.66	175.17 ± 1.38	0.245
Body Weight (kg)	86.79 ± 2.01	88.60 ± 2.51	0.585

* $p < 0.05$; # $p < 0.10$)

Table 6. Significant correlations between Experimental Deformation from the three-point bending evaluation of the femur with other femur characteristics and significant correlations between body weight and femur characteristics of piglets receiving daily 750 mL of milk or isocaloric amount of maltodextrin in Experiment 1 (n=6/group) and Experiment 2 (n=6/group). All correlations are with data from Experiment 2 unless labelled otherwise.

Correlation between Experimental Deformation and:	**Pearson Correlation Coefficient**	**p-value**
Femur Diameter	-0.75	**0.004***
Femur Area	-0.69	**0.013***
Femur BMC	-0.62	**0.032***
Femur Length	-0.56	*0.061#*
Correlation between Body Weight and:	**Pearson Correlation Coefficient**	**p-value**
Femur Length (Experiment 1)	0.77	**0.003***
Femur Length	0.58	**0.048***
Femur Area	0.58	**0.048***
Femur BMC	0.60	**0.040***

*- Statistically significant ($p < 0.05$)

#- Statistical tendency ($p < 0.10$)

Table 7. Experimental comparison for common data from the femur

Parameter	Maltodextrin	Milk	S.E.M.	p-value	Exp. 1	Exp. 2	S.E.M.	p-value	Treatment x Experiment p- value
Body Weight (kg)	74.79	78.60	9.21	0.177	60.21	87.69	3.89	**<0.001***	0.378
Femur BMC (g)	32.23	31.60	4.00	0.597	22.39	38.26	1.69	**<0.001***	0.562
Femur BMD (g/cm^2)	0.52	0.52	0.40	0.946	0.44	0.58	0.02	**<0.001***	0.462
Femur Area (cm^2)	60.27	59.16	4.60	0.421	50.82	65.64	1.94	**<0.001***	0.106
Medial Cortical Thickness (CT) (mm)	4.19	4.05	0.53	0.376	3.99	4.20	0.22	0.189	**0.043***
Lateral CT (mm)	6.12	6.92	1.28	**0.048***	6.10	6.81	0.54	*0.083*$^{\#}$	0.314
Dorsal CT (mm)	4.18	3.75	1.24	0.250	4.37	3.70	0.52	*0.089*$^{\#}$	0.884
Ventral CT (mm)	4.46	4.96	0.86	*0.062*$^{\#}$	4.24	5.02	0.36	**0.008***	0.863
Proximal Epiphysis (PE) Medial CT (mm)	4.30	4.09	0.80	0.367	4.24	4.16	0.35	0.685	0.754
PE Lateral CT (mm)	4.98	5.18	2.06	0.745	5.14	5.05	0.89	0.901	0.586
PE Ventral CT (mm)	5.08	5.50	0.88	0.112	4.9	5.54	0.38	**0.020***	0.568
Distal Epiphysis (DE) Medial CT (mm)	3.38	3.60	0.55	0.196	3.23	3.67	0.23	**0.016***	0.281
DE Lateral CT (mm)	6.96	7.19	1.25	0.532	6.53	7.44	0.53	**0.026***	0.833
DE Dorsal CT (mm)	3.39	3.09	0.98	0.315	3.30	3.19	0.41	0.710	0.281
DE Ventral CT (mm)	4.30	4.58	0.70	0.197	4.00	4.73	0.30	**0.003***	0.611
Medullary Cavity Area (mm^2)	183.19	176.85	59.58	0.864	142.95	201.38	25.80	**0.028***	0.825
Average CT (mm)	4.84	4.99	0.54	0.338	4.94	4.90	0.23	0.869	0.382
Cortical Area (mm^2)	315.38	312.23	45.36	0.807	294.21	325.10	19.64	**0.034***	0.676
Cross-Sectional Area (mm^2)	498.58	489.08	82.93	0.690	437.16	526.49	35.91	**0.002***	0.699
Femur Diameter (mm)	23.94	23.59	1.72	0.504	22.69	24.49	0.73	**0.003***	0.762
Femur Length (mm)	169.70	170.50	6.57	0.683	160.50	176.50	2.77	**<0.001***	**0.042***

* $p < 0.05$; # $p < 0.10$

APPENDIX A

Effect of milk supplementation on in-vivo bone growth and bone mineral density in pre-pubertal pigs

Brandon Batty, Angel Torres, Nina Enos, Katherine Swanson, Sebastiano Busato, Nicolas Aguilera, Efren Plancarte, Michelle Kutzler, and Massimo Bionaz

Milk consumption during childhood and into adulthood has been shown to protect against osteoporosis onset in aging adults, but the mechanism remains unclear. Our hypothesis is that milk improves the osteogenic capacity of bone marrow stem cells (BMSC). The objective of the study was to determine the effect of milk supplementation on BMSC using a pre-pubertal pig model. In the first part of the study, presented in this abstract, we assessed the effect of milk supplementation of bone growth. For this we used 24 pre-pubertal pigs randomly split into two groups to receive either up to 750 mL of whole milk supplementation or an isocaloric maltodextrin solution for 12 weeks. The experiment was run in 2 trials: trial 1 utilized 5-weeks old Yorkshire pigs of both sex while trial 2 used 7 weeks old male cross-bred (Duroc×Yorkshire). Ultrasonography was used to record in-vivo bone growth and measurements were taken of the 10^{th} rib and the right femur. At the end of the trial, the pigs were euthanized and several bones, including femur, tibia and fibula, the 10^{th} rib, radius, humerus, and the mandible, were collected. Bones were analyzed for dual energy x-ray absorptiometry. Cells from the bone marrow were isolated and quantified. Data were analyzed using GLM procedure of SAS with TRT and Experiment (and Sex for Trial 1) as fixed effect and pig as random. Significance was declared with $P \leq 0.05$. Among all the measurements taken, only the bone density of the mandible was significantly lower in piglets receiving milk. There proved to be no significant other effects of the milk supplementation on the other measurements taken.

Determination of the effect of milk supplementation on bone strengths and BMSC, including in vitro functional assays and transcriptome, are underway.

Effect of milk supplementation on in-vivo bone growth and bone mineral density in pre-pubertal pigs

Brandon Batty, Angel Torres, Nina Enos, Sebastiano Busato, Katherine Swanson, Efren Plancarte, Nicolas Aguilera, Michelle Kutzler, and Massimo Bionaz

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INTRODUCTION

- Osteoporosis, a degenerative bone disease, is responsible for millions of fractures and billions of dollars in healthcare costs each year (Hess et al., 2016)
- Increased calcium, phosphorus, and vitamin D consumptions has been shown to increase total bone mass and help protect against osteoporosis (Hess et al., 2016)
- Dairy products, especially milk, is high in these compounds and may contribute to increased peak bone mass and lower risk for osteoporosis (Pereira, 2014)
- Cow's milk contains micro-vesicles carrying RNA which may exert an effect on bone marrow mesenchymal stem cells (BMSC) (Izumi et al., 2015)
- Understanding the effects of milk on BMSC can be critical information for osteoporosis research

HYPOTHESIS

Milk supplementation increases bone density by affecting bone marrow stem cells

OBJECTIVES

To determine the effect of milk supplementation on:

- Bone density during time of peak bone accrual
- Bone marrow mesenchymal stem cells osteogenesis capacity

FURTHER ANALYSES

- Biomechanical testing → bone strength
- BMSC → colony forming unit, proliferation assay, gene expression, and transcriptomic effects of exosome isolated from milk

LITERATURE CITED

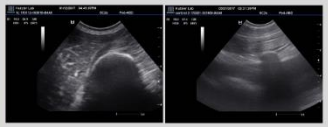
Hess, J.M., S.S. Jonnalagadda, and J.L. Slavin. 2016. Dairy Foods: Current evidence of their effects on bone, cardiometabolic, cognitive, and digestive health. Compr Rev Food Sci Food Saf. 15: 231-268.

Pereira, P.C. 2014. Milk nutritional composition and its role in human health. J Nutr. 30(6): 619-27.

Izumi, H., M. Tsuda, Y. Sato, N. Kosaka, T. Ochiya, H. Iwamoto, K. Namba, and Y. Takeda. 2015. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J Dairy Sci. 98: 2920-2933.

MATERIALS AND METHODS

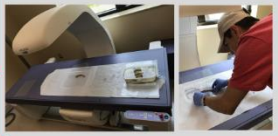
Pre-pubertal pigs
Treated for 12 wks.
Year 1 → 12 pigs 5-wks of age (8 males + 4 females)
Year 2 → 12 pigs 7-wks of age (males)
Two groups:
- **TRT** = 750 ml of milk
- **CTR** = maltodextrin



Ultrasounds taken 3 times during the experiment: 10th right rib and right femur

Femur, tibia and fibula, 10th rib, radius, humerus, and mandible were collected.

BMSC isolated from right femur

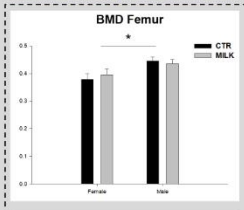


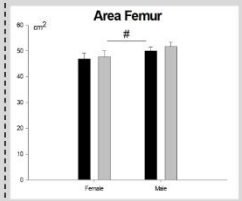
Dual-energy X-ray absorptiometry (DEXA)
Bone mineral content (BMC)
Bone mineral density (BMD)
Bone area

$$\text{BMD (g/cm}^2\text{)} = \frac{\text{Bone mineral content(g)}}{\text{Bone area (cm}^2\text{)}}$$

STATISTICAL ANALYSIS
GLM of SAS
Year 1: main effect = treatment, sex, treatment × sex; random = pig
Year 1 + Year 2 (only males): main effect = treatment, Year, treatment × Year; random = pig
Significant with P<0.05

RESULTS and DISCUSSION



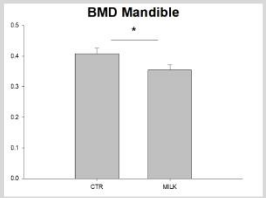


Year 1

Femur had a larger (* = P<0.05) bone density in males compared to females.

Females tended (# = P=0.11) to have smaller femurs compared to males, as expected.

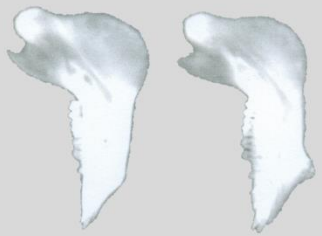
These data indicated that the difference between males and females is already evident prior to puberty.



The only difference observed was a lower bone density in the mandible of pigs receiving milk compared to control pigs.

All other analyses for BMD. BMC, and bone area for the humerus, radius, 10th rib, and tibia and fibula were not affected by feeding milk

Year 1 & 2



DEXA scans of the mandible
Left- Pig receiving control diet
Right- Pig receiving milk

CONCLUSIONS

Of all the measurements taken, only the bone density of the mandible was significantly lower in piglets receiving milk. This was surprising and the reason for the observed difference is still unknown.
It was expected that bones between males and female were different, but the observation that only the femur was different awaits an explanation. The lack of increase in bone density by feeding the USDA recommended 3 cups of milk/day was somewhat unexpected; however, this is a short term experiment that prevents making conclusions about long-term effects. More analyses are underway (see left).

ACKNOWLEDGMENTS The care of the animals was provided by the Swine crew lead by Matthew Kennedy. The work was supported by the USDA AFRI, NIFA Foundational Program: Exploratory Grant to M. Bionaz and M. Kutzler.

APPENDIX B

APPENDIX C

Effect of milk supplementation on Bone Growth in Pre-Pubertal Pigs

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Achieving peak bone mass during childhood and adolescence is associated with a decrease in the risk of osteoporosis and osteoporotic related fractures later in life. Milk has always been associated with increased bone health, strength, and development, but assessing the direct effect of milk on multiple properties of bone development is difficult with a human model. Therefore, this study aimed to use a pig model to evaluate the effect of milk consumption on the growing skeleton. For this, we used 24 pre-pubertal pigs randomly split into two groups to receive up to 750 mL of whole milk supplementation or an isocaloric maltodextrin solution for 12 weeks. The experiment was run in 2 trials: trial 1 utilized 5-week old Yorkshire pigs of 4 males and 2 females per group while trial 2 utilized 7-week-old Duroc×Yorkshire pigs of 6 males per group. Ultrasonography was used throughout the trial to record *in-vivo* bone growth. After 12 weeks, the pigs were euthanized and bones of the appendicular skeleton and the mandible were collected. All bones were analyzed with dual energy x-ray absorptiometry (DEXA). A three-point bending test was used for biomechanical testing. After fracture, the cortical bone thickness was taken at three regions of the femur. Data were analyzed using GLM procedure of SAS with TRT and Experiment (and Sex for Trial 1) as fixed effect and pig as random. To evaluate differences between groups, a Student's t-test was used with significance as $p = 0.05$. Of the

DEXA measurements taken, only the bone mineral density of the mandible was significantly lower in pigs receiving milk ($p = 0.045$). Upon further evaluation, the difference was in the mandibular body ($p = 0.03$). There was no difference in maximum force, stiffness, or extension of the femur from the biomechanical tests. The medial cortical thickness of the femur was higher in the control group (4.41±0.134 mm vs. 4.00±0.383 mm, $p = .03$), while the lateral thickness was higher in the group receiving milk (7.37±0.791 mm vs 6.25±0.946 mm, $p = 0.05$). Overall, there appears to be no effect of short-term milk supplementation on bone growth, mineral density, and strength This work was supported by the USDA, AFRI, NIFA Foundational Program: Exploratory Grant.