MONTMORENCY TART CHERRY SUPPLEMENTATION AND EXERCISE POSITIVELY AFFECT BONE MICROARCHITECTURE AND BIOMECHANICS IN THE GROWING SKELETON

By

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MONTMORENCY TART CHERRY

SUPPLEMENTATION AND EXERCISE POSITIVELY

AFFECT BONE MICROARCHITECTURE AND

BIOMECHANICS IN THE GROWING SKELETON

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Abstract:
Objectives: This study investigated the efficacy of Montmorency tart cherry (TC) alone and in combination with exercise on improving bone quality in young growing animals and the underlying mechanisms of action.
Methods: Six-week-old female C57BL/6 mice were randomly assigned to 4 groups (n=12 mice/group) in a 2x2 factorial design: control AIN-93G diet (CON), CON+exercise, TC (10% w/w), or TC+exercise. The exercise consisted of treadmill running for 30 min, 5 d/wk at 12 m/min and a 5° incline. Body weights were recorded weekly. After 8 wks of treatment, mesenchymal stem cells (MSC) in the tibial bone marrow were quantified via flow cytometry fluorescent activated cell sorting (FACS). Trabecular and cortical bone microarchitecture in the femur and lumbar vertebrae was assessed using micro-computed tomography. Biomechanical testing was performed using finite element analysis (FEA). The relative abundance of RNA for genes involved in osteoblast and osteoclast differentiation and activity was determined using RT-PCR. Data were analyzed using a 2-way ANOVA with TC and exercise as factors.
Results: At the end of the study, no differences in body weight were observed. Trabecular bone volume in the femur and spine was increased with exercise and TC ($p<0.05$), but there was no interaction. Cortical bone thickness in the vertebra was also increased by TC and exercise ($p<0.001$), but not in the femur. Trabecular bone strength and stiffness were increased in the vertebra in response to TC and exercise, but only in response to TC in the femur ($P<0.001$). An increase in bone marrow MSCs occurred in response to exercise ($p<0.01$), but not TC. However, the combination of TC and exercise reduced nuclear factor of activated T-cells 1 (*Nfatc1)* femur gene expression, a key regulator of osteoclastogenesis ($p<0.05$). TC also increased bone morphogenetic protein (*BMP)2* gene expression, a regulator of osteoblastogenesis.
Conclusion: Our data indicate that TC and exercise alone had positive effects on bone quality by suppressing regulators of osteoclastogenesis and increasing regulators of osteoblastogenesis. Even though the effects of TC and exercise were not synergistic, the effects of TC alone on bone were similar to and in some cases greater than exercise alone.

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CHAPTER I

INTRODUCTION

Introduction to the Problem

Osteoporosis is a degenerative musculoskeletal disease negatively affecting over 200 million people worldwide [1]. Low bone mass and microarchitectural deterioration are hallmark characteristics of osteoporosis, and consequently a marked increase in bone fragility and risk of fracture. Classic sites for osteoporotic fractures include those of the vertebrae, forearm, and proximal femur. Individuals who experience an osteoporotic fracture are at significantly higher risk of experiencing another fracture [2]. Osteoporotic fractures can be incapacitating, pose significant financial burden, and result in increased mortality rates [3]. Traditionally, white postmenopausal women have been considered one of the largest populations affected by osteoporosis; however men, other ethnicities (e.g. Asians and Hispanics), and all ages are susceptible depending on modifiable (e.g., sedentary, diet, or chronic inflammation) and non-modifiable (e.g., gender, age, or family history) risk factors [4]. Both types of risk factors contribute to one of two overarching determinants of osteoporosis, the ability to achieve optimal peak bone mass during growth and the loss of bone mass later in life.

Based on guidelines developed by the World Health Organization (WHO), bone mineral density (BMD) T-scores are used to define osteopenia (T-score -1.0 to -2.5) and

osteoporosis (T-score < -2.5) [5]. It is estimated that approximately 54% of white postmenopausal women have osteopenia and another 30% suffer from osteoporosis [6]. The prevalence of osteoporosis among women aged 50+ years is approximately 29.9%, but by the age of 80 years the prevalence increases to 77.1%. In contrast, for men the prevalence of osteoporosis increases from 16% at 50+ years to 46.3% at 80 years [6]. In 2010, prevalence of the disease in the United States was estimated to be approximately 10.2 million and by 2020 a projected 10.4 million more individuals will be affected [7]. Medical costs incurred by U.S patients with osteoporosis in 2005 were estimated to have been between 13.7-20.3 billion dollars [8]. Cumulative costs incurred by osteoporotic fractures are expected to increase to 22.8 billion dollars annually by 2016-2025 [7, 8].

The skeleton is a complex organ providing structure, locomotion, calcium storage, organ protection, and a compartment for the bone marrow. The skeleton is composed of two major subtypes of bone, trabecular and cortical, which are of significant interest in osteoporosis due to their potential influence on bone quality. Cortical bone, the dense outer layer of bone, provides a protective cover around the marrow space. The mature human skeleton is composed of approximately 80% cortical bone [9]. By comparison, trabecular or cancellous bone comprises 20% of the skeleton and forms a honeycomb-like structure within flat bones, such as the iliac crest, and in the ends of long bones [9]. Trabecular struts are organized into plates and rods and result in trabecular bone having a higher surface area to mass ratio than cortical bone. In general, cortical bone is stronger than trabecular bone, but has less elastic properties and can become brittle [10, 11]. Trabecular and cortical bone are composed of an organic matrix and inorganic salts [12]. The organic matrix is principally comprised of collagen, primarily

type I collagen, and also contains osteonectin, osteocalcin, bone sialoprotein II, bone morphogenetic proteins, and osteopontin, which are involved in regulating the catabolic and anabolic aspects of bone metabolism [13]. The inorganic portion of bone is composed largely of phosphate and calcium that form hydroxyapatite crystals within the collagen matrix and give structure to bone [12].

Bone is a dynamic tissue constantly undergoing complex processes, including bone modeling and remodeling. Bone remodeling, a cycle of bone resorption and subsequent formation that occurs within the bone multicellular units (BMU), prevents the accumulation of micro-damage within the mature bone and maintains its structural integrity. In contrast, bone modeling involves the shaping and sizing of bone by the independent activities of osteoblasts and osteoclasts, and is the primary process occurring in the young growing skeleton [14]. Both bone modeling and remodeling are carried out by osteoblasts, osteoclasts, and osteocytes. Osteoblasts, which differentiate from mesenchymal stem cells (MSCs), secrete the protein matrix (e.g. type 1 collagen, osteocalcin, osteonectin, and bone sialoprotein) that is then mineralized and thereby increases bone mass [15]. Osteoclasts on the other hand, originate from hematopoietic stem cells (HSCs) and resorb bone by creating an acidic micro-environment within the BMU and secreting proteases that catabolize proteins. Imbalances in the remodeling cycle that favor the catabolic activity of osteoclasts over the anabolic activity of osteoblasts contribute to the development of osteoporosis. Therefore, regulatory proteins of osteoclast and osteoblast differentiation and activity are highly relevant in the maintenance of healthy bone. Key regulators in osteoblast differentiation include runt related transcription factor 2 (Runx2) and Osterix, which promote the allocation of MSCs

to an osteogenic lineage [16]. The enzyme, alkaline phosphatase (ALP), which promotes extracellular matrix mineralization of bone by releasing phosphate, and procollagen I N-terminal peptide (PINP), a by-product of collagen formation are both considered indicators of osteoblast activity [17]. Furthermore, bone mineralization is regulated by proteins such as osteocalcin (OCN), phosphate-regulating neutral endopeptidase X (Phex), and peroxisome proliferator-activated receptor gamma (Ppar-γ) [17-19]. In terms of osteoclast differentiation, macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL), and nuclear factor of activated T-cells 1 (NFatc1) are key promoters of osteoclastogenesis while osteoprotegrin (OPG), acts as decoy receptor for RANKL [20, 21]. In contrast, cathepsin K and tartrate-resistant acid phosphatase (TRAP) are two of the major proteases involved in the degradation of the protein matrix [21, 22]. In addition to the regulator of osteoblast and osteoclast differentiation and mediators of their activity, pro-inflammatory cytokines such as interleukin (IL)-1 and IL-6, produced mainly by activated macrophages, increase the expression of M-CSF and RANKL that in turn promote osteoclastogenesis and bone resorption [21]. Osteocytes, which are bone cells formed when osteoblasts become embedded within the matrix, play an active role in regulating bone turnover and serve as the mechano-sensory cells in the bone with the capacity to stimulate osteogenesis via the Wnt/β-catenin pathway [23, 24].

Current FDA-approved pharmaceutical treatments for osteoporosis target either the osteoclast (i.e., anti-resorptive agents) or osteoblast (i.e., anabolic agents) activity. Anti-resorptive drugs (e.g. bisphosphonates or Denosumab) inhibit osteoclast differentiation and activity. Furthermore, bisphosphonates promote the induction of

osteoclast apoptosis [25]. The only FDA-approved anabolic option is Teriparatide, a recombinant human parathyroid hormone, that increases osteoblast differentiation and inhibits osteoblast apoptosis [26]. Anti-resorptive drugs are often considered a first line treatment option for osteoporosis, but in severe cases anabolic agents may be used alone or in combination with anti-resorptive agents [27]. While these drug therapies have shown to be effective in the reduction of fracture risk and consequently in the treatment of osteoporosis, they may not be ideal for long-term use due to side effects, cost and issues with compliance [28]. Therefore, investigation of novel alternatives is imperative.

One of the best predictors of osteoporosis risk is peak bone mass [29, 30]. Peak bone mass is defined as the quantity of bony tissue accrued by the end of skeletal maturation [29]. Females attain peak bone mass approximately 2 years after menarche, while males continue to accrue bone into their early twenties [30]. The higher the peak bone mass, the greater the margin for bone loss before becoming osteoporotic and at increased risk of facture. Identifying strategies for achieving an optimal peak mass would be a one ideal strategy to prevent osteoporosis.

Attainment of a higher peak bone mass may be achieved through adequate dietary intake (e.g., protein, calcium, and vitamin D), exercise, and avoidance of lifestyle behaviors that have negative effects on bone (e.g., smoking and alcohol). Exercise, namely weight-bearing exercise, is a well-established modifiable lifestyle factor that promotes the accumulation of bone mass [31]. It is widely understood that exercise that increases the forces experienced by bone resulting in micro-damage to bone tissue which necessitates repair or increased bone formation. The net effect is an increase in BMD. Other proposed mechanisms for enhanced bone formation associated with exercise

involve increased prostaglandin release, increased blood flow, and hormonal alterations [32]. Recently understanding the role of MSC in the response to weight-bearing exercise has become an area of interest. A study by Wallace et al. [33] showed that with short-term treadmill running (i.e., 30 min/d for 5 d) mice exhibit a decrease in bone marrow derived MSCs, thus suggesting their potential lineage allocation away toward osteoblasts. Another study by Maredziak et al. [34] examined the effects of a chronic treadmill running (i.e., 45 min/d, 3 d/wk, for 5 wks) and reported an increase in MSC populations in the exercising groups as well as a decrease in Ppar-γ signifying a reduction in the adipogenic lineage allocation of MSCs. These studies, taken together suggest that weight-bearing exercise not only increases the number of MSCs in the long-term, but may also increase their potential to differentiate into active osteoblasts [33, 34]. An increased population of osteoblasts likely promotes osteogenesis and consequently the accrual of a higher peak bone mass.

Likewise, dietary intake is paramount to the attainment of a high peak bone mass. Nutritional adequacy of protein, calcium, and vitamin D and their implications on osteoporosis are widely recognized by the scientific community as well as the general population. Less credited, but still critical nutrients, include: Zn, Cu, Mn, and vitamin K [35]. However, emerging evidence suggests a role of non-nutritive bioactive compounds (e.g., polyphenols) may play a pivotal role in skeletal health. Functional foods, including dried plum, green tea, and soy have shown promising osteoprotective effects [36-46]. The distinct ability of these foods to promote bone quality may be derived in part from their rich polyphenolic profiles [47, 48]. Polyphenols elicit potent anti-inflammatory and antioxidant effects and in some instances directly interact with functional proteins (e.g.

receptors, growth factors and cytokines). However, recent findings also suggest that the beneficial effects exerted by some of these functional foods may be due to other components such as non-digestible carbohydrates (e.g., fructooligosaccharides and galactooligosaccharides) [49]. Non-digestible carbohydrates elicit a number of favorable effects, including the promotion of beneficial bacteria (e.g. *Bifidobacterium)*, a reduction of pathogenic bacteria, and enhanced short chain fatty acid (SCFA) production [50]. In vitro and in vivo, SCFA supplementation with propionate and butyrate has been shown to downregulate TRAF6 and NFatc1 expression, osteoclastogenesis, and bone resorption [51].

Recently, our laboratory has investigated the osteoprotective effects of Montmorency tart cherries, which have a similar polyphenolic profile as dried plum containing high amounts of hydroxycinnamic acids and anthocyanins. Tart cherries are among the most polyphenol rich cultivars of cherries and they area also abundant in oligosaccharides including fructooligosaccharides and galactooligosaccharides [49]. Dietary supplementation with dried Montmorency tart cherry powder was shown to be efficacious in attenuating age-related bone loss and in some sites exhibit anabolic effects (*manuscript under review*). These findings provide evidence that suggests tart cherry supplementation suppresses the resorptive activity of osteoclasts, and may even promote the bone forming activity of osteoblasts. Whether or not these effects are mediated by altering the differentiation of osteoblasts or later phases of bone mineralization is not clear, but these findings have raised the question of whether or not combining tart cherry with exercise would promote bone accrual in the young growing skeleton.

Purpose and Hypothesis:

Therefore, the purpose of this study is to investigate the effects of tart cherry alone and in combination with exercise as a means to improve peak bone mass and bone quality in growing female C57BL/6 mice, while gaining insight into the underlying mechanisms of action. The hypothesis is that tart cherry and exercise in combination will yield a synergistic effect on bone quality in growing skeletons, which exceeds either treatment administered individually. The synergistic effect will result from an increase in MSC populations, in response to tart cherry and exercise, and their ability to differentiate into active osteoblasts.

Specific Aims and Working Hypotheses:

The hypothesis will be tested by accomplishing the following aims:

Specific Aim 1: To compare the effects of the tart cherry, exercise, and their combination on bone quality (i.e., BMD, trabecular and cortical microarchitecture, and biomechanical properties) in young growing animals.

Working hypothesis Aim 1: The combination of tart cherry and exercise will yield a synergistic effect on bone quality exceeding either variable administered individually.

Specific Aim 2: To determine the effect of tart cherry, exercise and their combination on bone marrow MSC populations and their progression towards an osteoblast lineage.

Working hypothesis for Aim 2: Tart cherry combined with exercise will result in a larger MSC population and a greater potential to form active osteoblasts than either variable individually.

Specific Aim 3: To assess the alterations in regulators of osteoblast (e.g. Runx2 and Osterix) and osteoclast (e.g. RANKL, OPG, and NFatc1) differentiation that occur in response to treatments.

Working hypothesis for Aim 3: The combination of tart cherry and exercise will, to a greater magnitude, promote osteoblastogenesis and suppress osteoclastogenesis compared to all other treatment groups.

Specific Aim 4: To evaluate the effects of tart cherry, exercise or their combination on osteoblast and osteoclast activity by examining circulating levels of biomarkers of resorption (i.e., CTX) and formation (i.e., P1NP), and local indicators of osteoblast activity and mineralization (e.g. type 1 collagen, OCN, Phex, and Ppar-γ), and osteoclast activity (e.g. CathK).

Working hypothesis for Aim 4: Tart cherry and exercise in combination will, to a greater magnitude, stimulate osteoblast activity and inhibit osteoclast activity above all other treatment groups.

Specific Aim 5: To assess alterations in antioxidant status indicated by gene expression of key enzymes involved in scavenging free radicals in bone (e.g. Gpx1 and SOD1) resulting from tart cherry, exercise or their combination.

Working hypothesis for Aim 5: Tart cherry will increase the levels of endogenous antioxidant indicators.

Limitations

As is the case with any research, the study presented here is not without limitations. These limitations include lack of information related to: 1) whether or not protein levels of key regulators and indicators is affected; 2) whether or not alterations in osteoblast and osteoclast activity occurred earlier in the intervention period; 3) whether or not gender and age could influence the response; and 4) whether or not the response observed here, especially in the vertebra would translate to bipedal animals. At this time, data supporting the site-specific mechanisms through which tart cherry and exercise alters bone quality are based on regulators of bone modeling and remodeling at the transcriptional level. It remains to be seen if tart cherry and exercise induce protein changes, which will be determined in subsequent analyses. Additionally, this study is limited to analysis of effects on bone metabolism after 8 weeks of treatment and changes occurring earlier may not be apparent. This study is also limited to providing evidence for the efficacy of tart cherry and exercise in young female mice. Subsequent studies will be necessary to determine the effects in both male and aged mice.

Lastly, the use of an animal model, specifically a quadruped animal model, in this study could be considered a limitation in some respects. Loading effects of exercise in this model are not likely to directly translate into biped animals. Therefore, while the study design simulates the effects of diet and exercise on the development of peak bone mass, the site-specific outcomes may not translate to humans. Subsequent clinical studies are needed to determine the skeletal response to exercise and tart cherry alone and in combination.

CHAPTER II

REVIEW OF LITERATURE

Introduction to Osteoporosis

Osteoporosis is a chronic degenerative bone disease affecting millions of individuals and causing an estimated 9 million fractures worldwide each year with 4.5 million occurring in the United States and Europe alone [52]. The World Health Organization (WHO) defines osteoporosis as a bone mineral density (BMD) more than 2.5 standard deviations below the population's mean BMD and osteopenia between 1 and 2.5 standard deviations below the mean BMD for young healthy women [2]. Low bone mass is the hallmark characteristic of osteoporosis; however, the disease is also marked by micro-architectural deterioration, bone fragility, and consequently fracture risk [2].

Traditionally, there have been two subtypes of osteoporosis considered, primary and secondary, which differ based on etiology. Primary osteoporosis is the result of classic risk factors (i.e. age related bone loss) (**Table 1**), whereas secondary osteoporosis is the result of endocrine, metabolic, or immune disorders [53]. Therefore, management of modifiable risk factors is critical in the prevention of osteoporosis in all individuals, but especially in those with multiple non-modifiable risk factors (e.g. age and gender). White postmenopausal women are one of the highest risk populations for developing osteoporosis; however, men, other ethnicities, and individuals of all ages are susceptible

when subjected to numerous risk factors (Table 1). Postmenopausal women are more susceptible to osteoporosis due to associated declining in estrogen. Perimenopausal rates of bone loss from the spine and hip are on average 0.018 and 0.10 g/cm^2 per year, respectively. However, postmenopausal rates increase to 0.022 and 0.13 g/cm^2 per year, respectively [54]. Overall, women lose approximately 50% of their trabecular bone mass and 30% of their cortical bone mass over their lifetime, but half of this loss occurs in the first ten years after menopause [54].

Table 1: Risk factors for the development of osteoporosis [4].

Non-Modifiable Risk Factors	**Modifiable Risk Factors**
Previous Fracture	Physical activity
Female	Vitamin D intake & sun exposure
Ageing	Calcium intake
Family history (maternal) of fracture	Alcohol consumption
Primary and secondary amenorrhea	Smoking
Early menopause	Corticosteroid use
Osteopenia	Low body weight
Malabsorption syndromes	
Primary hyperparathyroidism	

Screening and Prevalence of Osteoporosis

Screening and diagnosis of osteoporosis are of critical importance due to the increasing prevalence and the tendency for diagnosis to occur post fracture. The gold

standard for diagnosis of osteoporosis is dual-energy x-ray absorptiometry (DXA), which provides an aerial measurement of BMD. Based on the guidelines set for by the National Osteoporosis Foundation and College of Preventative Medicine, DXA scans are recommended for women beginning at 65 years of age and men beginning at 70 years of age when no risk factors are present. Both men and women who have risk factors (e.g. modifiable and non-modifiable) are advised to get DXA scans beginning at age 50. However, adherence to routine screening guidelines is uncommon [28, 55]. Therefore, osteoporosis often goes undiagnosed, which has negative consequences on fracture prevention [56]. While BMD is used to diagnose osteoporosis, a better indicator of fracture risk would include evaluation of trabecular and cortical bone microarchitecture. However, clinical screening using computed x-ray tomography would be required to evaluate bone microarchitecture and at the present it is neither economical or practical [53].

Based on BMD measurements it is estimated that approximately 54% of white postmenopausal women have osteopenia and 30% have osteoporosis. The prevalence of osteoporosis among women who are 50 years of age is approximately 5%, but by the age of 85, the prevalence of osteoporosis increases to 50%. By comparison, for men the increase is from 2.4% to 20% in these two age groups [57]. In 2010, the prevalence of osteoporosis in the United States was estimated to be approximately 10.2 million and was expected to increase to more than 10.4 million by 2020 [7]. Medical costs incurred by Americans with osteoporosis in 2005 were estimated to be between 13.7-20.3 billion dollars and cumulative costs incurred due to osteoporotic fractures were projected to increase to $228 billion for 2016-2025 [7, 8].

Osteoporotic Fractures

Classically, the most common sites for osteoporotic fractures include the spine, forearm, and hip. Individuals who endure one fracture have a significantly higher chance of experiencing another. Based on the prevalence of osteoporosis, current treatment options, and population demographic shifts, Wolfe and Pfleger estimated that more than 50% of women aged 50 years and older will experience an osteoporotic fracture in their lifetime [57]. Hip fractures are considered to be one of the most devastating and debilitating outcomes of osteoporosis and are a major public health concern [58]. An estimated 1.66 million hip fractures occurred worldwide in 1990 and this number is expected to rise to 6.26 million by 2050 [59, 60]. Since 1990, the incidence of hip fractures has increased with the highest rates occurring in North America and Scandinavian countries [59]. It has been well established that hip fractures lead to increased mortality rates. A study by Sernbo and Johnell [3] reported that 34% of men and 20% of the women died within the first year of hip fracture. Additionally, the study examined 1,429 cases of hip fracture worldwide and reported the average cumulative cost of a hip fracture, including surgery, to be $26,000 [3]. However, Panula and colleagues [61] followed hip fracture patients (n=428), 65 years of age and older, and showed a mortality rate of 27.3% after the first year and 79% at the end of a 9 year follow-up [61]. Fractures are costly, debilitating, and in the case of hip fracture may lead to increased mortality; therefore, fracture prevention is of paramount importance.

Based on the increasing prevalence of osteoporosis, the status of bone health is a global concern. The development of osteoporosis stems from two overarching determinants: the failure to develop a high peak mass early in life and loss of bone mass

with age or resulting from exposure to risk factors. The accrual of a high peak bone mass is regarded as the single greatest determinant in the development of osteoporosis [29, 62]. Consequently, targeting development (puberty) is favorable in prevention.

Peak Bone Mass and Osteoporosis

Peak bone mass is regarded as the greatest predictor in the development of osteoporosis [29]. Peak bone mass is considered the quantity of bone accrued by the end of skeletal maturation. Achievement of peak bone mass occurs after the cessation of puberty. For females this usually occurs between 15-20 years of age and between 20-25 years in males [63]. Peak bone density, a measure of bone mass per bone volume, measurements are similar between genders. However, peak bone mass, independent of volume, may differ depending on factors such as an individual's height and frame size [64]. During pubertal growth, sex steroids (e.g. estrogen and testosterone), growth hormone (GH), and insulin-like growth factor 1 (IGF-1) increase significantly [65]. GH is known to stimulate pre-chondrocyte and pre-osteoblast proliferation as well as IGF-1 production, which subsequently promotes chondrocyte and osteoblast differentiation and therefore cartilage and bone formation, respectively [65]. Growth plate osteoblasts contain androgen receptors, which testosterone and estrogen can bind to induce epiphyseal fusion resulting in the cessation of bone growth or elongation [64]. Weise and colleagues found that higher levels of estrogen resulted in increased rates of plate senescence and epiphyseal fusion [66]. Furthermore, estrogen acts to inhibit bone resorption through promoting osteoclast apoptosis [67]. A better indicator for the development of peak bone mass in females is the onset of menarche. During pubertal growth, menarche occurs concurrently with the elevation in estrogen, which promotes

epiphyseal fusion. Normally, no further gains in peak bone mass are observable in females within 2 years of menarche [62].

Rate of bone mass accrual changes throughout skeletal maturation. Based on the work of Soyka and colleagues [64], bone mass accrues throughout childhood with peak rates occurring during early to mid-puberty before slowing in late puberty **(Figure 1)**. The achievement of a high peak bone mass is critical as it allows for a greater margin of bone loss before an individual develops osteopenia and subsequently osteoporosis. Consequently, years 11-14 for females and 13-17 for males are critical periods to target for maximizing peak bone mass [64]. Lifestyle choices such as diet and exercise have a significant effect on the accrual of bone early in life, estimated to influence 20-40% of adult peak bone mass [68, 69]. Therefore, targeting modifiable lifestyle factors (i.e. diet and exercise) during development may promote the development of a high peak bone mass, and be efficacious in the prevention of osteoporosis.

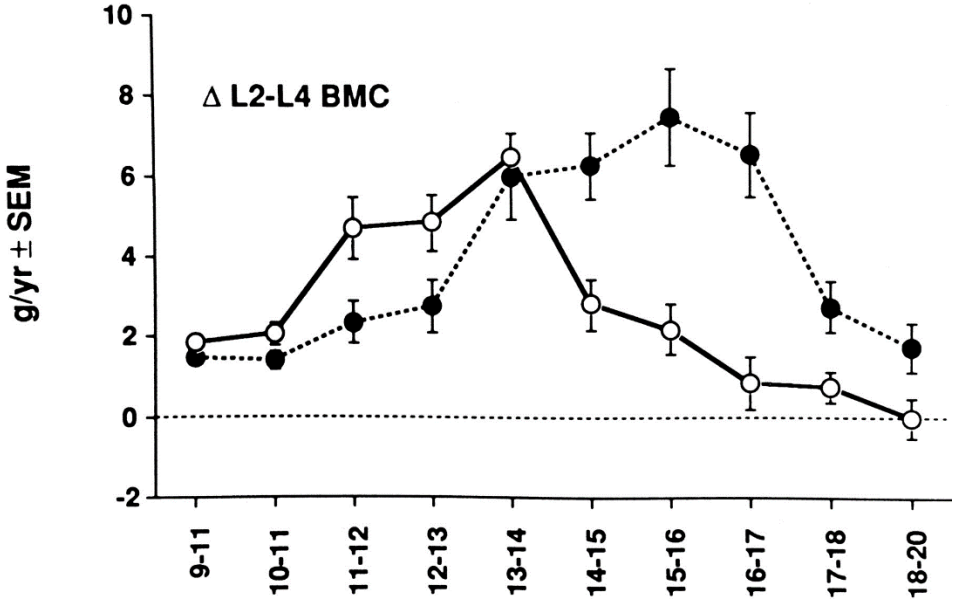

Figure 1: Rates of vertebral bone mass accrual by age females (○) and males (•) [64].

Bone Anatomy and Structure

The skeleton is a complex organ composed of inorganic salts and an organic matrix [12]. The skeleton provides structure, calcium storage, locomotion, organ protection, and houses the bone marrow. By weight, the inorganic salt phase of bone tissue makes up approximately 60% of the skeleton [70]. This phase is composed of hydroxyapatite, a crystalline structure of calcium and phosphorus $[Ca_3(PO_4)_2]_3Ca(OH)_2$ [70]. Hydroxyapatite crystals are plate shaped and contain numerous impurities including: 1) carbonate in place of phosphate, 2) potassium, strontium, magnesium, or sodium in place of calcium, and 3) chloride or fluoride in place of hydroxyls. These impurities interfere with many bone processes including mineral homeostasis and remodeling [70]. The organic phase of bone makes up for nearly 30% of total bone weight. This phase is composed of numerous proteins, of which type I collagen is the most abundant. Type I collagen is responsible for ~90% of the organic matrix with the remaining 10% being non-collagenous proteins [70]. Some of these non-collagenous proteins include osteocalcin, osteonectin, osteopontin, and bone sialoprotein [71]. However, type I collagen is the primary structural component of the protein matrix while the non-collagenous proteins contribute to other biological functions, including affecting osteoclast and osteoblast function, via cytokines or growth factors [70].

Bone may be further classified into two subtypes: cortical bone and trabecular (i.e., cancellous) bone. Cortical bone is the dense outer layer of bone surrounding and protecting the marrow space. Cortical bone in healthy adults has a porosity of less than 5%, but greater porosity is commonly seen in developing bones or in the aging skeleton [9]. The outer layer of cortical bone is sheathed by the periosteum while the inner surface

is covered by the endosteum. The periosteum is fibrous connective tissue anchored to the periosteal surface of the cortical bone via collagen fibers. The endosteum is a membrane on the endosteal surface of cortical bone which is in contact with the marrow space and trabecular bone. Bone formation on the periosteal surface exceeds resorption in growing skeletons resulting in an increased bone and medullary cavity diameter [9]. Cortical bone is dense and strong, but possesses few elastic properties, thus it has the potential become brittle. Cortical bone microarchitecture is assessed in terms of its thickness, area and porosity [10]. Trabecular bone, or cancellous bone, forms a complex meshwork resembling a honeycomb like structure. This trabecular network is composed of struts, which are can have a plate- and rod-like organization. Compared to cortical bone, cancellous bone possesses a much higher degree of anisotropy indicative of its greater elastic potential.

Collectively, the mature human skeleton is made up of approximately 80% cortical bone and 20% trabecular bone [9]. However, this ratio differs depending on the site. Long bones such as the femur are composed of three sections: the diaphysis, metaphysis, and epiphyses **(Figure 2)**. The diaphysis is regarded as the long hollow shaft residing between the proximal and distal ends of the bone. Consequently, this portion of the bone is primarily comprised of cortical bone. The metaphysis and epiphysis reside proximal to and distal to the growth plate at both ends of the long bone, respectively. Unlike the diaphysis the metaphysis and epiphysis contain significant amounts of trabecular bone, which reside within a thin layer of the cortices [9].

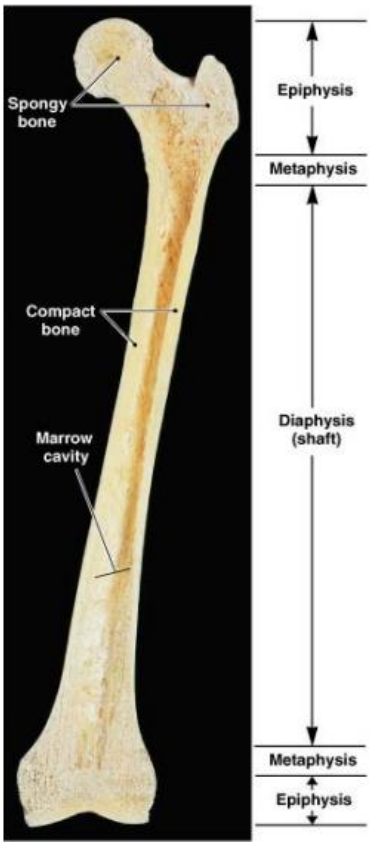

Figure 2: Bone diaphysis, metaphysis, and epiphyses[72].

Bone Quality Effectors

The skeleton is a dynamic organ that is constantly undergoing complex processes, including bone modeling and remodeling. Early in life, the skeleton primarily undergoes bone modeling [9]. During bone modeling, bones are shaped, reshaped, and positioned by the uncoupled anabolic activity of osteoblasts and catabolic activity of osteoclasts. Bone shape and size is altered in response to physiological factors (e.g. PTH and sclerostin) and/or mechanical loading [73]. Based on the magnitude and frequency of mechanical loading, bone modeling may be directed towards atrophy, maintenance, or hypertrophy of boney tissues [74] **(Figure 3)**. Kontulainen and colleagues [75] showed this bone-

modeling phenomenon in the dominant arm of tennis players, which exhibited a higher bone mass than the non-dominant arm due to exposure to repeated strain. Repeated strain elicited by the sport promotes hypertrophy where resorption (R) is less than formation (F). Promoting an anabolic state of bone modeling is essential in the attainment of an optimal peak bone mass during skeletal maturation and consequently the prevention of osteoporosis later in life. Therefore, the incorporation of frequent mechanical loading during development is likely to prove effective in the prevention of osteoporosis.

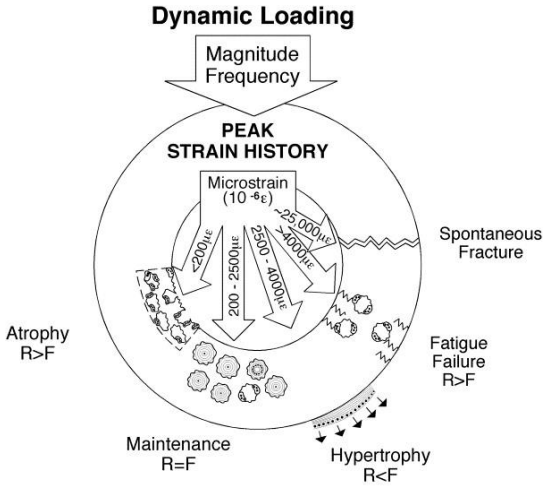

Figure 3: Effects of loading magnitude and frequency on bone modeling response [74]. Abbreviations: R resorption, F formation. Atrophy, resorption exceeds formation (R>F); Maintenance, resorption equals formation (R=F); Hypertrophy, formation exceeds resorption (R<F).

As the skeleton ages, the primary metabolic process within the bone shifts from modeling to remodeling. The function of bone remodeling is foremost to maintain bone integrity through the resorption of old bone and subsequent formation of new bone. This cycle prevents the accumulation of micro-damage and associated bone fragility. Additionally, bone remodeling is critical in mineral homeostasis [76]. The bone

remodeling cycle has been described as four distinct phases: activation, resorption, reversal, and formation. Each phase involves the recruitment and activation of specific cells, namely osteoclasts and osteoblasts, organized into coupled basic multicellular units (BMUs) [77]. At a single point in time, there are millions of active BMUs at different stages in the remodeling cycle throughout the skeleton.

During the activation phase of bone remodeling, multinucleated pre-osteoclasts are formed through the recruitment and subsequent fusion of mononuclear cells (e.g. monocytes and macrophages). These newly formed pre-osteoclasts bind to the bone matrix where they are signaled to differentiate into mature osteoclasts, a highly regulated process known as osteoclastogenesis. Osteoclastogenesis is closely regulated by the cytokines, receptor activator of nuclear factor kappa-β ligand (RANKL) and osteoprotegrin (OPG). RANKL, which is excreted by cells of the osteoblast lineage and T cells, binds with the RANK receptor on the surface of pre-osteoclasts, activating nuclear factor-activated T-cells-1 (NFatc1), the master transcription regulator of osteoclast differentiation [21]. This process occurs via TNF receptor associated factor-6 (TRAF6), nuclear factor- κβ (NF-κβ) and c-Fos signaling pathways [78]. NFatc1 can also bind to its own promoter region and increase expression via auto-amplification. While RANKL stimulates osteoclastogenesis, its soluble decoy receptor, OPG, prohibits the RANK-RANKL interaction and inhibits osteoclast formation. Both RANKL and OPG can be produced by osteoblasts in response to stimuli such as cytokines (e.g., tumor necrosis factor-alpha (TNF-α), IL-1, M-CSF and IL-6) and hormones (e.g., parathyroid hormone (PTH), 1,25-dihydroxy vitamin D, and calcitonin) [16]. Foods rich in bioactive components with anti-inflammatory properties such as dried plum's polyphenols have

been shown to downregulate expression of RANKL, TNF-α, and NFATc1 thereby inhibiting osteoclastogenesis and consequently the activation phase [47, 79, 80].

Initiation of the activation phase can also occur in response to micro-damage brought about by changes in mechanical loading from factors such as weight-bearing exercise [22]. The osteocytes, which are osteoblasts that become surrounded by bone matrix, are responsible for orchestrating this response [76]. These cells have cytoplasmic processes which maintain complex canalicular networks to surface cells, including osteoclasts and osteoblasts. Micro-damage or micro-fractures to bone result in the disruption of these osteocyte networks or osteocytes themselves. Damaged osteocytes secrete CSF and RANKL to promote osteoclastogenesis and subsequent resorption of the damaged bone [16]. Furthermore, osteocytes have a mechano-sensory function critical in detecting changes in mechanical loading [22]. Through site specific deletion of osteocytes it has been shown that bone does not respond to unloading, highlighting the important role that osteocytes play in these processes [24].

The maturation of pre-osteoclasts into mature osteoclasts marks the start of the resorption phase. During the resorption phase, mature osteoclasts form microscopic annular sealing zones on the bone surface and create an acidic microenvironment by secreting hydrogen ions **(Figure 4)**. The low pH promotes the mobilization of the bone mineral component of the tissue. Osteoclasts also secrete cathepsin K, matrix metalloproteinase 9, gelatinase, and tartarate resistant acid phosphatase to digest the organic matrix [9]. Cathepsin K is considered a key enzyme involved in the digestion of the proteins matrix of the bone, namely type I collagen resulting in carboxy-terminal and amino-terminal cross linked telopeptide of type I collagen, CTX and NTX, respectively

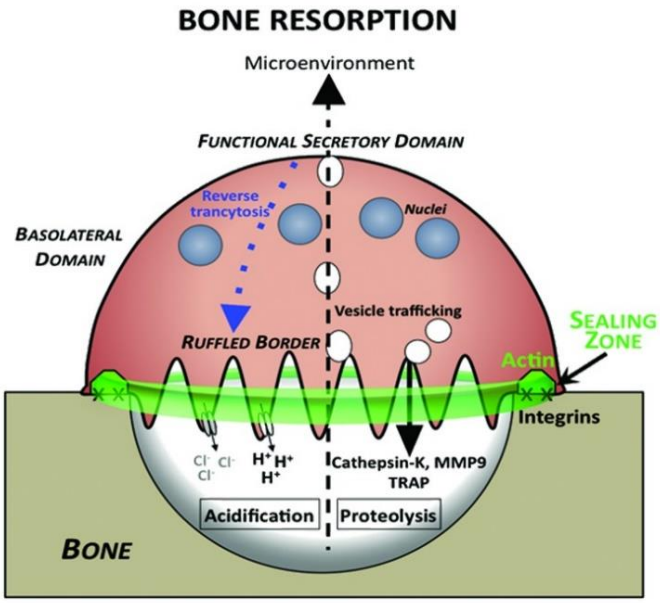

Figure 4: Annular sealing zone and bone resorption [81].

[76, 82]. The ensuing digestion of bone results in the formation of Howship's lacunae on cancellous bone surfaces and Haversian canals on cortical surfaces. Bone resorption in humans requires 2 to 4 weeks and culminates with osteoclast apoptosis [9].

The reversal phase occurs at the end of the resorption phase and transitions to the formation phase. Signaling required for this transition has not been well defined. A proposed mechanism is the release of TGF-β from the bone matrix during resorption that can inhibit osteoblast synthesis of RANKL, consequently downregulating osteoclastogenesis. Other proposed pathways that regulate this process include IGF-I and II, which are also released during resorption and are known to attract osteoblast precursors and promote their differentiation [83]. Overall during this phase an osteogenic environment is formed at remodeling sites [77].

The bone formation phase requires between 4 to 6 months in humans and is carried out chiefly by osteoblasts. Osteoblasts originating from pluripotent MSCs, which have the potential to differentiate into a number of different cell types including adipocytes, chondrocytes, and myoblasts [76]. Commitment of MSCs to the osteoblast lineage is mediated by the canonical Wnt/β-catenin pathway and its activation of the "master transcriptional regulator" Runx2 [9, 20]. The cell lineage progression known as osteoblastogenesis proceeds as follows: MSC, immature osteoprogenitor, mature osteoprogenitor, pre-osteoblast, and mature osteoblast **(Figure 5)**.

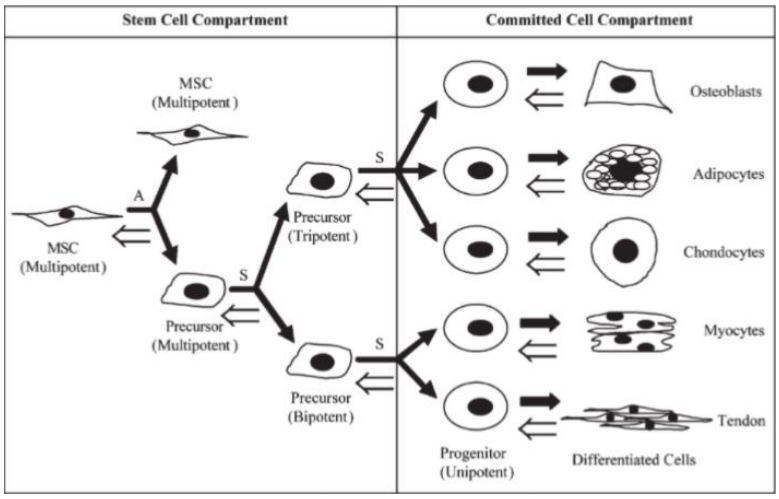

Figure 5: Mesenchymal stem cell lineages [84].

Following commitment to the osteoblast lineage, osteogenesis proceeds in three phases (i.e., proliferation, matrix maturation, and mineralization) with each phase exhibiting unique osteoblast markers **(Figure 6)** [85].

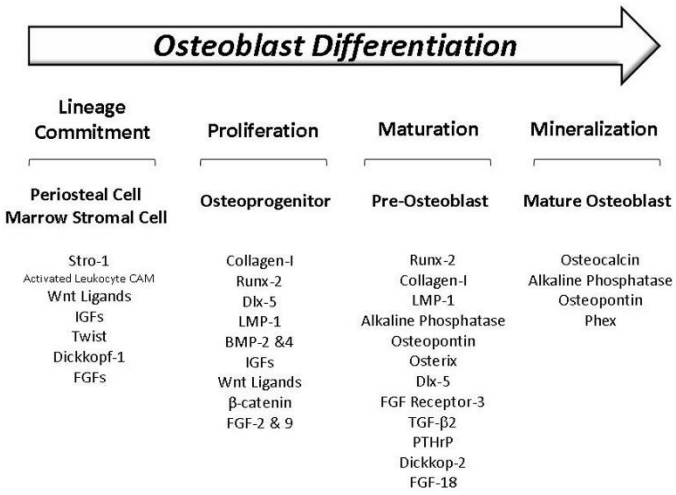

Figure 6: Phases of osteogenesis and protein expression [86].

The first phase is marked by continual proliferation of pre-osteoblasts. Osteoprogenitor cells in this stage express fibronectin, type 1 collagen, TGFβ receptor1, and osteopontin [20]. Transcriptional regulators of osteoblastogenesis include osterix and Runx2. Osterix, like Runx2, promotes differentiation of osteoblasts, but is only expressed by pre-osteoblasts and differentiated osteoblasts [85]. Runx2 is expressed at all phases of osteoblastogenesis and is considered the master transcriptional regulator due to its upstream expression compared to osterix. Another key factor in osteoblastogenesis is peroxisome proliferator activated receptor gamma (Ppar-γ), the key regulator in MSC differentiation into adipocytes [18]. Therefore, elevated Ppar-γ competes for MSCs thereby promoting their lineage allocation to adipocytes which normally corresponds with a decrease in osteoblastogenesis [18]. Also of recent interest in osteogenesis is bone morphogenic protein-2 (BMP2). Sun and colleagues [87] showed that treatment of rat bone marrow derived MSCs with BMP2 enhanced proliferation and induced an altered osteogenic phenotype characterized by increased expression ALP, OCN, OPN, and type-

1 collagen. Furthermore, BMP2 treatment of MSCs increased nuclear accumulation of Runx-2, the master regulator of osteogenesis [87]. Statin drugs and some bioactive components in foods have been shown to promote osteogenesis [88]. Our lab and others have shown that polyphenol-rich foods, such as dried plums and blueberries, promote the proliferation stage of osteogenesis through the upregulation of BMP2 and IGF-I which leads to increases in Runx2 and Osterix, [48, 79, 89].

Stage two of osteogenesis is marked by the halt of cell proliferation and ensuing start of differentiation [20]. The extracellular matrix in stage two develops through the expression of alkaline phosphatase (ALP) and type 1 collagen. Procollagen type I N-terminal and C-terminal propeptides (PINP and PICP) are produced from the cleavage of type I procollagen into type I collagen by N-proteases and C-proteases [90]. Linkhart and colleagues [91] demonstrated that circulating serum PINP is directly proportional to collagen formation.

The third and final stage is marked by the expression of Phex, Ppar-γ, and incorporation of osteocalcin (OCN) into the matrix and subsequent mineralization [20]. Phex, an osteoblast integral membrane protein, functions to degrade osteopontin, which is expressed in phase I and inhibits bone mineralization. Consequently, Phex degradation of osteopontin allows for increased bone mineralization [19]. Ppar-γ functions to down-regulate bone mineralization by inhibiting the expression of ALP and thereby decreasing the formation of hydroxyapatite [92]. The end of the formation stage is marked by the appearance of osteocytes within the BMU. During formation, osteoblasts can become entombed in bone matrix and develop the phenotype consistent with osteocytes [20].

The primary characteristic differing between bone modeling and remodeling is the uncoupled actions of osteoblasts and osteoclasts in bone modeling. The independent actions of osteoblasts and osteoclasts during bone modeling allows for greater influence on bone formation without first requiring bone resorption to occur. Bone modeling is the prominent process occurring in developing skeletons making it critical in the attainment of peak bone mass. Therefore, it stands to reason that factors that promote bone formation (e.g., mechanical loading and polyphenol-rich foods) should be considered important components of osteoporosis prevention strategies by their ability to enhance the anabolic bone modeling state and the subsequent attainment of an optimal peak bone mass.

Current Treatments in Osteoporosis

Current FDA-approved pharmaceutical treatments for osteoporosis target either anti-resorptive or anabolic mechanisms of action. Anti-resorptive drugs (e.g. bisphosphonates or Denosumab) are the first line of defense and consequently the most widely used drugs in the treatment of osteoporosis [93]. A common bisphosphonate, Alendronate, exerts its effects in a number of ways including: reducing osteoclast differentiation, reducing osteoclast activity, and promoting osteoclast apoptosis [94]. On the other hand, Denosumab is a monoclonal antibody which binds RANKL, thereby inhibiting osteoclast differentiation and activation. In response to bisphosphonate or Denosumab treatment, the resorptive phase of bone remodeling is significantly reduced, thereby reducing the rate of bone loss [27].

Teriparatide is the only approved anabolic treatment option for osteoporosis and it acts to promote bone formation as opposed to slowing the rate of bone loss. Teriparatide,

a synthetic form of human parathyroid hormone (PTH), functions by increasing osteoblast formation while also inhibiting osteoblast apoptosis [26]. This mechanism of action affects the reversal and formation phases of bone remodeling, ultimately leading to increased bone mineralization. In severe cases, such as individuals who have already suffered a hip fracture [95], anabolic agents may be used in combination with anti-resorptive drugs to effectively target all stages of bone remodeling and yield a synergistic effect [27].

While these drug therapies have shown notable efficacy in fracture risk reduction in both non-vertebral and vertebral fracture sites [96], they can be cost prohibitive and produce undesirable side effects [28]. Teriparatide is estimated to cost $800 per month which is ten to thirty times the cost of anti-resorptive agents and requires a daily self-administered injection [97]. Moreover, long-term safety of Teriparatide (>24 months) has not been established [98]. Anti-resorptive drugs such as bisphosphonates have numerous reported side effects, including gastrointestinal distress, musculoskeletal pain, hypocalcemia, increased risk of esophageal cancer and atypical femoral fractures [99]. Therefore, investigation and development of novel alternatives which are more effective is imperative in the prevention and treatment of osteoporosis.

Exercise and Bone

Exercise, namely weight-bearing exercise, has long been heralded as a critical modifiable lifestyle factor in the prevention of osteoporosis. Exercise has been shown to increase peak bone mass while also attenuating age associated bone loss [100]. Largely the effectiveness of exercise has been attributed to loading and micro-damage [101].

Other proposed mechanisms for enhanced bone formation associated with exercise involve prostaglandin release, increased blood flow, and hormonal alterations [32]. Loading and micro-damage is sensed by osteocytes activating the Wnt/β-catenin pathway which transmits signals to surface bone cells (i.e., osteoblasts and osteoclasts), thus altering bone cell activity [24]. Increased loading stimulates the Wnt/β-catenin pathway in osteoblasts and subsequent activation of Runx2. Runx2 promotes osteoblast differentiation and consequently increased bone formation. Equally important to the magnitude of the load is the frequency of loading [74]. Increased bone formation resulting from exercise is supported by Gönül and colleagues who found that treadmill running mice resulted in increased wet and dry weights of the femur and tibia while also increasing the Zn, Mg, and Ca mineral contents of bones [31].

Recently, some investigators have begun to explore the role of exercise-induced changes in MSC populations as a possible mechanism through which exercise improves bone quality. Emerging evidence suggests that exercise influences MSC populations and their lineage allocation. For example, Wallace et al., [33] exposed 4 week-old outbred female mice (Hsd:ICR) to treadmill running (12m/m for 30 minutes) for either 5 days or 5 days a week for 4 weeks. The skeletal effects of the 4-week exercise protocol were analyzed via μCT and bone marrow cells of mice that exercised for 5 days were subjected to fluorescent activated cell sorting (FACS) to characterize the MSC short-term response to exercise. μCT showed significant enhancements of the cortical bone and moments of area in the ulna, femur, and tibia, but not in the humerus or radius. Additionally, trabecular bone was enhanced by treadmill running in the femur and tibia. FACS analysis revealed a significant decrease in the MSC population in the femur after the exercise

period, but no difference was observed in the humerus. The authors concluded that the MSC populations' ability to differentiate towards osteoblasts may be a better indicator of bone adaptation than the external forces caused by exercise. Marwdziak et al. [34] also examined the effects of treadmill running on MSC populations using 4 week-old male C57Bl/6 mice. Mice were subjected to treadmill running for 5 weeks after which MSC populations of the tibia and femur were quantified using FACS. The exercised animals had a significantly higher population of bone marrow derived MSCs. Additionally, the authors reported elevated alkaline phosphatase activity, increased osteopontin and osteocalcin in cultured bone marrow derived MSCs, and decreased marrow cavity fat in response to treadmill running. The authors suggested that the data is indicative of exercise's ability to increase the MSC population and promote its osteogenic potential while inhibiting its adipogenic potential. Taken together, these two studies suggest that exercise influences the lineage progression of MSCs towards osteoblasts. However, the studies differ on the effects of exercise on MSC populations which may indicate that the decreased in MSCs observed by Wallace and colleagues [33] was a transient response to exercise while the results reported by Marwdziak et al [34] were an adaptive response to a prolonged exercise regimen. Although questions remain about the long-term effects of exercise on MSCs, these findings suggest that a chronic exercise regimen results in an increase in MSCs and osteogenesis making it an important component of strategies designed to increase peak bone mass.

Biomechanical Properties

Bone's ability to withstand stress induced by exercise or other forces may be assessed via its biomechanical properties. Bone tissue obtains a rigid structure due to its

inorganic mineral content; however, it also possesses elastic properties derived from its organic component, largely collagen. Bone's ability to handle a load is determined by its strength, hardness, and type of load [102, 103].

Terminology that is often used to describe bone include bone strength and hardness. The strength of bone refers to the maximum sustainable load before failure, while the hardness of bone refers to its ability to deform in response to force. Bone is an anisotropic material, meaning that depending on the direction of the applied load the response can differ. In general, bone possesses a greater capacity to withstand loads in the longitudinal direction due in part to the repeated exposure to forces in this direction as a result of gravity. [102]. Additionally, bone possesses viscoelastic characteristics, meaning it responds to loads differently based on the speed and duration at which they are applied. Bone tissue can handle higher loads when delivered fast [103]. This is due to the bone's ability to deform when a load is first applied, known as the elastic response. However, a load which is applied continually, exhausts this initial ability of bone to deform, which is known as the plastic response, and thus requires significantly less force to result in micro damage or fracture [103]. Therefore, bone is most able to withstand a high magnitude force which is administered quickly and in the longitudinal direction.

An important determinant in the bone's ability to handle a load is the type of the load. Types of load include: compression, tension, shear, torsion, and bending (**Figure 7**) [103]. A compression load may be produced by gravity, muscles, or an external force.

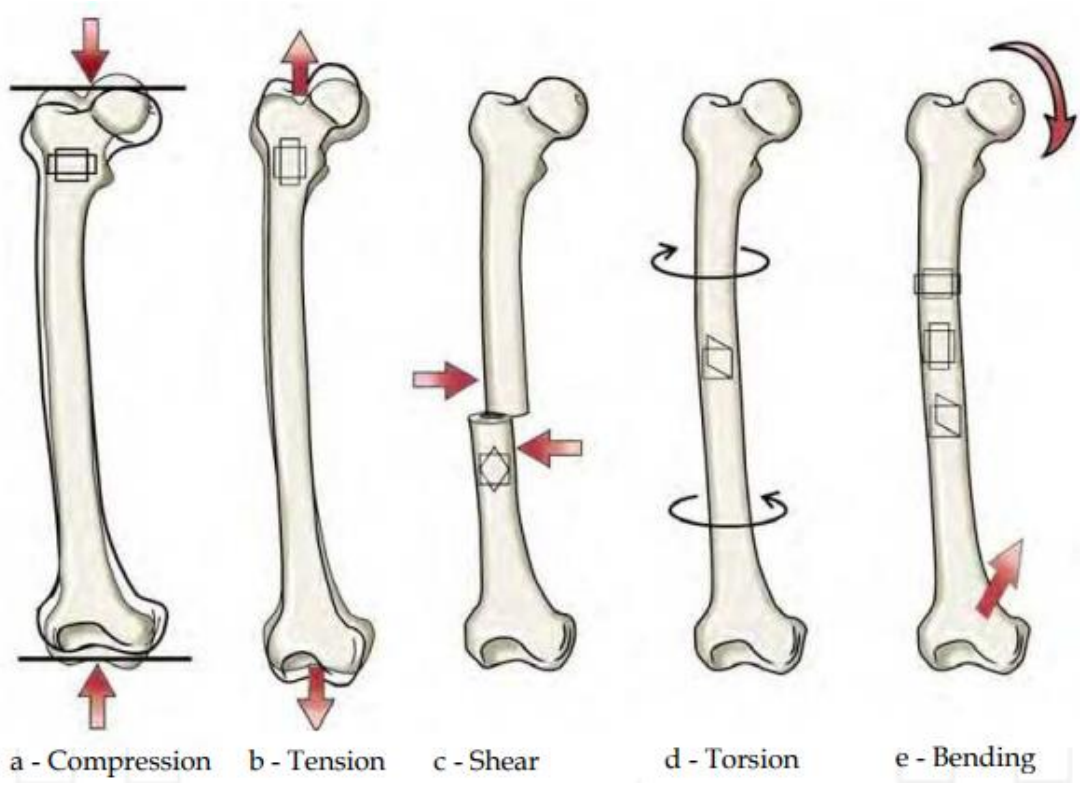

Figure 7: Types of Biomechanical Loads.

It is characterized by forces pressing simultaneously against opposite sides of the same bone [103]. Compressive force results in shortening of the bone in the plane of the applied force and extension of the bone in the perpendicular plane (i.e. shorter and thicker). Tensive stresses are opposite compressive stresses and are usually caused by muscles-tendon actions of pulling on the bone. Exposure to tensive forces causes the bone to elongate and narrow [103]. Shear forces occur when a bone is exposed to compressive or tensive forces which possess force that is also applied in the horizontal plane [102]. The bone fails quickly under shear forces due to anisotropic properties. Classically, shear forces cause issues in vertebral discs and femoral condyles [103]. Torsion of the bone is indicative of force being applied in a rotational manner. Torsion of

the bone (e.g. during exercise) produces shear, compressive, and tensive forces. Lastly, bending is a type of deformation that bone undergoes when a force is applied in a direction that doesn't have support offered by the bone's structure. Bending results in tensive and compressive stresses [103]. Ultimately, the bone mass or BMC, protein content, and micro-architectural phenotype (e.g. trabecular meshwork and cortical thickness) determine a bone's strength and hardness and consequently determine the magnitude, duration, and type of force tolerable. The greater the force a bone can endure, the less likely it is to fracture. Therefore, developing and maintaining an optimal BMC, protein content, and micro-architectural phenotype is critical in fracture prevention.

Nutrition and Bone Overview

Another critical factor in bone health and consequently the development of a high peak bone mass is nutrition. Proper bone development requires adequacy of key nutrients including vitamin D, calcium, phosphorus, vitamin K, and protein [104]. Bone hydroxyapatite is composed of calcium and phosphorus. Therefore, nutritional adequacy of the divalent cation calcium and phosphorus is critical in bone mineralization and the development of a high peak BM. Estimates suggest that between 60-70% of teenage girls and 70% of postmenopausal women consume less than the recommended amount of calcium [105]. Calcium absorption and excretion is a tightly regulated process modulated by PTH, calcitonin, vitamin D, and the renal system. In response to low blood calcium concentrations, the parathyroid gland secretes parathyroid hormone (PTH). PTH functions to stimulate resorption of Ca from bone, increase renal reabsorption, increase intestinal absorption, and increase 1,25-dihydroxy vitamin D synthesis [106]. Osteoclasts, which lack PTH receptors, are indirectly activated by PTH. Pre-osteoblasts possess PTH

receptors, which when activated promote their differentiation into osteoblasts. However, upon becoming mature osteoblasts PTH stimulates the secretion of IL-6 and IL-1, which in turn stimulate osteoblasts to synthesize CSF and RANKL. The net effect is the stimulation of osteoclastogenesis [107]. This increase in the formation of osteoclasts leads to enhanced bone resorption that normalizes plasma calcium, inhibits PTH synthesis and restores homeostasis.

The RDA for vitamin D is 600 IU per day for individuals between the ages of 1 and 70 years. Vitamin D may be acquired through the diet (e.g., dairy and fatty fish); however, it is primarily derived through subcutaneous sun exposure. Functions of vitamin D include increased intestinal absorption of calcium via TRPV6 and calbindin and increased osteoclast activity through the stimulation of RANKL expression in osteoblasts [108]. It has been estimated that approximately 70% of children in the United States are vitamin D insufficient or deficient [109]. This could have important long-term skeletal health consequences since vitamin D is essential in maintaining calcium homeostasis and bone formation.

By mass, one-third of bone is composed of protein [110]. The organic matrix of bone is largely made up of collagen, which continually undergoes turnover [9]. Post-translational modifications (e.g. hydroxylation) and remodeling of amino acids in collagen is necessary for crosslinking; however, it results in un-usable fragments protein thus increasing protein demand [110]. Inadequate dietary protein intake impairs IGF-1 production. IGF-1 is critical for longitudinal growth of bones as it promotes chondrocytes within the growth plates to proliferate as well as the activity of osteoblasts [111]. A study by Glick and colleagues [112] showed that chronic protein deficiency did not alter

mineral content of bone, but decreased the quantity of bone formed by significantly decreasing appositional bone growth in growing animals [112]. Therefore, an adequate supply of protein is necessary for proper collagen formation, IGF-1 production, and osteoblast activity. Furthermore, protein is even more critical due to its role in longitudinal bone growth in the growing skeleton.

Vitamin K, a fat-soluble vitamin, may be consumed in the diet as phylloquinone or synthesized endogenously by intestinal bacteria as menanquinone. Gamma glutamyl carboxylase (GGC) is a vitamin K-dependent enzyme that is involved in the carboxylation of the bone extracellular matrix protein, osteocalcin [113]. Osteocalcin can be found in carboxylated or un-carboxylated form. Carboxylated osteocalcin, the active form, promotes bone mineralization. Thus, low vitamin K intake resulting in undercarboxylated osteocalcin correlates with low BMD [114].

Emerging evidence suggests that the modulating effects of some foods may in part be due to non-digestible carbohydrates such as fructooligosaccharides and galactooligosaccharides. Non-digestible carbohydrates elicit a number of favorable effects, including the promotion of beneficial bacteria (e.g. *Bifidobacterium),* a reduction of pathogenic bacteria, and enhanced short chain fatty acid production (SCFAs) [50]. Oligosaccharides have been shown to increase cecal concentrations of SCFAs including acetate, propionate, and butyrate [115]. Recently Lucas and colleagues [51] showed that SCFAs (i.e. propionate and butyrate) significantly increase bone mass and prevent bone loss occurring post-menopause or resulting from inflammation. These researchers suggest these effects are exerted via a downregulation of the osteoclastogenesis genes TRAF6 and NFATc1 [51]. SCFAs are known to modulate the gut mucosal immune responses by

increasing T helper cells, macrophages, and neutrophils thereby increasing immune function. Furthermore, SCFAs have pre-biotic effects that can promote calcium absorption and increase BMD [116].

Nutritional adequacy of vitamin D, vitamin K, calcium, non-digestible carbohydrates, and protein are critical to the attainment of an optimal peak bone mass that can ultimately reduce the risk of osteoporosis. Nutrition also plays a role in bone health beyond macro and micro-nutrients. This role is evident when studying how the consumption of many plant-based foods affects bone loss and metabolism.

Functional Foods and Bone

Many fruits and vegetables are considered functional foods, which are defined as foods with health benefits beyond that which can be attributed to their nutrient components [117]. While functional foods can provide macronutrients and micronutrients that contribute nutritional adequacy and bone growth, they also contain bioactive compounds regarded as phytochemicals. The largest subclass of phytochemicals are the polyphenols [118]. Polyphenols may be the pigments in foods as well as the compounds that provide flavor, odor, and oxidative stability [118]. Over 8000 polyphenolic compounds have been identified and may be further classified as phenolic acids, stilbenes, lignans, and flavonoids based on the number of phenol rings, side groups (e.g. phenolic acids have carboxylic acid side groups), and structural binding **(Figure 8)** [118].

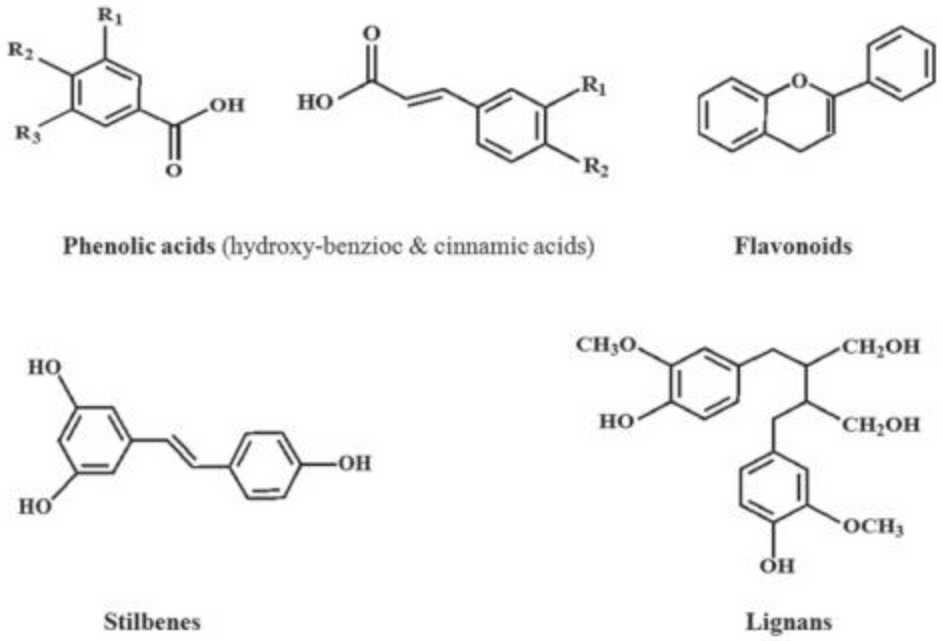

Figure 8: Chemical structures of polyphenols subclasses [84].

Characteristically, polyphenols possess potent anti-inflammatory and anti-oxidant capacities providing defense against ultraviolet radiation, pathogens, and other inflammation causing processes. Polyphenols exert their anti-oxidant capacity in a number of ways. Foremost, polyphenols quench free radicals via redox reactions, donating an electron and thereby reducing reactive species. Polyphenols may also function as metal chelators, chelating metals such as ferrous iron and thus reducing the rate of the Fenton reaction and its production of hydroxyl radicals [119]. Indirectly polyphenols may exert antioxidant effects by regenerating antioxidant vitamins (e.g. vitamin E) or promoting the synthesis of endogenous antioxidants such as glutathione peroxidase, catalase, and superoxide dismutase [120, 121]. Consequently, many studies support the efficacy of polyphenols in the attenuation or prevention of chronic diseases

(e.g., obesity, cardiovascular disease, Alzheimer's disease, cancer, diabetes, and osteoporosis) in which inflammatory processes are central to the pathophysiology [36, 121-124]. In particular, a number of studies using cell culture systems, animal models, and clinical trial protocols have shown the efficacy of foods with high polyphenolic profiles in the prevention or attenuation of osteoporosis. These functional foods include dried plums, green tea, soy, and blueberries to name a few.

A common fruit with a high flavonoid content is blueberries. Blueberries contain hippuric acid, phenylacetic acid, and hydoxybenzoic acid [125]. Using an *in vitro* model, Chen and colleagues showed that blueberry polyphenols stimulated Wnt signaling and subsequently the differentiation of osteoblast precursors into osteoblasts [126]. In ovariectomized rats, blueberry supplementation was found to prevent osteoblast apoptosis and the loss of collagen [127]. These results were supported further by Tao and colleagues who reported that blueberry prevented the development of osteoporosis in ovariectomized rats by inhibiting bone resorption and maintaining trabecular bone structure [128].

Soy and green tea flavonoids have shown marked benefit in the pathophysiology of osteoporosis. Studies have shown that soy isoflavones attenuate decreases in tibial BMD and BMC in ovariectomized rats, while improving architectural properties including trabecular thickness, separation, and number [44]. Furthermore, soy studies show significant increases in serum bone formation markers BSAP, IGF-1, and osteocalcin [129]. Soy isoflavones are classified as phytoestrogens and are able to bind estrogen receptors, eliciting estrogen-like effects (e.g. anti-resorptive), stimulate osteoblast activity via IGF-1 synthesis, and downregulate inflammatory cytokines (e.g.

TNF- α) [130-132]. By comparison, the primary phytochemicals in green tea are catechin derivatives such as gallocatechin, catechin gallate, gallocatechin gallate, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCG) with small amounts of phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acids) [133]. Isolated human osteoblasts subjected to oxidative stress and green tea extract showed improved mineralization and cell viability while also increasing expression of osteocalcin and type I collagen [134]. Long-term supplementation of EGCG exhibits an increase in the expression of osteogenic genes (e.g. Runx2) promoting MSC commitment to an osteoblast lineage [135]. These findings suggest that green tea, blueberries, and soy possess promising therapeutic potential in osteoporosis prevention.

Dried plums are a rich source of polyphenolic compounds (e.g., chlorogenic acid, caffeoylquinic acids, and quercetin) and non-digestible carbohydrates [136]. Rendina et al., [39] examined the effects of diets supplemented with 25% (w/w) of either dried plum, apricot, mango, or grapes on bone loss in ovariectomized C57BL/6 mice. Dried plum exhibited a unique capability to decrease osteoclastogenesis while increasing osteoblast and glutathione activity [39]. Franklin et al., [137] in an animal model using Sprague-Dawley rats reported that dried plum decreased osteoclastogenesis via the downregulation of RANKL expression and promoted bone formation via IGF-1 [138]. Using aged male mice, Halloran and colleagues [139] showed that dried plum supplementation results in an anabolic effect, causing increased bone volume and restoration of bone previously lost to aging. In the growing skeleton, Shahnazari and colleagues [140] found that dried plum increased peak bone mass by as much as 94%. These animal studies suggest dried plum decreases bone resorption and actually promotes

an anabolic effect on bone. Clinical studies on postmenopausal women by Hooshmand et al. [37, 141] show that dried plum attenuates bone loss, maintains BMD, and decreases serum resorption markers BAP and TRAP-5b. These clinical findings indicate the results of the animal studies translate to humans, suggesting that dried plum decreases bone resorption and promotes bone formation through the regulation of osteoclast differentiation and osteoblast activity.

The beneficial effects of functional foods are largely attributable to their polyphenolic content. In an *in vitro* model Bu and colleagues [79, 80] showed that isolated plum polyphenols decreased the effects of TNF-alpha on osteoblasts resulting in an up-regulation of Runx2, Osterix and IGF1 as well as inhibiting osteoclastogenesis via the downregulation of NFATC1 and inflammatory cytokines. Recently, Graef and colleagues [47, 48, 142] further demonstrated the ability of select polyphenols to independently modulate the activity of osteoblasts and osteoclasts. Bone-marrow derived osteoblast cultures treated with one of two crude polyphenolic fractions (i.e. DP-FrA or DP-FrB) showed that both fractions increased BMP2 and Runx2 expression [48]. Furthermore, treatment of osteoblast cultures with TNF-α, to elicit inflammatory conditions, resulted in increased Smad6 and suppressed ALP levels [48]. However, treatment with dried plum fractions resulted in an attenuation of this response. Treatment of bone-marrow derived osteoclast cultures with polyphenolic fractions (i.e. DP-FrE or DP-FrF) produced a decrease in osteoclast differentiation and activity via a downregulation of Nfatc1, osteoclast-associated receptor (Oscar), signaling regulatory protein β1 (Sirpb1), triggering receptor expressed on myeloid cells 2 (Trem2), extracellular signal-regulated kinase (Erk), and p38 MAPK [47]. Under inflammatory

conditions (i.e. TNF-α) only Sirpb1 was suppressed via polyphenolic fractions [47]. Graef and colleagues [142], further showed these results through translation into an animal model using osteopenic Sprague Dawley rats supplemented with dried plum polyphenolic extracts alone and in combination with vitamin K. Supplementation with the polyphenolic fractions, the combination of the polyphenolic fractions with vitamin K, and dried plum all restored whole body BMD to a similar extent. Trabecular bone loss in the vertebrae and cortical bone loss in the femur mid-diaphysis was reversed via the combination treatment [142]. Biomarkers of bone resorption (i.e. deoxypyridinoline, Dpd) were decreased by the fractions and combination treatment [142]. These outcomes reinforce the concept that some of these functional foods exert their favorable effects largely through their polyphenolic content, but that their fructooligosaccharides may also be a contributing factor.

Tart Cherries

Tart cherries are a well-known source of polyphenolic compounds, namely hydroxycinnamic acids and anthocyanins, and oligosaccharides (e.g., fructooligosaccharides and galactooligosaccharides) [143, 144]. Balaton cherries have the highest total anthocyanins while the Montmorency tart cherries are highest in total phenolics (**Table #2**) [143]. These phenolics include anthocyanins (e.g. cyanidins and pelargonidins), hydroxycinnamates (e.g. neochlorogenic acid and p-coumaroylquinic acid), and flavanols (e.g. catechins, quercetin, and kaempferol).

Cherry products	Total anthocyanins[a]	Total phenolics[b]
Montmorency cherry (dried-no sugar added)	173 ± 31	7813 ± 855
Balaton cherry (dried-no sugar added)	564 ± 65	6343 ± 776
Montmorency cherry (dried with sugar)	62 ± 5.3	5103 ± 455
Balaton cherry (dried with sugar)	273 ± 33	3522 ± 512
Montmorency cherry (frozen)	533 ± 47	12665 ± 1321
Balaton cherry (frozen)	1741 ± 287	6742 ± 786
Montmorency cherry concentrate	213 ± 41	4013 ± 578
Balaton cherry concentrate	722 ± 87	2541 ± 371
Montmorency cherry (IQF powder)	482 ± 56	10323 ± 1468
Balaton cherry (IQF powder)	1063 ± 178	7752 ± 932

Table 2: Anthocyanins and total phenolics of tart cherry products [143]. Total anthocyanins presented as µg/g dry weight of cyaniding 3-glucoside equivalents. Total phenolics presented as µg/g dry weight of gallic acid equivalents.

Tart cherries contain approximately 0.33 g of total fructooligosacharides per 100 g of tart cherry [49]. Previous studies suggest that tart cherry supplementation has favorable effects on muscle recovery, peripheral neuropathy, and arterial stiffness [145-147]. Levers and colleagues [148] reported that tart cherry supplementation increased performance in endurance athletes while attenuating muscle catabolism and inflammatory stress [148]. Similarly, this same group of investigators found that tart cherry supplementation reduced muscle catabolism and soreness during recovery from resistance training [149]. Moreover, tart cherry supplementation has been shown to aid in muscle recovery following strenuous activity by reducing inflammation and lipid peroxidation, and increasing antioxidant capacity [150].

Due to their rich source of phenolic acids and fructooligosaccharides, our lab recently showed that Montmorency tart cherries attenuate age-related bone loss in an animal model (*manuscript under review*). Further investigation showed that Phex gene expression was increased and Ppar-γ gene expression was decreased in the groups receiving cherry. Taken together these results suggest that tart cherry may in fact promote bone mineralization. Furthermore, at some skeletal sites tart cherry may have an anabolic effect on bone. To date no studies have been conducted on tart cherry on the growing

skeleton to determine if bone mineral content can be increased. Evidence suggesting that tart cherry promotes bone mineralization in the aged skeleton provides the rationale for using tart cherry as a means of increasing bone accrual to achieve a higher peak bone mass in the growing skeleton.

Based on this review of the literature, it is clear that osteoporosis is a global health concern with increasing prevalence. One of the most important factors in the prevention of osteoporosis is the optimization of peak bone mass early in life. Peak bone mass is heavily influenced by modifiable lifestyle factors such as diet and exercise. Research suggests that functional foods high in anti-inflammatory polyphenols help prevent or attenuate osteoporosis. Furthermore, studies conducted on growing animals show that polyphenol rich foods increase peak bone mass. Montmorency tart cherries are rich sources of these polyphenols and have been shown to have favorable effects on bone in the aging skeleton by enhancing mineralization. Exercise, namely weight-bearing exercise, is a well-established lifestyle factor that can enhance peak BM. Therefore, it stands to reason that Montmorency tart cherry supplementation is an excellent candidate to consider in bolstering peak mass (BMC) in the growing skeleton, and that tart cherries in combination with exercise may yield a more efficacious approach to preventing osteoporosis.

CHAPTER III

METHODS

Animal Care

Fifty-eight, four-week old female C57BL/6 mice (Jackson Labs) were purchased and housed in the OSU Laboratory Animal Research Facility under environmentally controlled conditions. Mice were housed 4-5 per cage and underwent a 2-week acclimation period during which they were fed a chow diet and acclimated to walking and running on a 6-lane treadmill (Columbus Instruments, Columbus Ohio). Next, mice were randomly assigned to one of four treatment groups as follows (n=12-14/group) in a 2x2 experimental design: control diet w/o exercise (Con), control diet + exercise (Con + Ex), tart cherry supplemented diet (10% w/w) w/o exercise (TC), and tart cherry supplemented diet + exercise (TC + Ex). The control diet was formulated according to AIN93-G specifications (**Table 2**) [151]. Tart cherry powder (Van Drunen Farms, IL) was sent to NPAL Analytical Laboratories (St. Louis, Missouri) for protein, fat, fiber, calcium, and phosphorus analysis. Diets were adjusted to have similar calcium and phosphorus content. Additionally, macronutrients (i.e. protein, fats, and carbohydrates) were adjusted to control for macronutrient provided by the addition of tart cherry to the experimental diet so that diets were isocaloric. Food intake was recorded and animals had free access to R/O water throughout the study. Mice were weighed once a week

Table 2: Formulation of Diet

Ingredients	Control Diet (per kg)	Tart Cherry Diet (per kg)
Cherry Powder	0	100
Carbohydrates		
Cornstarch (g)	397.5	318.0
Maltodextrin (g)	132	132
Sucrose (g)	100	100
From tart cherry (g)	NA	79.5
Total (g)	629.5	629.5
Protein		
Casein (g)	200	195.5
From tart cherry (g)	NA	4.6
Total (g)	200	200
Fat		
Soy bean oil (g)	70	69.4
From tart cherry (g)	NA	0.6
Total (g)	70	70
Fiber		
Cellulose (g)	50	45.7
From tart cherry (g)	NA	4.3
Total (g)	50	50
Vitamin mix (g)	10	10
Mineral		
Mineral mix (Ca-P Deficient) (g)	13.4	13.4
Calcium Carbonate (40.04%ca) (g)	12.5	12.4
Calcium from tart cherry (g)	NA	0.045
Sodium Phosphate, monobasic (25.81% P) (g)	4.2	4.1
Potassium Phosphate, monobasic (22.76% P)(g)	2.1	2.0
Phosphorus from tart cherry (g)	NA	0.1
Sucrose (g)	2.8	3
Total (g)	**35.0**	**35.0**
Choline Bitartrate (g)	2.5	2.5
L-Cysteine (g)	3	3

Exercise Protocol

Mice in the exercise groups were run 5 days/wk at 12 m/min on a 5° incline for 30 min. The exercise protocol was typically carried out at approximately 9:00 am to minimize interference with diurnal cycles. Although not required often, mice were gently prodded with a tongue depressor if needed to promote compliance with the running protocol [152]. Mice in the non-exercising groups were individually removed from cages and handled on exercise days for 5 minutes to control for the stress of handling.

After the 8-week treatment period, animals were anesthetized using a ketamine/xylazine cocktail at 100 mg and 10 mg per kg body weight. Mice were then exsanguinated by the carotid artery. Blood samples were collected and serum was separated by centrifugation of whole blood at 4,000 rpm for 20 minutes. Serum was then aliquoted and stored at -80º C. Bone samples were obtained and stored accordingly. Bone marrow from one femur was flushed with RNA later for gene expression analysis, while the other femur was stored in PBS for microarchitectural and biomechanical testing. The flushed femur hard tissue was snap frozen in liquid nitrogen for protein and gene expression assays. Tibias were both flushed with complete media for FACS analysis. The spine was removed and the 5^{th} lumbar vertebrae (L5) was isolated for micro-computed X-ray tomography (μCT) analysis. Tissues, including the liver, white adipose tissue, uterus, and spleen were harvested and weighed.

BMD Assessment Using DXA

Prior to necropsy, whole body DXA scans were performed using GE Medical Systems Lunar PixiMus (Madison, WI). These scans were used to determine the whole

body bone mineral area (BMA), bone mineral density (BMD), bone mineral content (BMC), lean mass, fat mass, and body fat percentage.

Assessment of Microarchitectural Parameters by µCT

Femurs and L5 vertebrae specimens, were stored in PBS, and then scanned using µCT (MicroCT40, SCANCO, Switzerland). Both trabecular and cortical bone micro-architectural analysis were performed on the femur and vertebrae. The trabecular bone was analyzed in the distal femur metaphysis and the cortical bone was assessed in the femur mid-diaphysis. Femur scans of the distal femur metaphysis were performed at a high resolution of 2048 x 2048 pixels and analyzed via evaluation of 125 slices (6 µm/slice or 0.75 mm volume of interest (VOI)) starting 0.09 mm from the growth plate. Cortical analysis was performed by evaluating 30 slices or a 0.18 mm VOI at the mid-shaft.

L5 trabecular analysis was performed by analyzing all medium resolution slices (1024 x 1024 pixels) starting 10 slice in from each growth plate, approximately 150-175 slices (12 µm/slice or 60 µm VOI). Vertebral cortical bone analysis was performed by evaluating ~100 slices or a 1.2 mm VOI. Trabecular bone evaluation provided the ratio of trabecular bone volume to total bone volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular spacing (TbSp), structural model index (SMI), connectivity density (ConnDens), apparent density, and material density. Cortical analysis provided cortical bone porosity, cortical area, medullary area, and cortical thickness. Cortical analysis of the femur was performed using a threshold of 260 and a sigma and support of 0.8 and 1, respectively. Trabecular analysis of the femur, trabecular

analysis of the vertebral body, and cortical analysis of the vertebral body was performed using a threshold of 400 and a sigma and support of 0.7 and 1.0, respectively.

Assessment of Bone Marrow MSC Populations by Flow Cytometry FACS

Bone marrow was flushed and pooled from both tibias using complete media which included DMEM, 0.1g BSA, 58.44 mg EDTA at the time of necropsy. Samples were kept on ice and then centrifuged at 750 g for 5 min at 4° C. Media was then aspirated and the red blood cells (RBC) lysed for 7 min (FACS Lysing solution, BD Biosciences, San Jose, CA). After RBC were lysed, samples were washed and re-suspended in complete DMEM media for counting. Cells 2 x 10^6 were aliquoted to 12 x 75 mm tubes and re-suspended in 100 µl of stain buffer (Stain Buffer, BD Biosciences, San Jose, CA). Samples were then incubated for 30 minutes at room temp with the following series of 5 antibodies: SCA-1, CD105, C-Kit, CD44, and CD90. After staining, samples were washed 3 times using ice cold PBS and re-suspended in 350 µl of stain buffer for subsequent analysis via a Becton Dickinson FACSCalibur. Cells were characterized based on the following cell surface markers: ($SCA\text{-}1^+$), ($CD105^+$), ($C\text{-}Kit^+$), ($CD44^+$), and ($CD90^+$). Analysis was performed using successive sub gating for positive markers. Final cell counts expressing positive readings for all 5 markers ($SCA\text{-}1^+$, $CD105^+$, $C\text{-}Kit^+$, $CD44^+$, $CD90^+$) were deemed as mesenchymal stem cells and expressed as a percentage of total cells analyzed.

Serum Biochemical Markers Analyses

Systemic markers of bone formation and resorption were analyzed via commercially available kits. Serum tartrate-resistant acid phosphatase form 5b (TRAcP 5b), which is secreted by osteoclasts to de-mineralize bone, is a marker of bone resorption and was assessed using MouseTRAP Elisa kit (Immuno Diagnostic Systems, United Kingdom). The sensitivity of the assay is 0.1 U/L and the inter and intra assay variability are <8% and <6.5% respectively. Serum N-terminal propeptide of type I procollagen (PINP), a byproduct of collagen formation from procollagen, is a marker of bone formation and was assessed using a PINP Elisa kit (Immuno Diagnostic Systems, United Kingdom). The sensitivity of the assay is 7 ng/mL and the inter and intra assay variability are <10% and <8% respectively. Serum osteocalcin (OCN), which promotes bone mineralization, was assessed using a mouse OCN ELISA kit (Immutopics Inc., San Clemente, CA). The sensitivity of the assay is 0.4 ng/mL and the inter and intra assay variability are <7% and <4% respectively.

Corticosterone, a glucocorticoid released by the adrenal cortex as part of the stress response, was measured via a corticosterone Elisa kit (Immuno Diagnostic Systems, United Kingdom) to assess stress resulting from the handling involved with the exercise protocol. The sensitivity of the assay is 0.55 ng/mL and the inter and intra assay variability are <9% and <7% respectively. All kits were run n=10 samples from each group in duplicate and read on a plate reader (Synergy HT, BioTek, Winooski, VT).

Biomechanical Analyses

Femurs were cleaned and stored in phosphate buffered saline (PBS) at 4º Celsius until biomechanical analysis via reference point indentation (RPI) (Bio-dent Active Life

Scientific, Inc, Santa Barbara, CA). Testing was performed at two sites. The first site was the mid-diaphysis, which is primarily composed of cortical bone. The second location was the femoral neck which is rich in trabecular bone but also has a thick cortex. Both sites were tested using 2 N force, 2 Hz, and 10 cycles. Testing of the diaphysis was performed using a BP2 probe while testing of the femoral neck used a BP3 probe.

μCT analyses allows for micromechanical finite element (FE) models to be constructed by converting bone voxels from the VOI into 8-node brick elements [153]. Simulated compression testing was performed on the ROI from the scan of the femur metaphysis and vertebral body. Selected apparent mechanical properties included: elastic, linear, and isotropic with a Young's modulus of 10 GPa and a Poisson's ratio of 0.3 [154]. FE analyses results were used to compute the apparent stiffness, strains, and stresses for a given force. These calculations are indicative of loading characteristics for individual elements within the bone.

RNA Extraction and Quantitative Real-Time PCR

RNA extraction was performed on both bone marrow and flushed bones. Whole frozen femurs were pulverized using a freezer mill (6770 freezer mill, SPEX, Metuchen, NJ). Trizol reagent (Life Technologies, Carlsbad, CA) was used to isolate total RNA from samples. Supernatant was placed into a micro-centrifuge tube containing chloroform and incubated prior to centrifugation and subsequent phase separation. The aqueous phase was transferred into a micro-centrifuge tube containing isopropanol and allowed to precipitate on ice for 30 minutes. Samples were then centrifuged, decanted, washed with 75% ETOH, and suspended in DEPC H_2O. A Nanodrop spectrophotometer

(Rockland, DE) was used to determine the concentration, A260/230, and A260/280 ratios. RNA quality was further verified via gel electrophoresis.

Total RNA (2 µg) was treated with DNase I and then cDNA synthesis was performed via reverse transcription (Superscript II, Invitrogen, Carlsbad, CA). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in duplicate using 50 ng of cDNA and SYBR green (Roche, Penzberg, Germany) on the PCR machine (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA). qRT-PCR used gene specific primers to investigate how treatments affected regulators of osteoblast differentiation (i.e. Runx2 and Osterix), regulators of osteoclast differentiation (i.e. RANKL, NFATC1, and OPG), indicators of bone formation (i.e. Col1al), indicators of osteoblast activity and mineralization (i.e. ALP, Phex, and Ppar-γ), and indicators of osteoclast activity (i.e. CathK). Primers were validated by performing qRT-PCR using a serial dilution of cDNA synthesized from tissue that abundantly expresses the gene of interest. The comparative cycle number at threshold (C_t) and the invariant control hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used to evaluate the results.

Table 4: Primer Sequence List for qRT-PCR

Symbol	Name	Sequence
RUNX2	Runt-related transcription factor 2	QF 5′- GGC GTC AAA CAG CCT CTT CA -3′ QR 5′- GCT CGG ATC CCA AAA GAA GTT -3′
Osterix	Osterix	QF 5’- GAA GTT CAC CTG CCT GCT CTG T-3’ QR 5’- CGT GGG TGC GCT GAT GT-3’
RANKL	Receptor activator for nuclear factor κ B ligand	QF 5′- TTT CAA GGG GCC GTG CAA AG -3′ QR 5′- AGC CAC GAA CCT TCC ATC ATA -3′

NFATC1	Nuclear factor of activated T-cells cytoplasmic 1	QF 5′- TGC AGC TAC ATG GTT ACT TGG AA -3′ QR 5′- CGT CAG CCG TCC CAA TG -3′
ALP	Alkaline Phosphatase	QF 5′- GCA CAA CAT CAA GGA CAT CG -3′ QR 5′- TGG CCT TCT CAT CCA GTT CA -3′
OPG	Osteoprotegrin	QF 5′- GTT CTT GCA CAG CTT CAC CA -3′ QR 5′- AAA CAG CCC AGT GAC CAT TC -3′
Col1al	Type 1 collagen	QF 5′- AGA TTG AGA ACA TCC GCA GCC -3′ QR 5′- TCC AGT ACT CTC CGC TCT TCC A -3′
CTSK	Cathepsin K	QF 5’- GCA GGA TGT GGG TGT TCA AGT -3’ QR 5’- TCC GGA GAC AGA GCA AAG CT -3’
PHEX	Phosphate Regulating Endopeptidase Homolog, X-Linked	QF 5’ – CTA ACC ACC CAC TCC CAC TT -3’ QR 5’ – CCA ATA GAC TCC AAA CCT GAA GA -3’
PPAR-λ	Peroxisome proliferator-activated receptor- λ	QF 5’ – GAA GCA GAA ACA GGG AGC AC -3’ QR 5’ – TGC AGC GGC CGC TAC CAG AGT CGG CAA GAA TC -3’
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	QF 5’ – GCC TAA GAT GAG CGC AAG TTG -3’ QR 5’ – TAC TAG GCA GAT GGC CAC AGG -3’
BMP2	Bone Morphogenic Protein 2	QF 5' – GGA CAT CCG CTC CAC AAA -3’ QR 5' – GGC GCT TCC GCT GTT T – 3’
c-Fos	Fos proto-oncogene	QF 5' – GGA CAG CCT TTC CTA CTA CCA TTC C – 3’ QR 5' – AAA GTT GGC ACT AGA GAC GGA CAG A – 3’
c-Fms	Colony Stimulating Factor 1 Receptor	QF 5’ – CCT CCT CTG GTC CTG CTG CTG G – 3’ QR 5’ – GCT CAC ACA TCG CAG GGT CAC C – 3’
TRAF6	TNF receptor associated factor 6	QF 5’ – CAG CAG TGT AAC GGG ATC TAC – 3’ QR 5’ – CTG TGT AGA ATC CAG GGC TAT G – 3’
BMP4	Bone Morphogenic Protein 4	QF 5' – TTA TGA GGT TAT GAA GCC CCC A – 3’ QR 5' – GCT CAC ATC GAA AGT TTC CCA C – 3’
RANK	Receptor activator for nuclear factor κ B	QF 5' – GCG TGC TGC TCG TTC CA – 3’ QR 5' – ATG CCT CTC CTG GGT GCA T – 3’
OPN	Osteopontin	QF 5' – ACT CCA ATC GTC CCT ACA GTC G – 3’ QR 5' – TGA GGT CCT CAT CTG TGG CAT – 3’
OCN	Osteocalcin	QF 5' – TGA GCT TAA CCC TGC TTG TGA CGA – 3’ QR 5' – AGG GCA GCA CAG GTC CTA AAT AGT – 3’
BSP	Bone sialoprotein	QF 5’ – ACA CCC CAA GCA CAG ACT TTT G – 3’ QR 5’ – TCC TCG TCG CTT TCC TTC ACT – 3’
Sost	Sclerostin	QF 5' – ACC GGG CGG AGA ATG G – 3’ QR 5' – GCT GTA CTC GGA CAC ATC TTT GG – 3’

SOD1	Super oxide dismutase-1	QF 5' – GCC CGG CGG ATG AAG A – 3' QR 5' – CGT CCT TTC CAG CAG TCA CA – 3'
Gpx1	Glutathione peroxidase-1	QF 5' – CGG TTT CCC GTG CAA TC – 3' QR 5' – GAG GGA ATT CAG AAT CTC TTC AT – 3'

Statistical Analysis

All data were analyzed using statistical analysis software version 9.4 (SAS Institute, NC). First a Shapiro Wilks test was run to assess whether data was normally distributed. Normally distributed data were analyzed for treatment main effects and interactions using a two-way ANOVA with diet and exercise as factors. Fischer's least square means was run as a *post hoc* analysis when F values were significant. Data was expressed as mean $\pm$ standard error and statistical significance was set at an $\alpha = 0.05$.

CHAPTER IV

RESULTS

Body Weight, Tissue Weight, Body Composition, and Food Intake

Food consumption by all treatment groups gradually increased throughout the study, but no significant differences were observed between groups in food intake (~2.7 g/mouse/d). Although there were no significant differences in body weight at baseline, final body weights of the exercising groups were lower ($P<0.05$) than the non-exercising groups (**Table 5**). Assessment of body composition via x-ray absorptiometry revealed that the differences in body weight were a result of a significant reduction in body fat mass in response to the exercise protocol (Table 5). However, no effects of exercise were observed on whole body lean mass and tart cherry had no effect on body weight or body composition after 8 weeks of intervention.

The effect of tart cherry and exercise on organ weights were determined at the end of the study and were expressed per g body weight. No effects of tart cherry or exercise were observed on the heart, liver, spleen, thymus, or uterine weights (Table 5). An exercise effect was exhibited on visceral WAT, resulting in a decrease in tissue weight and was consistent with the observed changes in body composition.

Whole Body and Femur Bone Mineral Area, Content and Density

BMD, BMC and BMA were assessed via DXA for the whole body and the femur. At the end of the 8-week treatment period, no effects of tart cherry or exercise were observed on whole body BMD or femur BMD (**Table 6**). However, a significant interaction was detected in whole body BMC and BMA. Mice fed the control diet exhibited an increase in whole body BMA with exercise, while mice consuming the tart cherry diet experienced a decrease in BMA. Whole body BMC was significantly elevated compared to Con in the TC group, however when combined with exercise the TC/Ex group was not different from Con. The TC group increased (*P<0.05)* whole body BMC and BMA compared to the Con group. Site-specific DXA of the femur showed a decrease in BMC and BMA in response to exercise (Table 6), but no effect of tart cherry or exercise were observed on femur length, suggesting that neither intervention alone or in combination altered bone growth.

Microarchitectural Properties of Femur and Vertebra

Morphometric (i.e. BVTV, TbN, TbTh, and TbSp) and non-morphometric parameters (i.e. SMI, apparent density, material density, and degree of anisotropy) of trabecular bone at the distal femur metaphysis were assessed using μCT as an indicator of treatments at a weight-bearing site. An effect of TC and exercise, resulted in an increase (*P<0.05*) in trabecular BVTV was observed in the femur (**Table 7**). The relative increase in femoral BVTV in TC/Ex compared to Con was ~34%. The increase in femoral trabecular bone was a result of tart cherry increasing (*P<0.05*) TbN and TbTh, and decreasing TbSp (*P<0.05*) (Table 7). Analyses of non-morphometric parameters of trabecular bone exhibited predominantly a diet effect in the femur metaphysis. The trabecular bone had a more plate-like structure resulting from exercise and TC as

indicated by the significant decrease in SMI (Table 7). Apparent density of the bone, increased in response to tart cherry. Material density of the trabecular bone, which unlike apparent density accounts for porosity, was increased in response to TC at this site. The degree of anisotropy exhibited no diet or exercise effects.

Analysis of the morphometric and non-morphometric parameters of trabecular bone micro-architecture in the lumbar vertebral body were also performed using μCT. Vertebral BVTV was increased by both tart cherry and exercise (**Table 8**). The relative increase in vertebral BVTV in TC/Ex compared to Con was ~26.6%. In the vertebral body, the increase in trabecular bone resulted from a TC and exercise main effects which improved TbTh ($P<0.05$) (Table 8). However, the effect of exercise on TbTh was blunted in the context of the tart cherry diet. Tart cherry tended to increase TbN ($P=0.07$) and decrease TbSp ($P=0.08$). Analysis of non-morphometric parameters revealed both tart cherry and exercise affect the vertebral body. SMI was decreased by tart cherry and exercise ($P<0.05$) resulting in a more plate-like structure (Table 8). Vertebral apparent density increased in response to exercise and to tart cherry, but no differences in the trabecular bone material density was detected. Similar to the femur, the degree of anisotropy in the vertebral body exhibited no diet or exercise effects.

Cortical analysis at the femur mid-diaphysis and vertebral cortex resulted in distinctly different responses to treatment. No effect of tart cherry or exercise was detected on cortical thickness in the mid-femur diaphysis, but an exercise effect resulting in a decrease in cortical area was observed (Table 7). However, no effects of treatments were observed on cortical bone porosity or medullary area (Table 7). The only cortical parameter generally reported for the vertebral body, cortical thickness, was increased by

tart cherry, exercise, and exhibited a trend toward an interaction (*P=0.06*). The effects of treatment in the TC/Ex group resulted in a 16.13% increase in vertebral cortical thickness compared to Con.

Taken together these data indicate tart cherry improved trabecular bone micro-architecture in both the femur and spine as well as enhanced cortical bone thickness in the spine.

Femur and Vertebral Biomechanical Testing

Finite element analysis was performed to assess trabecular bone biomechanical properties in the femur metaphysis and vertebral body via simulated compression testing. According to the results of these tests, tart cherry improved all of the trabecular bone biomechanical properties, including stiffness, total force, size independent stiffness, and corrected Von Mises, in the femur and vertebral body (**Table 9**). Additionally, favorable exercise effects were observed on all trabecular bone biomechanical parameter in the vertebral body, but improved biomechanical properties did not reach of the level of statistical significance in the femur metaphysis (Table 9). Enhanced bone strength in the TC/Ex compared to control was evident by a 101.43% and a 59.86% increase in total force required to fracture in the femur and vertebral body, respectively. Furthermore, average Von Mises, a measure of material stress under an established force, for TC/Ex decreased compared to Con by 45.07% and 29.82% in the femur and vertebral body, respectively. These data suggest that TC and exercise induce alterations in trabecular bone micro-architecture which result in enhanced bone strength.

In addition to trabecular bone biomechanical testing, reference point indentation of the femur mid-diaphysis was used to assess cortical bone biomechanical properties. In

agreement with the effects of treatments on cortical microarchitecture, no effect of tart cherry or exercise was observed on total indentation distance or indentation distance increase (**Table 10**). However, tart cherry tended to decrease (*P=0.08*) total indentation distance, which would be consistent with a stronger bone structure. Reference point indentation testing was also performed at the femoral neck, a trabecular rich site with a thick outer cortex, and exhibited profound effects. A tart cherry, exercise, and interaction effect decreased total indentation distance. Relative to Con the Con/Ex group exhibited a 21.95% decrease in total indentation distance, but this improvement in bone biomechanical properties did not occur in the mice consuming the tart cherry diet (TC/Ex). Exercise decreased the indentation distance increase and tart cherry tended (p=0.06) to produce a similar effect. Relative to Con the Con/Ex group exhibited a 27.5% decrease in indentation distance increase, but this improvement did not occur in mice consuming the tart cherry diet (TC/Ex).

FACS Assessment of Mesenchymal Stem Cells

Tibial bone marrow derived mesenchymal stem cell populations were assessed via FACS utilizing a progressive gating scheme. Sub-gating was performed to identify SCA-1^+, $CD90^+/CD44^+$, and $CD105^+$/c-Kit^+ cells, which were classified as MSCs (2-1) (**Figure 9A-C**). MSC populations were increased (p=0.001) in response to exercise (**Figure 9D**). Exercise significantly increased the percent MSC with the bone marrow (Figure 12). In contrast, tart cherry tended to decrease the MSC population (p=0.069). No interaction effect was observed between tart cherry and exercise; however, an ~25% decrease in MSCs in the TC/Ex group was observed in comparison to the Con/Ex group. These data indicate that while exercise increases the MSC population, TC inhibits this

response, suggesting that either the combination reduces the MSC in bone marrow, or promotes their allocation to another lineage.

Serum Corticosterone

Due to the effects that stress can have on bone metabolism and the need to eliminate the possibility that the exercise regimen elicited a stress response, serum corticosterone was assessed. Interestingly, there was no effect of exercise on serum corticosterone, but tart cherry reduced serum levels by 45.18% (**Figure 10A**). This indicates that the exercise protocol did not induce stress exceeding that of non-exercising groups, but that supplementing the diet with TC effectively lowered serum corticosterone.

Serum Bone Biomarkers

Serum OCN and P1NP were assessed to determine the systemic effects of TC and exercise on bone formation and turnover. Serum P1NP, which is indicative of type 1 collagen formation, was decreased in response to TC ($P<0.0001$) and tended to be decreased ($P=0.09$) in response to exercise (**Figure 10B**). In contrast, OCN, an indicator of bone mineralization, was not altered by either tart cherry or exercise (**Figure 10C**). Serum TRAP5b was assessed to determine the effects of TC and exercise on bone resorption. Neither diet nor exercise had a significant effect on serum TRAP (**Figure 10D**).

Gene Expression Associated with Osteoblast Differentiation and Activity

The differentiation of MSC into osteoblast is a highly regulated process an essential for the bone formation stage of bone remodeling. The relative abundance of the primary regulator of osteoblast differentiation, Runx2 was not altered by tart cherry or exercise effect was observed (**Table 11**). Osterix, another key regulator of osteogenesis downstream of Runx2, similarly exhibited no diet or exercise effects (Table 11). BMPs are important cytokines which function as growth factors and promote osteoblast differentiation were also assessed. BMP-2 was significantly increased (80%) by tart cherry, but there was no observed effect of exercise (Table 11). BMP-4 was not affected by tart cherry or exercise after 8 wks of treatment.

The organic matrix of bone is primarily composed of type 1 collagen. Type 1 collagen is formed through the cleavage of pro-type 1 collagen. qRT-PCR was performed to quantify the expressions of type 1 collagen. No tart cherry or exercise effects were observed on the expression of Col1a1 (Table 11). These data indicate that TC and Ex did not affect the formation of type 1 collagen. Mineralization of bone and resorption of minerals is a tightly controlled process that is critical to bone formation. Ppar-λ, which is a known inhibitor of bone mineralization, did not exhibit any differences in the femur (Table 11). No diet or exercise effects were observed on OPN, which is expressed during early stages of bone formation, or Phex, which degrades OPN during later stages to allow mineralization (Table 11). OCN and BSP, which stimulate bone mineralization, exhibited no changes in expression resulting from treatments (Table 11). These data suggest regulators of bone mineralization were not affected by TC or exercise in the femur in this study.

Sclerostin, secreted by osteocytes in response to mechanical unloading, is an inhibitor of Wnt signaling and consequently osteoblast bone formation. Exercise or mechanical loading suppresses the expression of sclerostin. qRT-PCR was performed to quantify the expressions (Table 11). No TC or exercise effects were observed on the expression of sclerostin.

Gene Expression Associated with Osteoclast Differentiation and Activity

Osteoclastogenesis, or the differentiation of osteoclasts from HSCs, is crucial to bone resorption during both bone modeling and remodeling. The relative abundance of RNA for signaling molecules (i.e. RANKL and OPG), cell surface receptors (i.e. RANK and c-FMS) and signaling cascades (i.e. TRAF6 and c-Fos) that regulate Nfatc1 expression and in turn osteoclastogenesis were assessed. No diet or exercise effects were observed on RANKL, its receptor RANK, or its competitive inhibitor OPG (**Table 12)**. c-Fms, the receptor for MCSF, did not exhibit a tart cherry main effect or an interaction (Table 12). However, exercise tended to decrease c-Fms gene expression (*P=0.05*) (Table 12). No effects were observed on TRAF6, but tart cherry tended to decrease the expression of c-Fos (*P=0.07*) in the bone (Table 12). The key regulator of osteoclastogenesis, Nfatc1, was significantly downregulated by TC/Ex compared to all other groups. Relative to Con, TC/Ex expressed ~36% decreased Nfatc1 expression. These data suggest that TC/Ex suppresses osteoclast differentiation locally within the femur via a downregulation of Nfatc1 which is mediated by a TC effect on c-Fos and exercise's effect on c-FMS.

Resorption of collagen is mediated by cathepsin-K, an osteoclast protease. qRT-PCR was performed to quantify the expressions of cathepsin-K. No tart cherry or exercise

effects were observed on the expression of Ctsk (Table12). These data indicate that TC and Ex did not affect the proteolysis of type 1 collagen.

Endogenous Antioxidant Gene Expression

Synthesis of endogenous antioxidants (i.e. GPX and SOD) is critical to eliminating free radicals. In terms of Gpx1, no main effects of tart cherry or exercise were observed (**Figure 11A**). However, Gpx1 was significantly increased in the TC group relative to control and the addition of exercise suppressed this response. Relative to Con, TC elicited a 23% increase in Gpx1 expression. No differences were observed in SOD1 expression in response to either the tart cherry or exercise treatments (Figure 11B).

Table 5
Body Weights, Tissue Weights, and Body Composition.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Body Weights							
Baseline (g)	16.07 ± 0.26	16.44 ± 0.28	16.49 ± 0.26	16.09 ± 0.34	0.9173	0.9245	0.1838
Final (g)	23.28 ± 0.27	22.55 ± 0.47	23.45 ± 0.55	22.18 ± 0.53	0.8714	0.0385	0.5696
Body Composition							
Lean mass (g)	17.73 ± 0.26	17.62 ± 0.28	18.01 ± 0.27	17.24 ± 0.29	0.8490	0.1130	0.2393
Fat mass (g)	5.75 ± 0.23	4.60 ± 0.23	5.52 ± 0.38	5.00 ± 0.27	0.7026	0.0055	0.2775
Percent Fat (%)	24.43 ± 0.81	20.58 ± 0.66	23.30 ± 1.28	22.30 ± 0.63	0.6765	0.0094	0.1138
Tissue Weight							
Heart (mg/g of bw)	4.49 ± 0.31	4.71 ± 0.32	4.24 ± 0.17	4.32 ± 0.10	0.1855	0.5289	0.7670
Liver (mg/g of bw)	47.59 ± 0.85	46.29 ± 0.93	45.40 ± 1.23	46.73 ± 1.01	0.4075	0.9687	0.2034
Spleen (mg/g of bw)	4.52 ± 0.12	4.96 ± 0.35	4.73 ± 0.17	4.32 ± 0.12	0.3055	0.9994	0.0516
Thymus (mg/g of bw)	2.82 ± 0.14	2.45 ± 0.14	2.50 ± 0.16	2.45 ± 0.11	0.1033	0.1923	0.3819
Visceral WAT (mg/g of bw)	27.92 ± 2.08	18.97 ± 1.41	24.48 ± 2.70	21.60 ± 1.07	0.9068	0.0037	0.1199
Uterus (mg/g of bw)	3.20 ± 0.39	4.39 ± 0.62	3.37 ± 0.49	3.09 ± 0.31	0.2131	0.3500	0.1237

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. Abbreviations: bw body weight; WAT white adipose tissue.

Table 6

Whole body DXA, Femur DXA, and Femur Length.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Whole Body DXA							
BMD (mg/cm^2)	51.95 ± 0.5	51.95 ± 0.4	52.39 ± 0.4	52.29 ± 0.3	0.3471	0.9010	0.9083
BMC (mg)	439.00 ± 9.2^{b}	459.46 ± 7.95^{ab}	466.68 ± 9.06^{a}	448.62 ± 5.99^{ab}	0.3240	0.9297	0.0213
BMA (cm^2)	8.45 ± 0.17^{c}	8.84 ± 0.10^{ab}	8.91 ± 0.13^{a}	8.51 ± 0.08^{bc}	0.6716	0.8909	0.0026
Femur DXA							
BMD (mg/cm^2)	52.31 ± 0.7	52.53 ± 0.8	54.41 ± 0.6	52.78 ± 0.8	0.1047	0.3407	0.2111
BMC (mg)	23.83 ± 0.37	22.92 ± 0.40	24.69 ± 0.40	23.08 ± 0.36	0.1503	0.0019	0.3666
BMA (cm^2)	0.457 ± 0.006	0.439 ± 0.003	0.455 ± 0.004	0.439 ± 0.008	0.9820	0.0058	0.8427
Femur							
Length (mm)	15.01 ± 0.14	15.02 ± 0.11	15.01 ± 0.08	14.76 ± 0.12	0.2823	0.2937	0.2755

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. BMD bone mineral density; BMC bone mineral content.

Table 7
Femur Microarchitecture of Trabecular and Cortical Bone Assessed Using μCT

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Distal-Femur Metaphysis							
BVTV (%)	9.11 ± 0.36	10.24 ± 0.53	11.71 ± 0.23	12.22 ± 0.42	<0.0001	0.0462	0.4433
Tb.N. (1/mm)	3.91 ± 0.07	3.96 ± 0.07	4.12 ± 0.04	4.26 ± 0.08	0.0004	0.1778	0.4928
Tb.Th. (mm)	0.045 ± 0.001	0.045 ± 0.001	0.049 ± 0.001	0.047 ± 0.001	0.0002	0.6614	0.1634
Tb.Sp. (mm)	0.26 ± 0.0112	0.25 ± 0.0105	0.24 ± 0.0091	0.24 ± 0.0116	0.0004	0.2202	0.6223
Connectivity Density (1/mm)	147.11 ± 9.16	172.75 ± 12.67	186.02 ± 9.81	216.05 ± 14.30	0.0010	0.0215	0.8519
SMI	2.28 ± 0.048	2.08 ± 0.057	2.00 ± 0.034	1.94 ± 0.045	<0.0001	0.0083	0.1146
Apparent Density (mg HA/ccm)	218.47 ± 6.07	231.40 ± 7.32	250.04 ± 3.27	258.92 ± 6.36	<0.0001	0.0737	0.7351
Material Density (mg HA/ccm)	1136.49 ± 4.29	1140.89 ± 3.41	1150.88 ± 2.81	1146.28 ± 2.78	0.0054	0.9770	0.1898
Degree of Anisotropy (%)	1.62 ± 0.03	1.64 ± 0.02	1.66 ± 0.02	1.62 ± 0.03	0.6663	0.5610	0.2132
Mid-Femur Diaphysis							
Cortical Thickness (mm)	0.201 ± 0.002	0.199 ± 0.001	0.201 ± 0.002	0.200 ± 0.001	0.8863	0.2255	0.9053
Porosity (%)	0.899 ± 0.045	1.007 ± 0.048	0.926 ± 0.044	0.93 ± 0.024	0.5124	0.1925	0.2179
Cortical Area (mm^2)	0.825 ± 0.009	0.809 ± 0.007	0.837 ± 0.010	0.812 ± 0.010	0.3641	0.0249	0.6172
Medullary Area (mm^2)	7351.3 ± 443.9	8347.7 ± 383.8	7767.5 ± 419.3	7664.6 ± 207.5	0.6790	0.2497	0.1474

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. BV/TV bone volume per unit of tissue volume; Tb.N. trabecular number; Tb.Th. trabecular thickness; Tb.Sp. trabecular separation; Conn Density connectivity density; SMI structural model index.

Table 8
Lumbar Vertebral Microarchitecture of Trabecular and Cortical Bone Assessed Using μCT

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Vertebral Body							
BVTV (%)	16.08 ± 0.35	18.04 ± 0.73	18.76 ± 0.54	20.35 ± 0.40	<0.0001	0.0015	0.7303
Tb.N. (1/mm)	4.46 ± 0.075	4.47 ± 0.066	4.49 ± 0.043	4.64 ± 0.045	0.0746	0.1789	0.2460
Tb.Th. (mm)	0.045 ± 0.001[b]	0.048 ± 0.001[a]	0.048 ± 0.001[a]	0.048 ± 0.001[a]	0.0178	0.0198	0.0339
Tb.Sp. (mm)	0.23 ± 0.004	0.22 ± 0.004	0.22 ± 0.002	0.21 ± 0.002	0.0815	0.1094	0.3633
Connectivity Density (1/mm)	191.96 ± 9.49[b]	190.86 ± 6.18[b]	204.78 ± 7.05[b]	232.99 ± 4.15[a]	0.0002	0.0653	0.0420
SMI	1.55 ± 0.034	1.38 ± 0.072	1.25 ± 0.054	1.14 ± 0.045	<0.0001	0.0134	0.5633
Apparent Density (mg HA/ccm)	344.02 ± 4.28	362.73 ± 7.85	371.27 ± 6.04	389.40 ± 4.63	<0.0001	0.0031	0.9605
Material Density (mg HA/ccm)	1137.24 ± 2.55	1140.81 ± 2.29	1137.05 ± 2.26	1134.36 ± 2.34	0.1610	0.8327	0.1933
Degree of Anisotropy (%)	1.84 ± 0.028	1.80 ± 0.015	1.80 ± 0.025	1.78 ± 0.017	0.2332	0.1317	0.7262
Vertebral Cortex							
Cortical Thickness (mm)	0.062 ± 0.001	0.069 ± 0.001	0.069 ± 0.001	0.072 ± 0.001	<0.0001	<0.0001	0.0606

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. BV/TV bone volume per unit of tissue volume; Tb.N. trabecular number; Tb.Th. trabecular thickness; Tb.Sp. trabecular separation; Conn Density connectivity density; SMI structural model index.

Table 9
Biomechanical Analysis of Trabecular Bone in Femur Metaphysis and Lumbar Vertebra Using Finite Element Analysis.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Femur Metaphysis FEA							
Stiffness ($Nx10^3/mm$)	344.95 ± 49.20	474.17 ± 73.84	633.27 ± 44.63	694.21 ± 49.66	<0.0001	0.0938	0.5417
Total Force (N)	179.49 ± 25.36	249.93 ± 38.26	329.64 ± 23.29	361.54 ± 25.86	<0.0001	0.0825	0.5071
Size Independent Stiffness (N/m)	112.48 ± 15.82	155.58 ± 23.57	197.97 ± 14.14	223.71 ± 17.40	0.0001	0.0636	0.6335
Corrected Von Mises (MPa)	6.59 ± 0.88	4.79 ± 0.31	3.69 ± 0.10	3.62 ± 0.19	0.0001	0.0563	0.0778
Vertebra FEA							
Stiffness (N/mm)	243.66 ± 22.47	312.73 ± 24.65	345.59 ± 34.56	370.50 ± 37.05	0.0006	0.0330	0.3113
Total Force (N)	322.91 ± 24.39	431.05 ± 30.60	460.66 ± 29.93	516.2 ± 23.89	0.0002	0.0042	0.3407
Size Independent Stiffness (N/m)	387.57 ± 22.24	514.74 ± 36.91	521.99 ± 30.93	584.63 ± 27.14	0.0012	0.0025	0.2847
Corrected Von Mises (MPa)	14.62 ± 0.64	12.31 ± 0.56	11.31 ± 0.46	10.26 ± 0.28	<0.0001	0.0017	0.2204

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other.

Table 10
Biomechanical Analysis of Cortical Bone in Femur Diaphysis and Femoral Neck Using Reference Point Indentation.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Mid Diaphysis							
Total Indentation Distance (µm)	33.1 ± 0.83	32.9 ± 0.42	31.8 ± 0.39	32.4 ± 0.39	0.0806	0.7009	0.4344
Indentation Distance Increase (µm)	5.11 ± 0.27	5.41 ± 0.23	4.93 ± 0.14	4.91 ± 0.27	0.1224	0.5580	0.4988
Femoral Neck							
Total Indentation Distance (µm)	30.62 ± 1.15^{b}	23.88 ± 0.88^{a}	25.13 ± 0.93^{a}	24.88 ± 0.52^{a}	0.0183	0.0006	0.0012
Indentation Distance Increase (µm)	4.4 ± 0.29^{b}	3.19 ± 0.09^{a}	3.36 ± 0.25^{a}	3.39 ± 0.16^{a}	0.0555	0.0084	0.0063

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other.

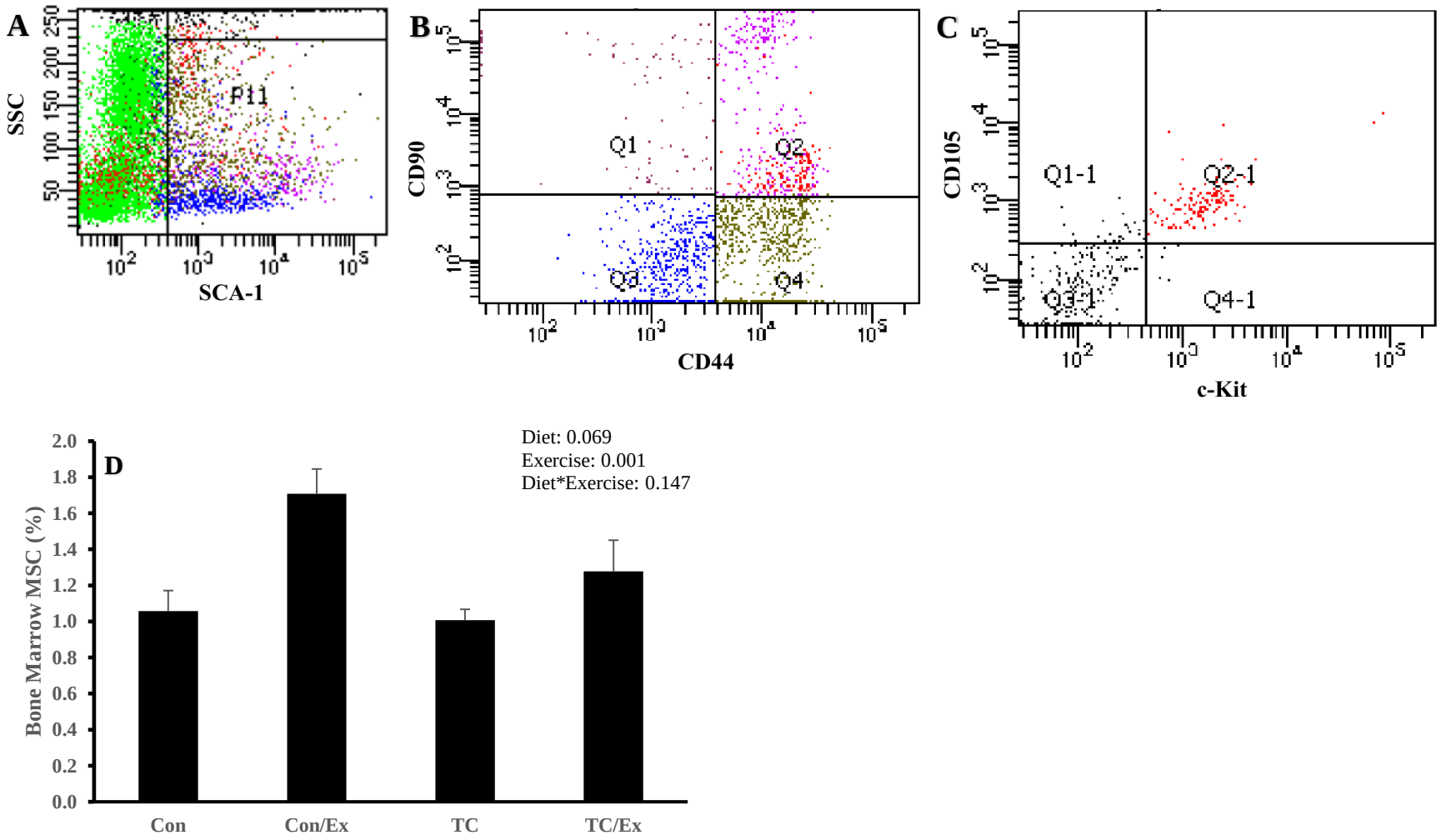

Figure 9. Representative figure of MSC population based on: SCA-1$^+$, CD90$^+$, CD44$^+$, CD105$^+$, and c-Kit$^+$. Events in p11 (**A**) were identified based on SSC and SCA-1$^+$, events in Q2 (**B**) CD90$^+$/CD44$^+$, and events in Q2-1 (**C**) CD105$^+$/c-Kit$^+$ indicating them as mesenchymal stem cells. (**D**) Effects of 60 days of exercise (Ex), tart cherry (TC), or their combination on tibial bone marrow derived mesenchymal stem cells (MSC). Bars represent the mean $\pm$ SE for each treatment group. Bars that share the same superscript letter are not significantly ($p<0.05$) different from each other.

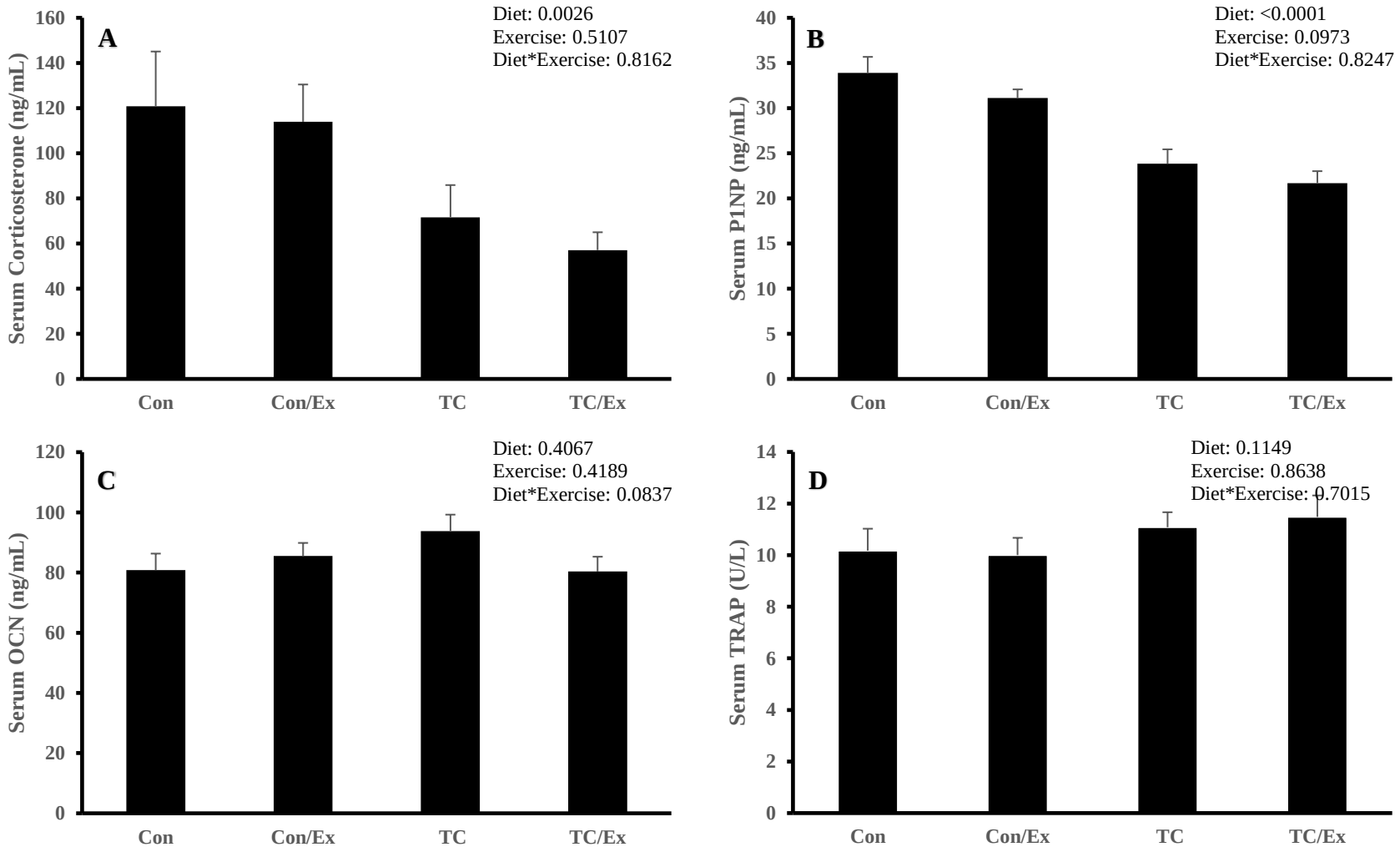

Figure 10. Effects of 60 days of exercise (Ex), tart cherry (TC), or their combination on serum markers of stress (i.e. Corticosterone), bone formation (i.e. P1NP and OCN), or bone resorption (i.e. TRAP). A) corticosterone; B) P1NP procollagen type 1 amino terminal pro-peptide; C) OCN osteocalcin; D) TRAP tartrate-resistant acid phosphatase. Bars represent the mean $\pm$ SE for each treatment group. Bars that share the same superscript letter are not significantly ($p<0.05$) different from each other.

Table 11
Relative Gene Expression Related to Osteoblastogenesis, Mineralization, Collagen Formation, and Osteocytes.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Osteoblastogenesis							
Osterix	1 ± 0.16	1.24 ± 0.27	0.92 ± 0.22	1.35 ± 0.50	0.9871	0.3060	0.7597
RUNX2	1 ± 0.16	1.25 ± 0.21	1.03 ± 0.21	1.06 ± 0.31	0.7366	0.5449	0.6380
BMP2	1 ± 0.12	0.92 ± 0.17	1.45 ± 0.20	2.00 ± 0.57	0.0266	0.4787	0.3223
BMP4	1 ± 0.11	0.99 ± 0.15	1.08 ± 0.21	0.91 ± 0.31	0.9505	0.6995	0.6992
Osteoblast Activity							
Col1a1	1 ± 0.16	0.80 ± 0.14	1.06 ± 0.16	0.90 ± 0.29	0.6304	0.3621	0.9329
Mineralization							
OPN	1 ± 0.23	0.82 ± 0.13	0.70 ± 0.10	0.90 ± 0.18	0.5206	0.9857	0.2755
OCN	1 ± 0.13	1.09 ± 0.22	0.94 ± 0.07	1.09 ± 0.29	0.8809	0.5431	0.8897
BSP	1 ± 0.21	0.83 ± 0.14	0.97 ± 0.11	0.89 ± 0.28	0.9359	0.5294	0.8150
Phex	1 ± 0.08	1.49 ± 0.22	1.06 ± 0.21	1.02 ± 0.44	0.3067	0.2088	0.1962
PPAR-λ	1 ± 0.25	0.95 ± 0.12	1.22 ± 0.22	0.81 ± 0.16	0.6224	0.2118	0.2816
Osteocyte							
Sost	1 ± 0.20	1.40 ± 0.24	1.06 ± 0.25	1.22 ± 0.38	0.8411	0.3240	0.6779

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. RUNX2 runt related transcription factor-2; BMP4 bone morphogenic protein-4; BMP4 bone morphogenic protein-2; OPN osteopontin; OCN osteocalcin; Phex phosphate regulating neutral endopeptidase; PPAR- λ peroxisome proliferator activated receptor-gamma; BSP bone sialoprotein; Col1a1 type 1 collagen; Sost sclerostin.

Table 12
Relative Gene Expression Related to Osteoclastogenesis and Collagen Resorption.

	Con	Con/Ex	TC	TC/Ex	P-Value Diet	P-value Exercise	P-value Diet*Exercise
Osteoclastogenesis							
Nfatc1	1 ± 0.07[b]	0.93 ± 0.07[b]	1.10 ± 0.12[b]	0.64 ± 0.05[a]	0.0972	0.0075	0.0287
MCSF	1 ± 0.08	1.07 ± 0.10	0.71 ± 0.06	0.65 ± 0.05	0.0002	0.9872	0.3908
c-Fos	1 ± 0.13	1.20 ± 0.22	0.76 ± 0.17	0.79 ± 0.10	0.0707	0.4866	0.6171
c-Fms	1 ± 0.13	0.88 ± 0.06	1.35 ± 0.19	0.84 ± 0.05	0.6249	0.0543	0.2846
TRAF6	1 ± 0.08	0.97 ± 0.12	1.07 ± 0.14	0.90 ± 0.07	0.9932	0.3608	0.5037
RANK	1 ± 0.10	0.95 ± 0.06	1.12 ± 0.14	0.93 ± 0.06	0.7490	0.2452	0.4817
RANKL	1 ± 0.15	0.97 ± 0.21	1.18 ± 0.12	0.91 ± 0.18	0.6709	0.4113	0.4941
OPG	1 ± 0.09	1.39 ± 0.24	1.28 ± 0.25	1.13 ± 0.33	0.9505	0.5840	0.2821
Activity							
Ctsk	1 ± 0.12	1.43 ± 0.21	1.64 ± 0.29	1.30 ± 0.28	0.3170	0.7946	0.1229
MMP2	1 ± 0.15	1.20 ± 0.12	0.93 ± 0.09	0.70 ± 0.07	0.0430	0.9735	0.0993
MMP3	1 ± 0.26	0.47 ± 0.13	1.12 ± 0.53	0.80 ± 0.36	0.4982	0.2615	0.7763
MMP8	1 ± 0.07[a]	1.29 ± 0.11[b]	0.94 ± 0.05[a]	0.75 ± 0.09[a]	0.0023	0.5852	0.0105
MMP9	1 ± 0.04	1.12 ± 0.10	0.87 ± 0.07	0.70 ± 0.08	0.0019	0.7004	0.0688

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other. Nfatc1 nuclear factor of activated t-cells 1; MCSF macrophage colony stimulating factor; c-Fos; c-Fms colony stimulating factor-1 receptor; TRAF6 TNF receptor associated factor-6; RANK receptor activator of nuclear factor-kappaβ; RANKL receptor activator of nuclear factor-kappaβ ligand; OPG osteoprotegrin; Ctsk cathepsin-K; MMP matrix metalloproteinase.

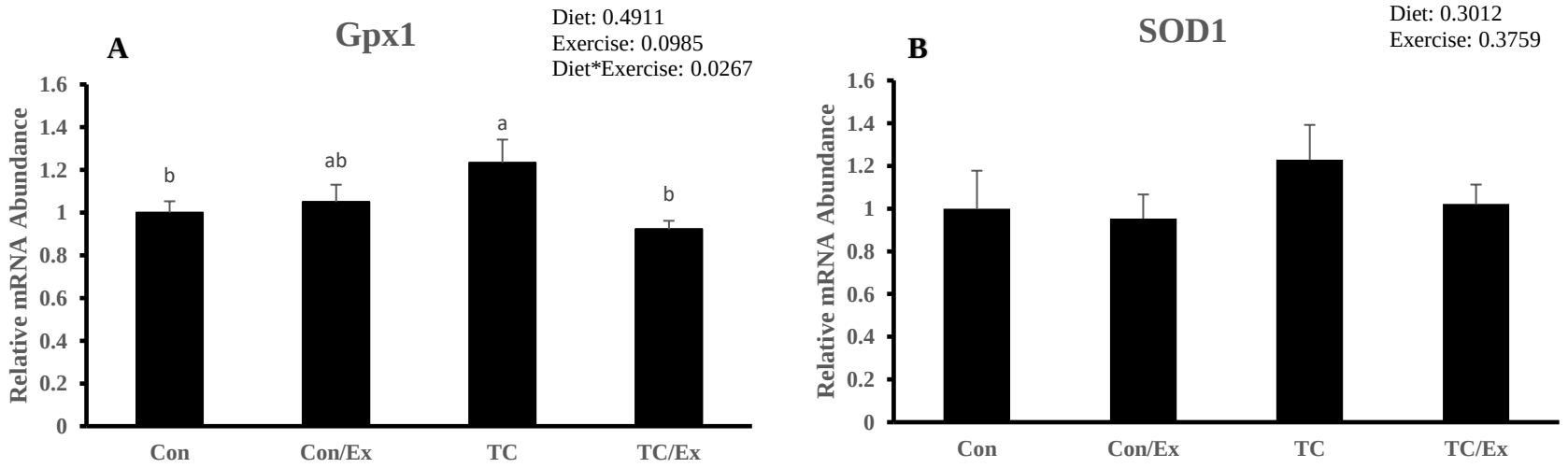

Figure 11. Effects of 60 days of exercise (Ex), tart cherry (TC), or their combination compared to control on endogenous antioxidant expression. A) Gpx1 glutathione peroxidase-1; B) SOD1 super-oxide dismutase-1. Bars represent the mean $\pm$ SE for each treatment group. Bars that share the same superscript letter are not significantly ($p<0.05$) different from each other.

CHAPTER V

DISCUSSION

This is the first study to assess the effects of tart cherry alone and in combination with exercise on the accrual of bone mass in a model of the growing skeleton. A previous study in our lab demonstrated the efficacy of tart cherry (5 and 10%) in the prevention of bone loss resulting from aging (*manuscript under review*). The results of the current study demonstrate that tart cherry and exercise exert significant benefit on trabecular and cortical bone in the growing skeleton, even though the combination did not elicit a synergistic or additive effect on most bone structural or biomechanical parameters. Moreover, it is worth noting, the magnitude of the response of bone parameters (e.g. whole body BMC, femoral and vertebral trabecular bone volume) was greater in terms of tart cherry than the response to exercise.

Peak bone mass has been identified as one of the single greatest predictors of osteoporosis risk [30]. The bone mass achieved in the growing animals used in this study after 8 weeks of treatment, was increased by 6.31% in response to tart cherry as indicated by whole body BMC. However, the addition of exercise did not enhance this response. Site-specific assessment of BMC in the femur, a site loaded by the treadmill running exercise regimen used in the study, suggested that exercise suppressed BMC accrual

compared to controls. To better understand the effects of tart cherry and exercise, trabecular and cortical bone compartments were assessed utilizing μCT. Trabecular bone analysis of the distal femur metaphysis revealed a ~34% increase in trabecular bone volume in response to exercise and tart cherry combined. This effect of tart cherry on trabecular bone resulted from an increase in both the number and thickness of trabeculae. In terms of the cortical bone, the only cortical parameter in the femur that was affected by exercise or tart cherry was cortical area, which was reduced by exercise. The reduction in cortical area was not anticipated as several studies have reported no alterations in cortical area in growing female C57BL/6 mice in response to treadmill running [155-157]. Wallace and colleagues [157] utilized a similar exercise protocol as the one used in the present study, showed no differences in cortical parameters in either the femur or tibia of female mice. This response raised the question of whether or not an exercise-induced stress response could be responsible. However, serum corticosterone was not altered by exercise in this study. Unexpectedly, trabecular bone of the spine, at a site not directly loaded by treadmill running, exhibited a more robust response to exercise and tart cherry with a 12.2% and 16.7% increase in BV/TV, respectively. Synergistic effects of the tart cherry and exercise on trabecular bone of the spine were noted and resulted from an increase in trabecular thickness resulting in a 26.6% increase in trabecular volume. Unlike the cortical bone in the femur, cortical thickness in the vertebrae was improved by 16.1% from the combination of exercise and tart cherry. It would be expected that exercise would more significantly affect the femurs due to increased mechanical loading; however, these results suggest a greater effect in the vertebral body. Data regarding the effects of treadmill running on the vertebral body is limited, but Iwamoto and colleagues

[158] reported a similar improvement in lumbar bone mass in response to a long-term exercise regimen. This response in the vertebrae could be due to the incline of the treadmill or from the forces resulting from flexion involved in running for quadrupedal animals, but that cannot be determined for certain from this study.

To date, no other studies have investigated the effects of tart cherry in the young growing skeleton. However, dried plum, which has a similar polyphenolic and oligosaccharide composition as tart cherry, has been investigated in the growing skeleton. Shahnazari and colleagues [140] reported that 5%, 15%, and 25% dried plum supplementation in growing male mice resulted in 12%, 36%, and 64% increases in trabecular bone volume, respectively. Chen and colleagues [126] reported that blueberry supplementation (10% w/w), which is also a rich source of polyphenolic compounds, resulted in a ~30% increase in tibial trabecular bone volume in growing female rats. By comparison, 10% tart cherry supplementation resulted in a ~28.5% increase in femoral trabecular bone volume. Zhu and colleagues [159] reported that in growing male mice trabecular bone volume was 3-fold higher in alendronate-treated mice, 2-fold higher in Zoledronate-treated mice, and 1.3- to 1.6-fold higher in Clodronate- and Pamidronate-treated mice. In terms of trabecular bone volume, tart cherry had comparable effects to dried plum, blueberry, and Pamidronate; however, unlike bisphosphonates, tart cherry elicited no unfavorable side effects and amounts to a fraction of the cost**.**

As a result of the alterations in bone structural properties, the effects of tart cherry and exercise were examined on bone biomechanical properties. Finite element analysis of trabecular bone within the distal femur revealed a 101.4% increase in the total force in the group receiving the tart cherry and exercise compared to the control group. Both

exercise and tart cherry improved the structural model index, which likely contributes to this response. Tart cherry alone improved stiffness and Von Mises stresses at this site and the effects of exercise only tended to have favorable effects. Similar to our observations in the structural parameters of trabecular bone within the spine, there was a robust response in non-morphometric parameters to exercise compared to the femur. The vertebral body exhibited a more plate like structure and increased apparent density in response to tart cherry and exercise alone. Trabecular bone strength was markedly improved with an ~60% increase in total force exhibited by the mice treated with the combination of tart cherry and exercise. Despite the more robust bone structural change in the spine, the effects on the trabecular bone biomechanical properties were not as great as those observed in the femur. Evaluation of cortical bone biomechanical parameters in the femur utilizing reference point indentation testing revealed no effects on bone biomechanical properties indicating that any structural changes that did occur in the femur, did not affect cortical bone strength. However, in the femoral neck a trabecular rich site with a thick cortex, total indentation distance was decreased suggesting a positive response to tart cherry, exercise, and their combination. A previous study examining the effects of treadmill running (15m/m for 30 min 5 d/wk) on bone biomechanical properties resulted in no alterations in stiffness or bearable load in 8 week old male C57BL/6 mice [160]. However, no studies on the effects of tart cherry on bone biomechanical have been published to date. The effects of other functional foods have revealed improvements in bone biomechanical properties soy protein, flaxseed, and their combination in young growing animals [161]. Our findings suggest that tart cherry and exercise may individually enhance bone biomechanical properties, the primary goal of

osteoporosis treatment. Additionally, tart cherry exerted improvements in mechanical properties independent of mechanical loading while exerting no side effects, thus supporting it as a potentially beneficial treatment in osteoporosis prevention.

To gain mechanistic insight into the bone microarchitectural changes that occurred in response to treatment, systemic and local indicators of osteoblast related bone formation and osteoclast related bone resorption were assessed. Serum TRAP, a systemic indicator of osteoclastic activity, showed no effects of either treatment. At the tissue level, evaluation of regulators of osteoclastogenesis within the femur revealed that the key regulator of osteoclastogenesis, Nfatc1, was suppressed by the combination of tart cherry and exercise. Only a trend was detected in the down-regulation of c-Fos, and c-Fms in response to tart cherry and exercise, respectively. Serum OCN, considered a systemic indicator of bone turnover and potentially mineralization, was not altered with treatment while serum P1NP, a systemic marker of collagen formation, was suppressed by tart cherry. The literature suggests that a decrease in serum P1NP concurrent with improved bone quality is not uncommon. For example, our lab has reported that dried plum supplementation in male mice resulted in a 43% suppression of serum P1NP simultaneous with significant increases in BMD, BMC, and trabecular bone volume [162]. Investigation of local indicators of osteoblast activity showed that tart cherry increased BMP-2 expression by ~80%. BMP-2 is known to stimulate MSC differentiation towards an osteoblast lineage via increasing the Runx-2 transcription factor [87]. However, Runx2 was not altered after 8 wks of treatment in this study. Polyphenolic compounds from functional foods with similar profiles as tart cherry have been shown to upregulate BMP-2 [48] and suppress Nfatc1 *in vitro* [47]. Thus, it stands to reason that

the polyphenolic compounds in tart cherry may be contributing to its effects on bone. The incongruence of the local and systemic findings suggests that local responses to tart cherry as well as exercise may differ, depending on the site that is being studied. Furthermore, the metabolic state of the bone after 8 wks of treatments may not reflect the early changes in osteoblast and osteoclast activity that lead to improvements in bone structural and biomechanical properties. Serial studies appear to be warranted on order to better understand the effects of tart cherry and exercise on bone.

Functional foods are believed to largely exert their effects via their antioxidant and anti-inflammatory properties. Montmorency tart cherries possess a higher total phenolic content relative to other cultivars of cherries [143] which is largely composed of anthocyanins and hydroxycinnamic acids. The oxygen radical absorption capacity (ORAC) for fresh tart cherries was ranked 14th out of 50 foods in terms of antioxidant capacity per serving [163]. Ou and colleagues [164] reported that dried tart cherries have an ORAC of 6120 per ½ cup (68/gram) serving which was second only to dried plums with an ORAC of 6850 per ½ cup serving (81/gram). In terms of indicators of antioxidant activity, the relative gene expression of Gpx1 increased by 23% in response to tart cherry. However, this response in Gpx1 was blunted when tart cherry was combined with exercise. An increase in the relative abundance of Gpx1 may result in an increase in glutathione peroxidase which reduces hydrogen peroxide radicals into water thereby preventing oxidative stress. Free radicals, which result in oxidative stress, have been shown to degenerate bone via a stimulation of IL-1 and subsequent osteoclastic resorption [165]. Furthermore, RANKL binding produces free radicals to stimulate osteoclastogenesis [166]. Consequently, an increase in glutathione peroxidase may result

in enhanced quenching of free radicals and the prevention of RANKL induced upregulation of Nfatc1.

In the current study, FACS analysis of MSC populations showed that it is significantly elevated in the exercise groups. These results are in agreement with Maredziak and colleagues [34] who reported increased bone marrow MSC populations resulting from a chronic exercise regimen. Although not statistically significant, tart cherry combined with exercise resulted in a 25% reduction in MSCs compared to exercise alone. Considering this decline in MSCs seen with tart cherry, the tart cherry upregulation of BMP-2, and reduction of corticosterone, indicate that tart cherry promotes MSCs towards an osteogenic lineage. This notion is supported by Pereira and colleagues [167] who reported BMP-2 enhanced MSC osteogenic potential and cortisol enhanced MSC adipogenesis. Other sources of polyphenols (e.g., EGCG) have been shown to promote osteogenesis of MSCs *in vitro*, thereby supporting the potential role of functional foods to stimulate osteoblast differentiation [135]. Based on the results of the current study, we surmise that exercise increased MSC populations in the bone marrow while tart cherry promoted their osteogenic lineage allocation via BMP-2 signaling and a suppression of corticosterone. This proposed mechanism would need to be confirmed by additional analyses but would provide mechanistic insights into the improved bone microarchitecture.

The combination of tart cherry and exercise as a means to prevent osteoporosis is an appealing alternative to pharmaceutical options. In this study tart cherry and/or exercise enhanced trabecular and cortical bone and biomechanical properties. These effects occurred in conjunction with alterations in key regulators of osteogenesis (BMP-

2) and osteoclastogenesis (Nfatc1), suppression of the stress hormone, corticosterone, and the upregulation of Gpx1, a potent antioxidant. Further studies are warranted to clearly delineate the role of each of these in the skeletal response to tart cherry and exercise. Moreover, clinical trials on the ability of tart cherry and exercise to enhance peak bone mass in children are needed. The 10% w/w dosage of tart cherry used in this study is a feasible quantity for human consumption (<1/2cup dried tart cherries), supporting potential for translation into clinical studies. Overall, tart cherry was at least as effective as exercise in its ability to improve bone quality, indicating that it may provide novel means of enhancing peak bone mass in the young growing skeleton and ultimately reducing lifetime risk of osteoporosis.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

This study was designed to determine whether tart cherry affects accrual of bone mass in growing animals and whether tart cherry would elicit a synergistic effect with exercise. Following a 2-week acclimation period, 4-week-old female C57BL/6 mice were assigned to one of four treatment groups (n=12-14mice/group): control (Con), control plus exercise (Con/Ex), tart cherry (TC;10% w/w), or TC plus Ex (TC/Ex). Animals were subjected to treatment for 8 weeks. At the end of the study the whole body and femur bone densitometry, FACS analysis of tibial bone marrow MSC, bone microarchitecture, bone biomechanical properties, and systemic and local indicators of osteoblast and osteoclast activity and differentiation were assessed. Findings indicate that tart cherry primarily affects trabecular bone of long bones and cortical bone of the spine. Systemic markers of resorption (i.e. TRAP) and mineralization (i.e. OCN) were not altered by treatment, but serum P1NP decreased in response to tart cherry suggesting decreased collagen formation. Within the bone tissue, tart cherry upregulated BMP-2, a key regulator of osteogenesis and tart cherry combined with exercised downregulated Nfatc1, suggesting a local suppression of osteoclastogenesis. MSC populations were increased by exercise and tended to be decreased in response to tart cherry. Importantly, tart cherry also increased in Gpx1 expression and suppressed serum corticosterone, which may

provide additional insight into its effects on the bone microenvironment. These observations in conjunction with gene expression data may suggest that exercise increases MSC populations and tart cherry promotes their differentiation toward an osteoblast lineage via BMP2; however, this would need to be confirmed with additional analyses.

Conclusions

The following is a list of aims and working hypothesis that were proposed for this study:

Specific Aim 1: To compare the effects of the tart cherry, exercise, or their combination on bone quality (i.e., BMD, trabecular and cortical microarchitecture, and biomechanical properties) in young growing animals.

Working hypothesis Aim 1: The combination of tart cherry and exercise will yield a synergistic effect on bone quality exceeding either variable administered individually.

We reject the hypothesis. Tart cherry and exercise improve bone quality and biomechanical properties, but their combination is not synergistic. Findings showed no change in BMD after treatment while BMC was increased (6.31%) by tart cherry. Both exercise and tart cherry increased trabecular bone volume in the femur (34%) and spine (26.6%), however not synergistically. Similarly, cortical bone in the vertebral body was improved (16.1%) by tart cherry and exercise individually. Trabecular biomechanical parameters (i.e. stiffness, total force, and Von Mises) in the femur were improved by tart cherry while vertebral body parameters were improved by tart cherry and exercise, but again not synergistically. Reference point indentation (RPI) of the femoral neck produced

synergistic effects on total indentation distance and indentation distance increase, however the combination was not significantly improved compared to either variable individually.

Specific Aim 2: To determine the effect of tart cherry, exercise or their combination on bone marrow MSC populations and their progression towards an osteoblast lineage.
Working hypothesis for Aim 2: Tart cherry combined with exercise will result in a larger MSC population and a greater potential to form active osteoblasts than either variable individually.

We reject the hypothesis. The combination of tart cherry and exercise did not result in a greater MSC population than either variable individually. Exercise significantly increased the MSC populations (61%) in the bone marrow of the tibia. In contrast, the combination of tart cherry and exercise resulted in 25% fewer MSCs than the exercise group. Whether the combination promotes a greater potential to form active osteoblasts as a potential explanation for the improved bone parameters remains unclear from these findings.

Specific Aim 3: To assess the alterations in regulators of osteoblast (e.g. Runx2 and Osterix) and osteoclast (e.g. RANKL, OPG, and NFatc1) differentiation that occur in response to treatments.
Working hypothesis for Aim 3: The combination of tart cherry and exercise will, to a greater magnitude, promote osteoblastogenesis and suppress osteoclastogenesis compared to all other treatment groups.

We reject the hypothesis. The combination of tart cherry and exercise suppressed Nfatc1 relative to all other groups. This coincided with trends toward suppression of c-Fos with tart cherry and c-Fms with exercise which could lead to the suppression of Nfatc1 and osteoclastogenesis, but neither c-Fos nor c-Fms reached the level of statistical significance. Tart cherry alone enhanced BMP2 expression (80%) which could promote osteogenesis.

Specific Aim 4: To evaluate the effects of tart cherry, exercise or their combination on osteoblast and osteoclast activity by examining circulating levels of biomarkers of resorption (i.e., CTX) and formation (i.e., P1NP) and local indicators of osteoblast activity (e.g. ALP, type I collagen), and mineralization (e.g., OCN, Phex, and Ppar-γ), and osteoclast activity (e.g. CathK).

Working hypothesis for Aim 4: Tart cherry and exercise in combination will, to a greater magnitude, stimulate osteoblast activity and inhibit osteoclast activity above all other treatment groups.

We reject the hypothesis. Serum P1NP was the only activity indicator altered by treatment. However, P1NP was suppressed (43%) by tart cherry suggesting reduced osteoblast activity. Serum TRAP was unaffected by treatments suggesting no alterations in resorption. However, relative abundance of Nfatc1 mRNA was suppressed in the femur from the combination treatment suggesting reduced osteoclastogenesis. Lastly, the relative abundance of BMP-2 mRNA was decreased in the femur from tart cherry suggesting increased osteoblastogenesis.

Specific Aim 5: To assess alterations in antioxidant status indicated by gene expression of key enzymes involved in scavenging free radicals in bone (e.g. Gpx1 and SOD1) resulting from tart cherry, exercise or their combination.

Working hypothesis for Aim 5: Tart cherry will increase the levels of endogenous antioxidant indicators.

We accept the hypothesis. Tart cherry increased the expression of Gpx1 by 23%, however the addition of exercise suppressed the tart cherry induced increase in Gpx1.

Recommendations

This study suggests some very positive effects of tart cherry supplementation in the growing skeleton, however a number of queries remain unanswered. What systemic and local alterations are occurring at different time-points throughout the study? What local gene effects are occurring at other sites (i.e. vertebral body)? Are transcriptional alterations being translated into alterations in protein levels and do tart cherry and exercise regulate post-transcriptionally? Do these effects occur in humans? To address these questions, future animal studies designed to include a time series study (e.g. baseline, 2 wks, 4 wks, and 8 wks) are needed to determine if alterations in systemic and local indicators are changing over the progression of treatment. Investigation of local indicators of bone formation and resorption in the vertebral body is warranted to provide insight relevant to the mechanism by which exercise is exerting a greater effect than in the femur. Analysis of protein to determine whether the altered transcription of BMP-2, Gpx1, and Nfatc1 is carried out translationally. Furthermore, investigation of protein is

needed to assess whether local indicators of osteoblastogenesis (i.e. Runx2), osteoclastogenesis (i.e. MCSF), bone formation (i.e. OCN), and bone resorption (i.e. CathK) are being translationally regulated as opposed to transcriptionally. This study provides evidence that in female growing mice, tart cherry and exercise cause beneficial effects on bone quality. However, further studies are needed to delineate the effects of tart cherry and exercise on translational regulation, different time points, and different sites before conducting clinical studies.

LITERATURE CITED

1. Reginster, J.Y. and N. Burlet, *Osteoporosis: a still increasing prevalence.* Bone, 2006. **38**(2 Suppl 1): p. S4-9.
2. *NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, March 7-29, 2000: highlights of the conference.* South Med J, 2001. **94**(6): p. 569-73.
3. Sernbo, I. and O. Johnell, *Consequences of a hip fracture: a prospective study over 1 year.* Osteoporos Int, 1993. **3**(3): p. 148-53.
4. Benjamin, R.M., *Bone Health: Preventing Osteoporosis.* Public Health Reports, 2010. **125**(3): p. 368-370.
5. Karaguzel, G. and M.F. Holick, *Diagnosis and treatment of osteopenia.* Rev Endocr Metab Disord, 2010. **11**(4): p. 237-51.
6. Wright, N.C., et al., *The impact of the new National Bone Health Alliance (NBHA) diagnostic criteria on the prevalence of osteoporosis in the USA.* Osteoporos Int, 2017. **28**(4): p. 1225-1232.
7. Wright, N.C., et al., *The Recent Prevalence of Osteoporosis and Low Bone Mass in the United States Based on Bone Mineral Density at the Femoral Neck or Lumbar Spine().* J Bone Miner Res, 2014. **29**(11): p. 2520-6.
8. Burge, R., et al., *Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025.* J Bone Miner Res, 2007. **22**(3): p. 465-75.
9. Clarke, B., *Normal Bone Anatomy and Physiology.* Clin J Am Soc Nephrol, 2008. **3**(Suppl 3): p. S131-9.
10. Brandi, M.L., *Microarchitecture, the key to bone quality.* Rheumatology (Oxford), 2009. **48 Suppl 4**: p. iv3-8.
11. Dalle Carbonare, L. and S. Giannini, *Bone microarchitecture as an important determinant of bone strength.* J Endocrinol Invest, 2004. **27**(1): p. 99-105.
12. Florencio-Silva, R., et al., *Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells.* BioMed Research International, 2015. **2015**: p. 421746.
13. Fuchs, R.K., S.J. Warden, and C.H. Turner, *2 - Bone anatomy, physiology and adaptation to mechanical loading*, in *Bone Repair Biomaterials*. 2009, Woodhead Publishing. p. 25-68.
14. Seeman, E., *Bone modeling and remodeling.* Crit Rev Eukaryot Gene Expr, 2009. **19**(3): p. 219-33.
15. Caetano-Lopes, J., H. Canhao, and J.E. Fonseca, *Osteoblasts and bone formation.* Acta Reumatol Port, 2007. **32**(2): p. 103-10.
16. Eriksen, E.F., *Cellular mechanisms of bone remodeling.* Reviews in Endocrine & Metabolic Disorders, 2010. **11**(4): p. 219-227.

17. Tanaka, Y., S. Nakayamada, and Y. Okada, *Osteoblasts and osteoclasts in bone remodeling and inflammation.* Curr Drug Targets Inflamm Allergy, 2005. **4**(3): p. 325-8.
18. Viccica, G., C.M. Francucci, and C. Marcocci, *The role of PPARgamma for the osteoblastic differentiation.* J Endocrinol Invest, 2010. **33**(7 Suppl): p. 9-12.
19. Barros, N.M., et al., *Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia.* J Bone Miner Res, 2013. **28**(3): p. 688-99.
20. Rutkovskiy, A., K.-O. Stensløkken, and I.J. Vaage, *Osteoblast Differentiation at a Glance.* Medical Science Monitor Basic Research, 2016. **22**: p. 95-106.
21. Katagiri, T. and N. Takahashi, *Regulatory mechanisms of osteoblast and osteoclast differentiation.* Oral Dis, 2002. **8**(3): p. 147-59.
22. Rucci, N., *Molecular biology of bone remodelling.* Clinical Cases in Mineral and Bone Metabolism, 2008. **5**(1): p. 49-56.
23. Aarden, E.M., E.H. Burger, and P.J. Nijweide, *Function of osteocytes in bone.* J Cell Biochem, 1994. **55**(3): p. 287-99.
24. Bonewald, L.F. and M.L. Johnson, *Osteocytes, mechanosensing and Wnt signaling.* Bone, 2008. **42**(4): p. 606-615.
25. Das, S. and J.C. Crockett, *Osteoporosis – a current view of pharmacological prevention and treatment.* Drug Design, Development and Therapy, 2013. **7**: p. 435-448.
26. Bodenner, D., C. Redman, and A. Riggs, *Teriparatide in the management of osteoporosis.* Clinical Interventions in Aging, 2007. **2**(4): p. 499-507.
27. Gupta, A. and L. March, *Treating osteoporosis.* Australian Prescriber, 2016. **39**(2): p. 40-46.
28. Kling, J.M., B.L. Clarke, and N.P. Sandhu, *Osteoporosis Prevention, Screening, and Treatment: A Review.* Journal of Womens Health, 2014. **23**(7): p. 563-572.
29. Bonjour, J.P., et al., *Peak bone mass.* Osteoporos Int, 1994. **4 Suppl 1**: p. 7-13.
30. Bonjour, J.P., et al., *The importance and relevance of peak bone mass in the prevalence of osteoporosis.* Salud Publica Mex, 2009. **51 Suppl 1**: p. S5-17.
31. Gönül, B., G. Baltacı, and M. Koz, *Effects of Run Training on Bone Development and Bone Mineralization in Growing Mice.* Biology of Sport, 2011. **28**(2): p. 129-132.
32. Chilibeck, P.D., D.G. Sale, and C.E. Webber, *Exercise and bone mineral density.* Sports Med, 1995. **19**(2): p. 103-22.
33. Wallace, I.J., et al., *Focal enhancement of the skeleton to exercise correlates with responsivity of bone marrow mesenchymal stem cells rather than peak external forces.* J Exp Biol, 2015. **218**(Pt 19): p. 3002-9.
34. Marędziak, M., et al., *Physical Activity Increases the Total Number of Bone-Marrow-Derived Mesenchymal Stem Cells, Enhances Their Osteogenic Potential, and Inhibits Their Adipogenic Properties.* Stem Cells Int, 2015. **2015**: p. 379093.
35. New, S.A., *Bone health: the role of micronutrients.* Br Med Bull, 1999. **55**(3): p. 619-33.

36. Arjmandi, B.H., et al., *Dried plums improve indices of bone formation in postmenopausal women.* J Womens Health Gend Based Med, 2002. **11**(1): p. 61-8.
37. Hooshmand, S., et al., *The effect of two doses of dried plum on bone density and bone biomarkers in osteopenic postmenopausal women: a randomized, controlled trial.* Osteoporos Int, 2016. **27**(7): p. 2271-9.
38. Smith, B.J., et al., *Effects of dried plum supplementation on bone metabolism in adult C57BL/6 male mice.* Calcif Tissue Int, 2014. **94**(4): p. 442-53.
39. Rendina, E., et al., *Dried plum's unique capacity to reverse bone loss and alter bone metabolism in postmenopausal osteoporosis model.* PLoS One, 2013. **8**(3): p. e60569.
40. Smith, B.J., et al., *A comparative study of the bone metabolic response to dried plum supplementation and PTH treatment in adult, osteopenic ovariectomized rat.* Bone, 2014. **58**: p. 151-9.
41. Shen, C.L., et al., *Synergistic effects of green tea polyphenols and alphacalcidol on chronic inflammation-induced bone loss in female rats.* Osteoporos Int, 2010. **21**(11): p. 1841-52.
42. Shen, C.L., et al., *Dietary polyphenols and mechanisms of osteoarthritis.* J Nutr Biochem, 2012. **23**(11): p. 1367-77.
43. Arjmandi, B.H., et al., *Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion.* J Clin Endocrinol Metab, 2003. **88**(3): p. 1048-54.
44. Devareddy, L., et al., *Soy moderately improves microstructural properties without affecting bone mass in an ovariectomized rat model of osteoporosis.* Bone, 2006. **38**(5): p. 686-93.
45. Khalil, D.A., et al., *Soy isoflavones may protect against orchidectomy-induced bone loss in aged male rats.* Calcif Tissue Int, 2005. **76**(1): p. 56-62.
46. Wong, W.W., et al., *Soy isoflavone supplementation and bone mineral density in menopausal women: a 2-y multicenter clinical trial.* Am J Clin Nutr, 2009. **90**(5): p. 1433-9.
47. Graef, J.L., et al., *Osteoclast Differentiation is Downregulated by Select Polyphenolic Fractions from Dried Plum via Suppression of MAPKs and Nfatc1 in Mouse C57BL/6 Primary Bone Marrow Cells.* Current Developments in Nutrition, 2017. **1**(10): p. e000406-e000406.
48. Graef, J.L., et al., *Select polyphenolic fractions from dried plum enhance osteoblast activity through BMP-2 signaling.* The Journal of Nutritional Biochemistry, 2017.
49. Jovanovic-Malinovska, R., S. Kuzmanova, and E. Winkelhausen, *Oligosaccharide Profile in Fruits and Vegetables as Sources of Prebiotics and Functional Foods.* International Journal of Food Properties, 2014. **17**(5): p. 949-965.
50. Slavin, J., *Fiber and Prebiotics: Mechanisms and Health Benefits.* Nutrients, 2013. **5**(4): p. 1417-1435.
51. Lucas, S., et al., *Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss.* Nature Communications, 2018. **9**(1): p. 55.

52. *Osteoporosis Facts and Statistics.* *[http://www.osteoporosis.ca/index.php/ci_id/8867/la_id/1.htm]*.
53. Bernabei, R., et al., *Screening, diagnosis and treatment of osteoporosis: a brief review.* Clin Cases Miner Bone Metab, 2014. **11**(3): p. 201-7.
54. Finkelstein, J.S., et al., *Bone Mineral Density Changes during the Menopause Transition in a Multiethnic Cohort of Women.* The Journal of Clinical Endocrinology and Metabolism, 2008. **93**(3): p. 861-868.
55. Kling, J.M., B.L. Clarke, and N.P. Sandhu, *Osteoporosis Prevention, Screening, and Treatment: A Review.* Journal of Women's Health, 2014. **23**(7): p. 563-572.
56. Vestergaard, P., L. Rejnmark, and L. Mosekilde, *Osteoporosis is markedly underdiagnosed: a nationwide study from Denmark.* Osteoporos Int, 2005. **16**(2): p. 134-41.
57. Woolf, A.D. and B. Pfleger, *Burden of major musculoskeletal conditions.* Bull World Health Organ, 2003. **81**(9): p. 646-56.
58. Kanis, J.A., et al., *Risk of hip fracture according to the World Health Organization criteria for osteopenia and osteoporosis.* Bone, 2000. **27**(5): p. 585-90.
59. Bacon, W.E., et al., *International comparison of hip fracture rates in 1988-89.* Osteoporos Int, 1996. **6**(1): p. 69-75.
60. Kannus, P., et al., *Epidemiology of hip fractures.* Bone, 1996. **18**(1 Suppl): p. 57s-63s.
61. Panula, J., et al., *Mortality and cause of death in hip fracture patients aged 65 or older - a population-based study.* BMC Musculoskeletal Disorders, 2011. **12**(1): p. 105.
62. Bonjour, J.-P., et al., *The importance and relevance of peak bone mass in the prevalence of osteoporosis.* Salud Pública de México, 2009. **51**: p. s5-s17.
63. Raisz, L.G. and E. Seeman, *Causes of age-related bone loss and bone fragility: an alternative view.* J Bone Miner Res, 2001. **16**(11): p. 1948-52.
64. Soyka, L.A., W.P. Fairfield, and A. Klibanski, *Clinical review 117: Hormonal determinants and disorders of peak bone mass in children.* J Clin Endocrinol Metab, 2000. **85**(11): p. 3951-63.
65. Ackerman, K.E. and M. Misra, *Bone Health in Adolescent Athletes with a Focus on Female Athlete Triad.* The Physician and sportsmedicine, 2011. **39**(1): p. 131-141.
66. Weise, M., et al., *Effects of estrogen on growth plate senescence and epiphyseal fusion.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(12): p. 6871-6876.
67. Vaananen, H.K. and P.L. Harkonen, *Estrogen and bone metabolism.* Maturitas, 1996. **23 Suppl**: p. S65-9.
68. Weaver, C.M., et al., *The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations.* Osteoporosis International, 2016. **27**: p. 1281-1386.
69. Heaney, R.P., et al., *Peak Bone Mass.* Osteoporosis International, 2000. **11**(12): p. 985-1009.

70. Feng, X., *Chemical and Biochemical Basis of Cell-Bone Matrix Interaction in Health and Disease.* Current chemical biology, 2009. **3**(2): p. 189-196.
71. Roach, H.I., *Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption.* Cell Biol Int, 1994. **18**(6): p. 617-28.
72. Markings, B., *The skeletal system.* 2004.
73. Langdahl, B., S. Ferrari, and D.W. Dempster, *Bone modeling and remodeling: potential as therapeutic targets for the treatment of osteoporosis.* Therapeutic Advances in Musculoskeletal Disease, 2016. **8**(6): p. 225-235.
74. Roberts, W.E., S. Huja, and J.A. Roberts, *Bone modeling: biomechanics, molecular mechanisms, and clinical perspectives.* Seminars in Orthodontics, 2004. **10**(2): p. 123-161.
75. Kontulainen, S., et al., *Effect of long-term impact-loading on mass, size, and estimated strength of humerus and radius of female racquet-sports players: a peripheral quantitative computed tomography study between young and old starters and controls.* J Bone Miner Res, 2002. **17**(12): p. 2281-9.
76. Clarke, B., *Normal Bone Anatomy and Physiology.* Clinical Journal of the American Society of Nephrology : CJASN, 2008. **3**(Suppl 3): p. S131-S139.
77. Delaisse, J.-M., *The reversal phase of the bone-remodeling cycle: cellular prerequisites for coupling resorption and formation.* BoneKEy Rep, 2014. **3**.
78. Kim, J.H. and N. Kim, *Regulation of NFATc1 in Osteoclast Differentiation.* J Bone Metab, 2014. **21**(4): p. 233-41.
79. Bu, S.Y., T.S. Hunt, and B.J. Smith, *Dried plum polyphenols attenuate the detrimental effects of TNF-α on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I.* The Journal of Nutritional Biochemistry, 2009. **20**(1): p. 35-44.
80. Bu, S.Y., et al., *Dried Plum Polyphenols Inhibit Osteoclastogenesis by Downregulating NFATc1 and Inflammatory Mediators.* Calcified Tissue International, 2008. **82**(6): p. 475-488.
81. Georgess, D., et al., *Podosome organization drives osteoclast-mediated bone resorption.* Cell Adhesion & Migration, 2014. **8**(3): p. 192-204.
82. Wheater, G., et al., *The clinical utility of bone marker measurements in osteoporosis.* Journal of Translational Medicine, 2013. **11**: p. 201-201.
83. Kawai, M. and C.J. Rosen, *The Insulin-Like Growth Factor System in Bone: Basic and Clinical Implications.* Endocrinology and metabolism clinics of North America, 2012. **41**(2): p. 323-vi.
84. Baksh, D., L. Song, and R.S. Tuan, *Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy.* J Cell Mol Med, 2004. **8**(3): p. 301-16.
85. Huang, W., et al., *Signaling and transcriptional regulation in osteoblast commitment and differentiation.* Frontiers in bioscience : a journal and virtual library, 2007. **12**: p. 3068-3092.
86. Strohbach, C.A., D.D. Strong, and C.H. Rundle, *Gene Therapy Applications for Fracture Repair*, in *Gene Therapy Applications*, C. Kang, Editor. 2011, InTech: Rijeka. p. Ch. 12.

87. Sun, J., et al., *Role of bone morphogenetic protein-2 in osteogenic differentiation of mesenchymal stem cells.* Molecular Medicine Reports, 2015. **12**(3): p. 4230-4237.
88. Wang, Z., et al., *Effects of Statins on Bone Mineral Density and Fracture Risk: A PRISMA-compliant Systematic Review and Meta-Analysis.* Medicine, 2016. **95**(22): p. e3042.
89. Zhang, J., et al., *Feeding Blueberry Diets in Early Life Prevent Senescence of Osteoblasts and Bone Loss in Ovariectomized Adult Female Rats.* PLOS ONE, 2011. **6**(9): p. e24486.
90. Hale, L.V., et al., *PINP: A serum biomarker of bone formation in the rat.* Bone, 2007. **40**(4): p. 1103-1109.
91. Linkhart, S.G., et al., *Synthetic peptide-based immunoassay for amino-terminal propeptide of type I procollagen: application for evaluation of bone formation.* Clin Chem, 1993. **39**(11 Pt 1): p. 2254-8.
92. Khan, E. and Y. Abu-amer, *Activation of peroxisome proliferator–activated receptor-γ inhibits differentiation of preosteoblasts.* Journal of Laboratory and Clinical Medicine, 2003. **142**(1): p. 29-34.
93. Lim, S.Y. and M.B. Bolster, *Current approaches to osteoporosis treatment.* Curr Opin Rheumatol, 2015. **27**(3): p. 216-24.
94. Cosman, F., et al., *Clinician's Guide to Prevention and Treatment of Osteoporosis.* Osteoporosis International, 2014. **25**(10): p. 2359-2381.
95. Cosman, F., *Anabolic and antiresorptive therapy for osteoporosis: combination and sequential approaches.* Curr Osteoporos Rep, 2014. **12**(4): p. 385-95.
96. Hopkins, R.B., et al., *The relative efficacy of nine osteoporosis medications for reducing the rate of fractures in post-menopausal women.* BMC Musculoskeletal Disorders, 2011. **12**(1): p. 209.
97. Sikon, A. and P. Batur, *Profile of teriparatide in the management of postmenopausal osteoporosis.* International Journal of Women's Health, 2010. **2**: p. 37-44.
98. Dore, R.K., *Long-term safety, efficacy, and patient acceptability of teriparatide in the management of glucocorticoid-induced osteoporosis.* Patient preference and adherence, 2013. **7**: p. 435-446.
99. Kennel, K.A. and M.T. Drake, *Adverse Effects of Bisphosphonates: Implications for Osteoporosis Management.* Mayo Clinic Proceedings, 2009. **84**(7): p. 632-638.
100. Bailey, C.A. and K. Brooke-Wavell, *Exercise for optimising peak bone mass in women.* Proc Nutr Soc, 2008. **67**(1): p. 9-18.
101. Czarkowska-Paczek, B., K. Wesolowska, and J. Przybylski, *[Physical exercise prevents osteoporosis]*. Przegl Lek, 2011. **68**(2): p. 103-6.
102. Natali, A.N. and E.A. Meroi, *A review of the biomechanical properties of bone as a material.* Journal of Biomedical Engineering, 1989. **11**(4): p. 266-276.
103. Bankoff, A., *Biomechanical Characteristics of the Bone*. 2012.
104. Price, C.T., J.R. Langford, and F.A. Liporace, *Essential Nutrients for Bone Health and a Review of their Availability in the Average North American Diet.* The Open Orthopaedics Journal, 2012. **6**: p. 143-149.

105. Bailey, R.L., et al., *Estimation of Total Usual Calcium and Vitamin D Intakes in the United States.* The Journal of Nutrition, 2010. **140**(4): p. 817-822.
106. Augustine, M. and M.J. Horwitz, *Parathyroid Hormone and Parathyroid Hormone-related Protein Analogs as Therapies for Osteoporosis.* Current osteoporosis reports, 2013. **11**(4): p. 10.1007/s11914-013-0171-2.
107. Kroll, M.H., *Parathyroid hormone temporal effects on bone formation and resorption.* Bull Math Biol, 2000. **62**(1): p. 163-88.
108. Takahashi, N., N. Udagawa, and T. Suda, *Vitamin D endocrine system and osteoclasts.* BoneKEy Rep, 2014. **3**.
109. Kumar, J., et al., *Prevalence and Associations of 25-Hydroxyvitamin D Deficiency in US Children: NHANES 2001–2004.* Pediatrics, 2009. **124**(3): p. e362-e370.
110. Heaney, R.P. and D.K. Layman, *Amount and type of protein influences bone health.* The American Journal of Clinical Nutrition, 2008. **87**(5): p. 1567S-1570S.
111. Zhang, W., et al., *Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: differential signalling via Akt and ERK.* Cell Biochem Funct, 2012. **30**(4): p. 297-302.
112. Glick, P.L. and D.J. Rowe, *Effects of chronic protein deficiency on skeletal development of young rats.* Calcified Tissue International, 1981. **33**(1): p. 223-231.
113. Pearson, D.A., *Bone Health and Osteoporosis: The Role of Vitamin K and Potential Antagonism by Anticoagulants.* Nutrition in Clinical Practice, 2007. **22**(5): p. 517-544.
114. Bugel, S., *Vitamin K and bone health.* Proc Nutr Soc, 2003. **62**(4): p. 839-43.
115. Pan, X.-d., et al., *Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel.* Journal of Zhejiang University. Science. B, 2009. **10**(4): p. 258-263.
116. Weaver, C.M., *Diet, Gut Microbiome, and Bone Health.* Current osteoporosis reports, 2015. **13**(2): p. 125-130.
117. Contor, L., *Functional Food Science in Europe.* Nutr Metab Cardiovasc Dis, 2001. **11**(4 Suppl): p. 20-3.
118. Pandey, K.B. and S.I. Rizvi, *Plant polyphenols as dietary antioxidants in human health and disease.* Oxidative Medicine and Cellular Longevity, 2009. **2**(5): p. 270-278.
119. Perron, N.R. and J.L. Brumaghim, *A review of the antioxidant mechanisms of polyphenol compounds related to iron binding.* Cell Biochem Biophys, 2009. **53**(2): p. 75-100.
120. Zhou, B., et al., *Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles.* Free Radic Biol Med, 2005. **38**(1): p. 78-84.
121. Banihani, S., S. Swedan, and Z. Alguraan, *Pomegranate and type 2 diabetes.* Nutr Res, 2013. **33**(5): p. 341-8.
122. Kim, H.M. and J. Kim, *The Effects of Green Tea on Obesity and Type 2 Diabetes.* Diabetes & Metabolism Journal, 2013. **37**(3): p. 173-175.
123. Ravindran, J., S. Prasad, and B.B. Aggarwal, *Curcumin and Cancer Cells: How Many Ways Can Curry Kill Tumor Cells Selectively?* The AAPS Journal, 2009. **11**(3): p. 495-510.

124. Li, F., et al., *Resveratrol, a neuroprotective supplement for Alzheimer's disease.* Curr Pharm Des, 2012. **18**(1): p. 27-33.
125. Weaver, C.M., et al., *Flavonoid Intake and Bone Health.* Journal of nutrition in gerontology and geriatrics, 2012. **31**(3): p. 239-253.
126. Chen, J.R., et al., *Dietary-induced serum phenolic acids promote bone growth via p38 MAPK/beta-catenin canonical Wnt signaling.* J Bone Miner Res, 2010. **25**(11): p. 2399-411.
127. Zhang, J., et al., *Blueberry consumption prevents loss of collagen in bone matrix and inhibits senescence pathways in osteoblastic cells.* Age (Dordr), 2013. **35**(3): p. 807-20.
128. Li, T., et al., *Rabbiteye blueberry prevents osteoporosis in ovariectomized rats.* Journal of Orthopaedic Surgery and Research, 2014. **9**: p. 56-56.
129. Arjmandi, B.H., et al., *One year soy protein supplementation has positive effects on bone formation markers but not bone density in postmenopausal women.* Nutrition Journal, 2005. **4**: p. 8-8.
130. Zheng, X., S.-K. Lee, and O.K. Chun, *Soy Isoflavones and Osteoporotic Bone Loss: A Review with an Emphasis on Modulation of Bone Remodeling.* Journal of Medicinal Food, 2016. **19**(1): p. 1-14.
131. Arjmandi, B.H., et al., *Role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone deficiency in rats.* Am J Clin Nutr, 1998. **68**(6 Suppl): p. 1358s-1363s.
132. Droke, E.A., et al., *Soy isoflavones avert chronic inflammation-induced bone loss and vascular disease.* Journal of Inflammation (London, England), 2007. **4**: p. 17-17.
133. Lorenzo, J.M. and P.E.S. Munekata, *Phenolic compounds of green tea: Health benefits and technological application in food.* Asian Pacific Journal of Tropical Biomedicine, 2016. **6**(8): p. 709-719.
134. Vester, H., et al., *Green Tea Extract (GTE) improves differentiation in human osteoblasts during oxidative stress.* Journal of Inflammation, 2014. **11**(1): p. 15.
135. Chen, C.-H., et al., *Green tea catechin enhances osteogenesis in a bone marrow mesenchymal stem cell line.* Osteoporosis International, 2005. **16**(12): p. 2039-2045.
136. Wallace, T.C., *Dried Plums, Prunes and Bone Health: A Comprehensive Review.* Nutrients, 2017. **9**(4): p. 401.
137. !!! INVALID CITATION !!! {}.
138. Franklin, M., et al., *Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway.* Bone, 2006. **39**(6): p. 1331-1342.
139. Halloran, B.P., et al., *Dietary dried plum increases bone mass in adult and aged male mice.* J Nutr, 2010. **140**(10): p. 1781-7.
140. Shahnazari, M., et al., *Dietary dried plum increases bone mass, suppresses proinflammatory cytokines and promotes attainment of peak bone mass in male mice.* J Nutr Biochem, 2016. **34**: p. 73-82.
141. Hooshmand, S., et al., *Comparative effects of dried plum and dried apple on bone in postmenopausal women.* Br J Nutr, 2011. **106**(6): p. 923-30.

142. Graef, J.L., et al., *Dried plum polyphenolic extract combined with vitamin K and potassium restores trabecular and cortical bone in osteopenic model of postmenopausal bone loss.* Journal of Functional Foods, 2018. **42**: p. 262-270.
143. Kirakosyan, A., et al., *Chemical profile and antioxidant capacities of tart cherry products.* Food Chemistry, 2009. **115**(1): p. 20-25.
144. Blando, F., C. Gerardi, and I. Nicoletti, *Sour Cherry (Prunus cerasus L) Anthocyanins as Ingredients for Functional Foods.* J Biomed Biotechnol, 2004. **2004**(5): p. 253-258.
145. Connolly, D.A., et al., *Efficacy of a tart cherry juice blend in preventing the symptoms of muscle damage.* Br J Sports Med, 2006. **40**(8): p. 679-83; discussion 683.
146. Lynn, A., et al., *Effect of a tart cherry juice supplement on arterial stiffness and inflammation in healthy adults: a randomised controlled trial.* Plant Foods Hum Nutr, 2014. **69**(2): p. 122-7.
147. Carson, C.A., *Tart Cherry Juice as a Treatment for Peripheral Neuropathy.* Integrative Medicine: A Clinician's Journal, 2015. **14**(1): p. 48-49.
148. Levers, K., et al., *Effects of powdered Montmorency tart cherry supplementation on acute endurance exercise performance in aerobically trained individuals.* Journal of the International Society of Sports Nutrition, 2016. **13**: p. 22.
149. Levers, K., et al., *Effects of powdered Montmorency tart cherry supplementation on an acute bout of intense lower body strength exercise in resistance trained males.* Journal of the International Society of Sports Nutrition, 2015. **12**: p. 41.
150. Howatson, G., et al., *Influence of tart cherry juice on indices of recovery following marathon running.* Scand J Med Sci Sports, 2010. **20**(6): p. 843-52.
151. Reeves, P.G., *Components of the AIN-93 Diets as Improvements in the AIN-76A Diet.* The Journal of Nutrition, 1997. **127**(5): p. 838S-841S.
152. Conner, J.D., T. Wolden-Hanson, and L.S. Quinn, *Assessment of Murine Exercise Endurance Without the Use of a Shock Grid: An Alternative to Forced Exercise.* Journal of Visualized Experiments : JoVE, 2014(90): p. 51846.
153. Keyak, J.H., et al., *Automated three-dimensional finite element modelling of bone: a new method.* Journal of Biomedical Engineering, 1990. **12**(5): p. 389-397.
154. Newitt, D.C., et al., *In Vivo Assessment of Architecture and Micro-Finite Element Analysis Derived Indices of Mechanical Properties of Trabecular Bone in the Radius.* Osteoporosis International, 2002. **13**(1): p. 6-17.
155. Hollinski, R., et al., *Young and healthy C57BL/6 J mice performing sprint interval training reveal gender- and site-specific changes to the cortical bone.* Scientific Reports, 2018. **8**: p. 1529.
156. Koenen, K., et al., *Sprint Interval Training Induces A Sexual Dimorphism but does not Improve Peak Bone Mass in Young and Healthy Mice.* Scientific Reports, 2017. **7**: p. 44047.
157. Wallace, J.M., et al., *Exercise-Induced Changes in the Cortical Bone of Growing Mice Are Bone and Gender Specific.* Bone, 2007. **40**(4): p. 1120-1127.
158. Iwamoto, J., T. Takeda, and Y. Sato, *Effect of treadmill exercise on bone mass in female rats.* Exp Anim, 2005. **54**(1): p. 1-6.
159. Zhu, E.D., et al., *Effect of Bisphosphonates on the Rapidly Growing Male Murine Skeleton.* Endocrinology, 2014. **155**(4): p. 1188-1196.

160. Gardinier, J.D., et al., *Bone adaptation in response to treadmill exercise in young and adult mice.* Bone Reports, 2018. **8**: p. 29-37.
161. A Power, K., et al., *Flaxseed and Soy Protein Isolate, Alone and in Combination, Differ in their Effect on Bone Mass, Biomechanical Strength, and Uterus in Ovariectomized Nude Mice with MCF-7 Human Breast Tumor Xenografts*. Vol. 70. 2007. 1888-96.
162. Smith, B.J., et al., *Effects of Dried Plum Supplementation on Bone Metabolism in Adult C57BL/6 Male Mice.* Calcified Tissue International, 2014. **94**(4): p. 442-453.
163. Halvorsen, B.L., et al., *Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States.* American Journal of Clinical Nutrition, 2006. **84**(1): p. 95-135.
164. Ou, B., et al., *Processed Tart Cherry Products—Comparative Phytochemical Content, in vitro Antioxidant Capacity and in vitro Anti-inflammatory Activity.* Journal of Food Science, 2012. **77**(5): p. H105-H112.
165. Garrett, I.R., et al., *Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo.* Journal of Clinical Investigation, 1990. **85**(3): p. 632-639.
166. Kyung, T.-W., et al., *Rutin inhibits osteoclast formation by decreasing reactive oxygen species and TNF-α by inhibiting activation of NF-κB.* Experimental & Molecular Medicine, 2008. **40**(1): p. 52-58.
167. Pereira, R.C., A.M. Delany, and E. Canalis, *Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression.* Bone, 2002. **30**(5): p. 685-91.

APPENDIX

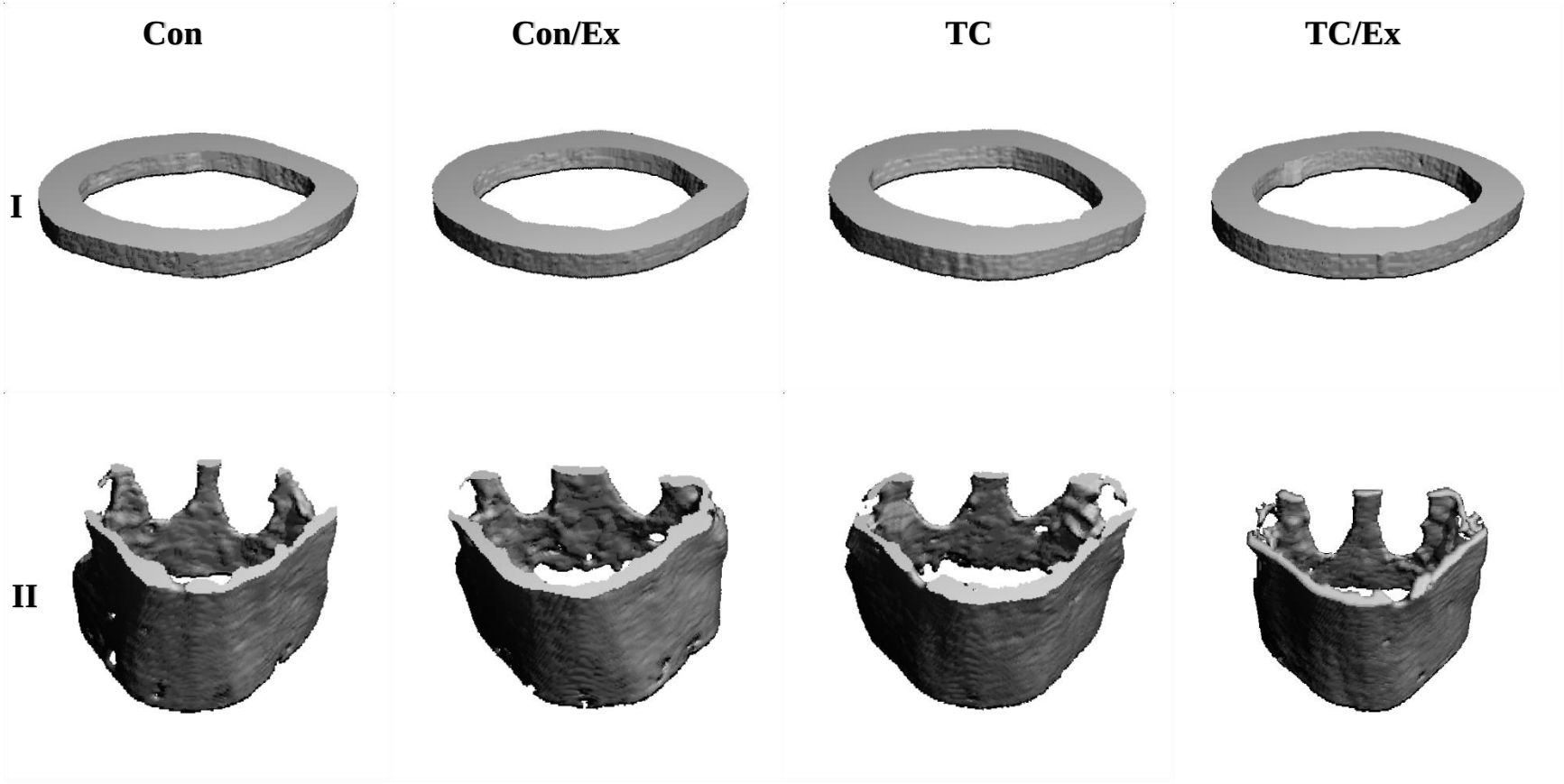

Figure 12. 3D representative images of cortical bone micro-architecture in the (Row I) femoral mid-diaphysis and (Row II) vertebral body.

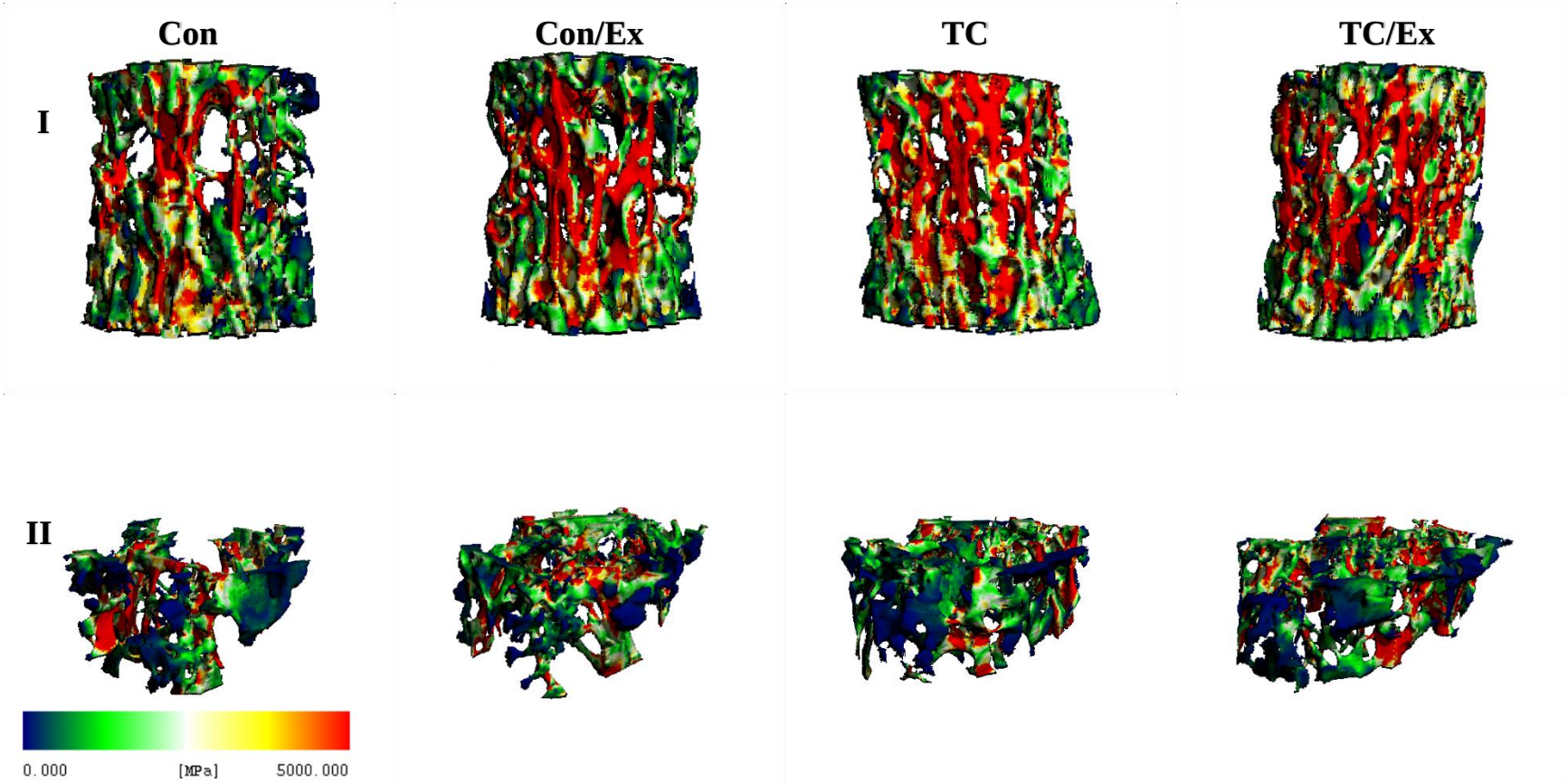

Figure 13. 3D representative images of trabecular bone micro-architecture with FEA overlay in the (Row I) vertebral body and (Row II) femoral distal-metaphysis.

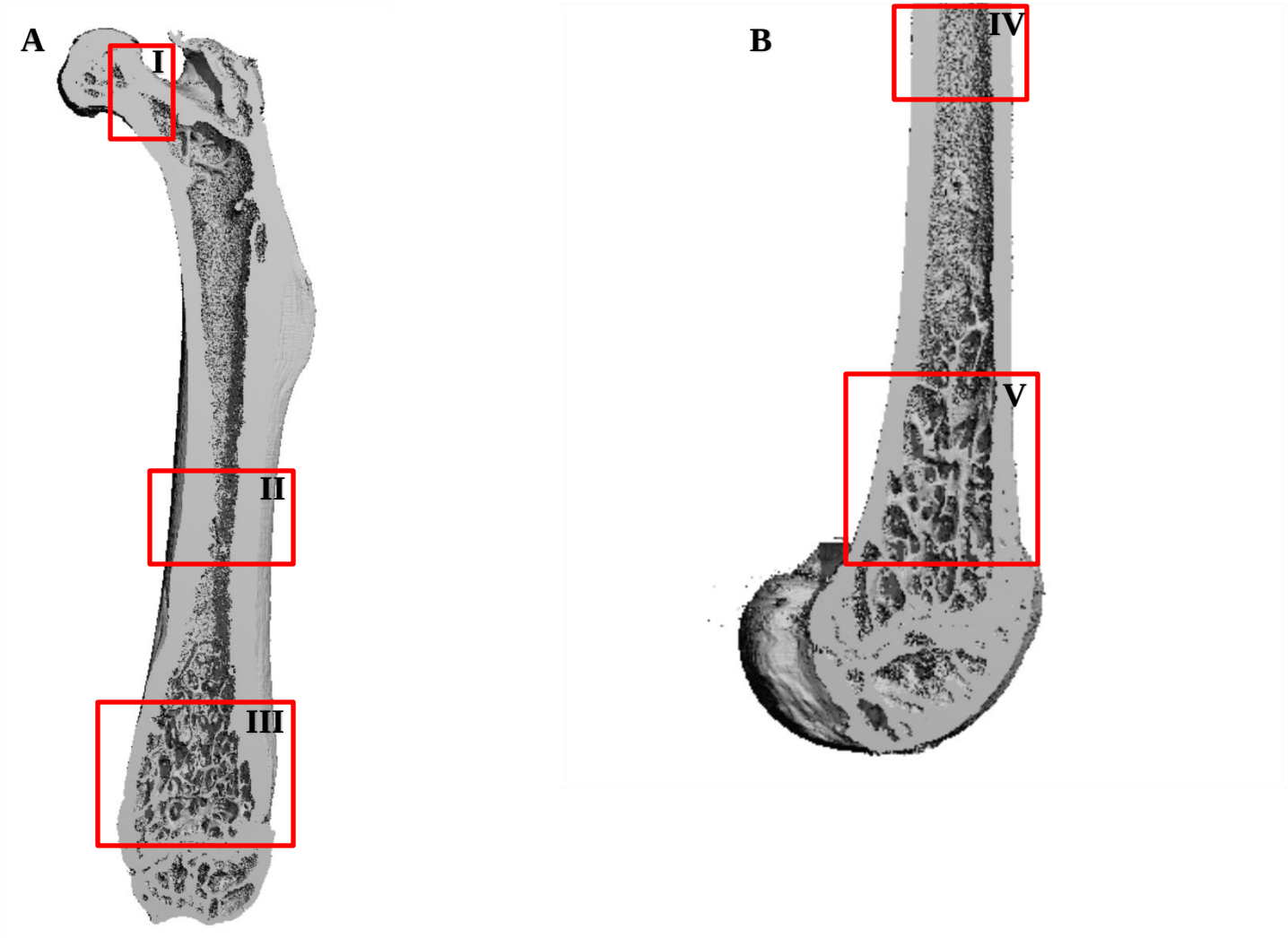

Figure 14. 3D representative images of (A) whole femur rear cut plane and (B) distal femur metaphysis side cut plane. I) femoral neck; II) mid-diaphysis rear view; III) distal metaphysis rear view; IV) mid-diaphysis side view; V) distal metaphysis side view.

VITA

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