THE EFFECTS OF TART CHERRY JUICE SUPPLEMENTATION ON BIOCHEMICAL MARKERS OF BONE METABOLISM IN WOMEN AGED 65-80 YEARS

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THE EFFECTS OF TART CHERRY JUICE

SUPPLEMENTATION ON BIOCHEMICAL MARKERS

OF BONE METABOLISM IN WOMEN

AGED 65-80 YEARS

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Abstract:

Objectives: This study examined the dose-dependent effects of supplementation with a commercially available Montmorency tart cherry juice on biomarkers of bone metabolism in older women.
Methods: Women 65-80 years of age (n=27) were randomly assigned to consume one of two doses of tart cherry juice (8 fl. oz. once or twice per day) for 90 d. Serum at baseline and final visits were assessed for biomarkers of bone formation and resorption, endocrine status, inflammation, and oxidative stress. Other data collected include relevant medical history, anthropometrics, calcium intake, physical activity, dietary intake, and sun exposure. Whole body, right hip, and lumbar spine bone mineral density (BMD) was evaluated at baseline with dual x-ray absorptiometry (DXA). Data were analyzed with paired t-test, Chi square analyses followed by confirmation with Fisher's exact test for categorical data, and Pearson's correlation.
Results: Biomarkers of bone resorption (i.e., tartrate resistant acid phosphatase type 5b [TRAcP 5b]) were decreased in the high but not the low dose group from baseline. Markers of bone formation (i.e, bone specific alkaline phosphatase) and bone turnover (i.e., osteocalcin [OCN]) were not different from baseline or between treatment groups. C-reactive protein was negatively correlated with TRAcP 5b (r = *-0.32*) and TBARS was negatively correlated with OCN (*r = -0.30*).
Conclusion: Supplementation with the higher dose of tart cherry juice for 90 d decreased biomarkers of bone resorption from baseline, but biomarkers of bone formation and mineralization were unchanged in this cohort of older women. Indicators of inflammation and oxidative stress were also unchanged from baseline. Future studies are needed to determine effect of tart cherry supplementation in forms other than juice and on outcomes such as bone mineral density.

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CHAPTER I

INTRODUCTION

Osteoporosis is a debilitating progressive skeletal disease, predominantly afflicting older adults, which can lead to costly fracture and devastating health complications. It is characterized by low bone mineral density (BMD), deterioration of bone microarchitecture, and/or occurrence of non-traumatic fracture.[2] Over 10 million Americans currently suffer from osteoporosis and more than 43 million have low bone mass (i.e., osteopenia).[3] Prevalence among older adults with this widespread condition is projected to increase two-fold by the year 2020 and three-fold by the year 2030.[3] The extensive impact is underlined by the more than two million non-traumatic fractures that occur annually, of which osteoporosis and low bone mass is the main contributor in adults over the age of 50 years,[5] and $19 billion in healthcare costs associated with osteoporotic fracture.[6] A hip fracture at age 60 years or older leads to reduced independence and initiates a rapid decline in health, ultimately reducing life expectancy by more than 11 years.[7] Importantly, women are at as much as three times greater risk for osteoporosis and fracture than men, which also increases with age for both.[3,8] As a result, women account for more than 75% of healthcare costs associated with the treatment of osteoporosis-related fracture.[6]

Two of the key determinants of osteoporosis risk are attainment of optimal peak bone mass and age of menopause onset in women.[9,10] Bone mass peaks in late puberty for males and

females; however, the later onset of puberty in males (13-15 years) compared to females (11-13 years) allows an additional 1-2 years of pre-pubertal bone accrual.[11,12] The result is a greater peak bone mass on average in men than women.[11] Further, women lose more bone over their lifetime than men due primarily to a sharp decline in the bone protective hormone, estrogen, following menopause.[13,14] Estrogen suppresses resorption, or degradation of bone, largely by its interaction with receptors on the surface of bone cells that modulate bone formation and degradation.[15,16] Estrogen also has potent anti-inflammatory properties, down-regulating the production of inflammatory mediators (e.g., tumor necrosis factor [TNF]-α and interleukin [IL]-6) known to increase bone resorption.[17] In early menopause, the activity of osteoblasts, or bone forming cells, initially increases in an attempt to compensate for accelerated bone resorption; however, due to the greater time required for formation and mineralization, resorption by osteoclasts dominates the remodeling cycle.[18] Taken together, estrogen deficiency, as occurs with menopause, increases bone turnover favoring bone resorption, therefore leading to postmenopausal bone loss.

Bone loss due to estrogen deficiency compounds age-related bone loss.[17] Age-related bone loss, which begins as early as the third decade of life, and postmenopausal bone loss, are recognized as processes driven by inflammatory cytokines and oxidative stress.[19-21] Cytokines are glycoproteins released by a wide variety of cells (e.g., immune cells, endothelial cells, and various stromal cells) that modulate cellular responses to tissue damage, pathogens, and toxins. They can have anti-inflammatory activity, thus down-regulating immune responses, or pro-inflammatory activity, signaling recruitment and activation of immune cells. Activated immune cells increase their production of free radicals (i.e., reactive oxygen and nitrogen species) which are known to damage organic molecules (e.g., DNA, lipids, and proteins), therefore altering their structure and function.[22] Oxidative stress occurs when there is an imbalance between the production of free radicals and the body's ability to neutralize them with its endogenous antioxidant defense systems. Inflammation and oxidative stress suppress osteoblast activity and

increase the activity of osteoclasts.[23,24] Consequently, the bone remodeling cycle (i.e., catabolism of existing bone and formation of new bone) is dysregulated. Age-related bone loss differs from postmenopausal bone loss in that, the overall rate of bone remodeling (i.e., bone turnover) is decreased, but still bone resorption is greater than bone formation.

Current FDA-approved pharmacological treatment options for osteoporosis largely target inhibiting bone resorption, with only one option for stimulating formation. For example, bisphosphonates and Denosumab both inhibit osteoclast activity by decreasing osteoclast differentiation and/or inducing osteoclast apoptosis, or cell death, thereby decreasing the rate of bone turnover.[25,26] While these anti-resorptive drugs have proven beneficial in reducing bone loss and the incidence of fractures,[27] they are not without side effects. Atypical fractures of the femur and necrosis of the jaw or mandible can occur with extended use of these drugs due to altered bone remodeling and death of bone tissue.[28] Further, bioavailability of orally administered bisphosphonates are sensitive to meal timing and can produce severe gastrointestinal upset leading to poor patient compliance. Hormone replacement therapy and selective estrogen receptor modulators (SERMs) are considered less effective alternative anti-resorptive agents and not suitable for all women. To that end, they are not considered a first-line treatment option due to concerns over efficacy in decreasing fracture risk and increased risk for certain cancers and other diseases.[25] The only FDA-approved therapy that has anabolic effects on bone, and thus stimulates bone formation, is Teriparatide, a recombinant form of human parathyroid hormone (PTH). Teriparatide increases osteoclast and osteoblast activity, but has a more pronounced effect on the osteoblast. This treatment requires daily injections, has been associated with side-effects such as dizziness and nausea, and is cost-prohibitive for many patients.[25] Additionally, lack of evidence of its safety for extended use limits administration. Thus, there is a need for, and current pursuit of, low-risk effective alternatives to treat or augment treatment of osteoporosis that are affordable and produce fewer side-effects.

Current research indicates certain foods rich in compounds known to reduce inflammation and oxidative stress may provide alternative or complementary approaches to prevent and treat both age-related and postmenopausal bone loss. Among some of the most studied functional foods, or foods which offer health benefits beyond nutritional sustenance, soy is rich in isoflavones. Isoflavones are structurally similar to estrogen, and modulate cellular inflammation and bone metabolism by binding to estrogen receptors.[29] In multiple pre-clinical and clinical studies examining both age-related and postmenopausal bone loss, soy isoflavones are shown to decrease markers of bone resorption and increase markers of formation as well as decrease expression of osteoclastogenic genes in animal models; however soy isoflavones failed in clinical trials to prevent bone loss.[30] The rich phenolic profile of olive oil is particularly anti-inflammatory and has potent antioxidant qualities. In animal models of postmenopausal bone loss, olive oil was effective in reducing markers of inflammation and oxidative stress while also reducing markers of osteoclast activity demonstrating its ability to protect against bone loss by preserving bone mineral density (BMD) compared to controls in mice.[31]

Research is ongoing with other functional foods that have been found to have osteoprotective effects in aging including wine, various teas, and fruits.[32-36] A particularly promising functional food, which has demonstrated a potential to reverse bone loss in animal models of postmenopausal and age-related bone loss, is dried plum.[37,38] Dried plum was shown to prevent bone loss in postmenopausal women in clinical trial.[39] Given the potent osteoprotective effects of dried plum, it begs the question if other related fruits may provide similar benefit. Tart cherry, being of the same *Prunus* genus as dried plum, has a similar phenolic and nutritional profile, but has been far less studied for its effects on bone health despite its demonstrated potent anti-inflammatory and antioxidant capabilities in pre-clinical and clinical trials.[40,41]

Animal studies from our lab have recently shown that tart cherries are a promising candidate as an osteoprotective functional food. Tart cherries are shown to have antioxidant and

anti-inflammatory properties and are a rich source of beta carotene, polyphenols, and fructooligosaccharides, an indigestible carbohydrate known to support beneficial gut bacteria.[42,43] Particularly rich in tart cherries are the polyphenolic compounds, hydroxycinnamic acids and anthocyanins, which contribute to the fruit's bright red color and have potent bioactivity including antioxidant and anti-inflammatory properties.[43] Whole tart cherry and other forms (i.e., juice and freeze-dried powder) have been studied for their health benefits, demonstrating the potential to reduce muscle damage and improve recovery after exercise, treat symptoms of gout, improve sleep quality, and protect against neurodegenerative diseases, cancer, and cardiovascular disease.[44-50]

Tart cherry's health benefits are believed to be, in part, due to its potent anti-inflammatory and antioxidant properties. Further, investigation in cell culture (e.g., rat microglial cells, breast and mammary gland cancer cells, and adipose stem cells) systems have shown treatments with polyphenols extracted from tart cherry decrease markers of inflammation and oxidative stress challenged with inflammatory cytokines or toxins.[41,51,52] Similar findings have been shown in animal models and clinical trials investigating the anti-inflammatory and antioxidant capacity of the fruit *in vivo*. In particular, tart cherry lowers serum markers of inflammation, including TNF-α, C-reactive protein (CRP), IL-1, and IL-6.[40,48,53,54] Additionally, tart cherry reduces serum indicators of oxidative stress such as total lipid hydroperoxide (LOOH), inducible nitric oxide synthase (iNOS), and F2-isoprostane (F2-IsoP).[48,53-57] Given its ability to reduce markers of inflammation and oxidative stress which are known to influence bone metabolism, tart cherry is a salient candidate for examining the influence of supplementation on bone health. Our laboratory recently showed that the consumption of a diet supplemented with tart cherry (i.e., five and 10% w/w freeze-dried tart cherry powder) protected against bone loss in animal models of age-related and postmenopausal osteoporosis.[58] In light of these promising findings, clinical studies investigating the effects of tart cherry on bone metabolism are needed.

The **purpose** of this research was to determine the dose-dependent effect of 90-day tart cherry juice supplementation on biochemical markers of bone formation and resorption in women aged 65-80 years. Due to reports that tart cherry juice has potent anti-inflammatory and antioxidant activity and the role that these biological processes play in bone loss, we also investigated the effects of two different doses of tart cherry juice on serum indicators of oxidative stress and inflammation. The **hypothesis** to be tested was ninety-days of tart cherry juice supplementation would increase biochemical markers of bone formation and decrease markers of bone resorption in a dose-dependent manner. These alterations in biomarkers of bone metabolism would coincide with a decrease in markers of oxidative stress and inflammation. The hypothesis was tested by accomplishing the following specific aims:

***Aim 1*:** Determine the change in serum biomarkers of bone metabolism (i.e., bone-specific alkaline phosphate [BAP], tartrate resistant phosphates [TRAP-5b], and osteocalcin [OCN]) after 90 days of consuming one of two doses of tart cherry juice (i.e., 8 fl oz once or twice per day) in a population of healthy women aged 65-80 years.

***Aim 2*:** Assess the change in serum indicators of inflammation (i.e., high sensitivity C-reactive protein or hsCRP) and oxidative stress (i.e., thiobarbituric acid reactive species or TBARS) after 90 days of consuming one of two doses of tart cherry juice (i.e., 8 fl oz once or twice per day) in a population of healthy women aged 65-80 years.

***Aim 3*:** To correlate the effects of tart cherry juice treatment on indicators of oxidative stress and inflammation with changes in biochemical markers of bone metabolism.

Limitations:

- This study utilized a tart cherry juice supplementation; however, a pre-clinical trial from our lab utilized a lyophilized tart cherry powder which showed that tart cherry supplementation restored bone loss due to aging and ovarian hormone deficiency.

- Power calculations were based on previous studies of dried plum to detect differences in biomarkers of bone metabolism; however, this study may be underpowered to detect subtle changes that occurred with the lower dose of the juice or to account for the variability that were observed with the inflammatory and oxidative stress biomarkers.

CHAPTER II

REVIEW OF LITERATURE

Defining osteoporosis and risk-factors

Osteoporosis is a degenerative bone disease characterized by low bone mineral density (BMD), microarchitecture deterioration, and/or the occurrence of non-traumatic fracture. Alarmingly, 53% of the U.S. population over the age of 50 years has osteoporosis or osteopenia.[3,59] Fragility due to low bone mass is a major contributor to costly non-traumatic fracture in older adults.[60] More than 2 million osteoporosis-related fractures occur annually in the U.S. with a single fracture incurring as much as $10,000 in direct medical care costs.[6] Current estimates indicate a staggering one in two women and one in four men, aged 50 years and older, will suffer an osteoporotic fracture in their lifetime.[61] As a result, osteoporosis is a serious public health concern in the United States that specifically impacts women; surpassing the incidence of both cardiovascular events and invasive breast cancer combined.[62]

The major criteria for an osteoporosis diagnosis is defined by the World Health Organization (WHO) as persons having a BMD T-score more than 2.5 standard deviations (SDs) below the mean for a healthy young adult (30 years of age) of the same sex and race.[63] In contrast, osteopenia is defined as a T-score between one to 2.5 SDs below the same population average. BMD is calculated from measures of bone mineral area (BMA) and bone mineral

content (BMC) assessed using dual-energy x-ray absorptiometry (DXA), the gold standard for bone densitometry.[64] BMC is a measure of the quantity of mineralized tissue in grams, whereas BMA is a measure of area in square centimeters. This allows calculation of the 2-dimensional or areal bone density. Measurements to diagnose osteoporosis are typically taken at the lumbar spine (L1-L4), hip, and at times the forearm, as fractures at these sites are most common in osteoporosis.[63] The rich quantity of trabecular, (**Figure 1**) or cancellous bone at these anatomical sites is particularly susceptible to rapid bone turnover and therefore, bone loss. Cortical bone, the dense outer mineralized layer of bone that provides structural rigidity, can also thin with age thus contributing to fracture risk. Although DXA is the best clinical measure of fracture risk that is currently available, thinning of the trabeculae in cancellous bone may be difficult to detect in some patients given the 2-dimensional limitation of measuring BMD with DXA.

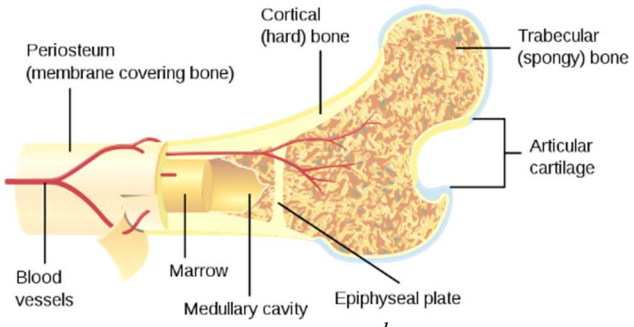

Figure 1. *Bone Cross-Section*[1]

To further define osteoporosis diagnosis, clinical guidelines put forth by the National Osteoporosis Foundation (NOF) include BMD T-scores at the lumbar spine and femoral neck while the International Society for Clinical Densitometry (ISCD) include these measures along with total hip BMD.[26,65] However, BMD is not the only indicator for risk of osteoporosis-related fracture. A fracture resulting from bone fragility, regardless of T-score, would clinically be considered an osteoporosis diagnosis requiring intervention treatment.[26] Therefore, guidelines by the NOF now include results from the Fracture Risk Algorithm (FRAX), adapted from the WHO absolute fracture risk model, in assessing patients for osteoporosis treatment.[26] The FRAX algorithm predicts the probability of an osteoporotic fracture and hip fracture within 10 years. It considers the T-score at the femoral neck as well as modifiable and non-modifiable risk factors

for osteoporosis such as age, sex, weight, height, personal and family fracture history, current smoking, glucocorticoid use, rheumatoid arthritis, secondary osteoporosis (i.e., presence of medical condition or treatment that disrupts normal bone metabolism), and excess alcohol intake. These factors are included for their known impact on skeletal growth and/or bone turnover and remodeling in the adult skeleton.

Although certain modifiable lifestyle factors may help slow or delay bone loss, they may not preclude individuals from developing osteoporosis. Genetic predisposition and other risk factors that accelerate bone loss contribute to disease progression.[66] The generally lower peak bone mass of women than men and hormonal changes in women post-menopause make them particularly susceptible to bone loss. Further, diseases such as cancer, diabetes, and chronic kidney disease, and their treatments are known to compound the risk for osteoporosis and fracture, especially in women.[27] Therefore, it is vital that women adopt recommendations for modifiable risk factors to conserve bone mass; including adequate nutrition, maintaining physical activity throughout the lifespan, and other measures (i.e., regular screenings, preventative care, and lifestyle factors such as avoiding drinking and smoking) to prevent development of chronic diseases.[26]

Adequate nutrition at every stage of life, with emphasis on nutrients such as vitamin D, calcium, phosphorus and other micronutrients, provides the necessary components to form and maintain healthy bone tissue. Further, diets sufficient in a variety of plant and/or animal proteins provide the materials to build and maintain the collagenous framework to facilitate mineralization of hard tissue as well as maintain muscle mass.[67] Aside from the better known dietary recommendations to support optimal bone health, diets high in a variety of fruits and vegetables are shown to be protective against osteoporosis.[68,69] Studies have shown a positive association of fruit and vegetable intake with BMD and certain measures of bone area (i.e., trabecular, tibial shaft) at various stages of life (i.e., adolescence, 40-75 years of age).[68,69] This effect was more

pronounced for fruit than vegetable intake in a study of Chinese men and women with low body mass index (BMI), a known risk factor for osteoporosis.[69]

A high intake of fruits and vegetables ensures a rich supply of dietary vitamin C and vitamin K among other osteoprotective compounds. Vitamin C participates in several processes involved in bone metabolism, both directly and indirectly. It is an important cofactor for the rate limiting step in collagen synthesis, type I collagen being the major extracellular matrix protein in bone. It is also an antioxidant, scavenging free radicals which can influence bone resorption. Vitamin K is a cofactor to enzymes responsible for carboxylating osteocalcin (OCN), an extracellular bone matrix protein, thereby changing its affinity for calcium and facilitating mineralization. Calcium is the primary component of hydroxyapatite, the crystalline component of mineralized bone matrix, followed by other minerals such as phosphorus and magnesium. The NOF and the Institute of Medicine (IOM) recommend 1000 mg of calcium per day for men 50-70 years of age and 1200 mg per day for women over the age of 50 years and men over the age of 70 years.[26,70]

Important for regulating calcium homeostasis, vitamin D increases calcium absorption in the intestines and reabsorption in the kidneys when serum calcium is low. Food is not typically a major source of vitamin D and the ability of the body to synthesize it from sun exposure decreases with age. Therefore, the NOF recommends adults 50 years and older supplement 800-1000 IU of vitamin D2 (ergocalciferol) or vitamin D3 (cholecalciferol) per day.[26] Alternatively, the IOM recommends 600 IU of vitamin D2 or D3 until 70 years of age then 800 IU thereafter.[70] Inadequate intake or disruption in the balance of these important nutrients for bone health can compound existing risk for bone loss with aging.

Other compounds found in fruits and vegetables, such as certain non-digestible carbohydrates and polyphenolic compounds, have no established guidelines for daily intake, but

have been identified as playing a role in slowing or even preventing the deterioration of bone with aging.[34,71,72] Intake of non-digestible carbohydrates found in fruits and vegetables are shown to suppress bone resorption attributed to increased intestinal absorption of minerals important for bone mineralization such as calcium and magnesium.[73,74] Additionally, non-digestible carbohydrates may help modulate inflammation within the gastrointestinal tract. The ability of whole foods and food extracts high in polyphenols shown to prevent decreases in bone mineral density may be related to the capacity of polyphenols to reduce inflammation and oxidative stress; two major components of age-related bone loss known to influence the process of resorbing and replacing bone throughout the lifespan, or bone remodeling.

Cellular processes involved in bone remodeling

After peak bone density is achieved during skeletal maturation, a process dominated by bone modeling involving appendicular and axial bone growth, the adult skeleton undergoes ongoing cycles of bone remodeling. Remodeling is a normal process that facilitates the repair and replacement of old or damaged bone and maintenance of mineral homeostasis (i.e., calcium, phosphate). Bone remodeling has been described as occurring in five distinct cyclic phases: activation, resorption, formation, mineralization, and resting.[75,76] Bone remodeling occurs within temporary anatomical structures known as bone multicellular units (BMUs) by the coupled activity of osteoclasts and osteoblasts, a process coordinated by osteocytes. The balance of the osteoclasts' and osteoblasts' activity, as well as the rate at which remodeling takes place (i.e., bone turnover), determines whether the net result is loss or gain of bone.

Osteoclasts

Osteoclasts are myeloid lineage cells that are responsible for the catabolic phase of bone remodeling, or bone resorption. They are distinguishable, histologically, by their giant size, multiple nuclei, and tartrate-resistant acid phosphatase (TRAcP) staining.[77] In the resorption

phase of bone remodeling, pre-osteoclasts are recruited to the resorption site by osteocyte signaling (e.g., increased high mobility group box protein 1 [HMGB1] and decreased osteoprotegerin [OPG]) or endocrine factors (e.g., PTH binding to its receptor on osteoblasts). This initiates the production of the chemoattractant monocyte chemoattractant protein-1 (MCP-1), attracting monocytes to the bone for differentiation to osteoclasts.[4] The cytokine macrophage colony stimulating factor (M-CSF), produced by various cells including osteoblasts and activated T cells, binding its receptor, colony-stimulating factor-1 receptor (c-fms), is integral for the commitment of hematopoietic stem cells (HSCs) to the myeloid lineage.[78] M-CSF is also important for the proliferation and survival of osteoclasts.[79] One pathway M-CSF induces to support osteoclastogenesis is expression of receptor activator of nuclear factor-κB (RANK) on mononuclear osteoclast precursors.[79] The binding of RANK ligand (RANKL), a member of the TNF superfamily, to RANK is essential to osteoclastogenesis and initiates fusion of pre-osteoclasts derived from monocytic macrophages.[80] Activated T-cells are the primary source of RANKL within the bone marrow; however other cells, such as bone marrow stromal cells, T cells, and osteoblasts, also produce RANKL.[81] OPG, produced primarily by B cells and osteoblasts, inhibits osteoclastogenesis by binding RANKL and hindering RANK-RANKL signaling.[82] Consequently, osteoclastogenesis is regulated by the relative abundance of RANKL to OPG. RANK is one of several receptors whose ligand activates a downstream signaling cascade targeting the transcription factor, nuclear factor of activated T-cells cytoplasmic-1 (NFATc1), which is critical to generate functional osteoclasts. NFATc1 regulates the expression of proteins necessary for resorption activities, including adaptor proteins, TNF receptor-associated factors (TRAF), cathepsin K, calcitonin receptor, ATPase subunit, and NF-κB, while also amplifying its own expression.

As the initiators of bone remodeling, osteoclasts produce matrix metalloproteinases (MMPs) that degrade the outer osteoid protein matrix of bone.[83,84] Adhesion sites within the

mineralized matrix are exposed. Integrin proteins within the osteoclast cell membranes are then able to recognize and bind arg-gly-asp (RGD) amino acid motifs on the extracellular matrix proteins osteopontin (OPN) and bone sialoprotein (BSP). The accumulation of actin-rich adhesive structures, or podosomes, at the binding sites closes off the bone-facing osteoclast plasma membrane facilitating formation of the "sealed zone." Subsequent microtubule and microfilament reorganization create folds in the bone-facing osteoclast cell membrane, optimizing bone resorbing surface area and forming the bone resorbing organelle known as the ruffled border.[75,83] The sealed zone allows osteoclasts to generate an acidic microenvironment by secreting hydrogen ions from the ruffled border to solubilize the hydroxyapatite mineralized matrix. Also secreted into the sealed zone are proteases (e.g., cathepsin K and TRAcP) that are optimized for low pH environments.[85] These enzymes degrade the bone protein matrix, or osteoid. As resorption takes place, osteoclasts take up and release minerals and protein fragments into the blood. Thus, certain forms of extracellular bone matrix proteins or fragments, such as undercarboxylated osteocalcin (OCN) and collagen type 1 cross-linked C-telopeptide (CTX), can be used as indicators of osteoclast activity.[86,87] As osteoclast activity declines within an active BMU due to cellular apoptosis, osteoprogenitor cells begin to accumulate, signaled by transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 released from the bone matrix during resorption, to prepare for the formation of new bone.[88-91]

Osteoblasts

Osteoblasts are bone forming cells that differentiate from pluripotent mesenchymal stem cells (MSCs) found within the bone marrow.[92] These cells lay the osteoid, facilitating mineralization by exhibiting a high affinity for hydroxyapatite and other minerals deposited in bone. Osteoid is made up predominantly of type 1 collagen. Other proteins found within the osteoid include OPN, BSP, and OCN.[75] Some of these proteins and other enzymes involved in mineralization, similar to those released during bone resorption, are released into the blood during

formation and can serve as surrogate biochemical markers of osteoblast activity such as bone-specific alkaline phosphatase (BAP), OPN, procollagen type 1 N-terminal propeptide (P1NP), and OCN. In particular, BAP is an indicator of early formation and OCN can be indicative of bone turnover or late formation (e.g., mineralization). During resorption, OCN fragments are released into the blood in an undercarboxylated form. Carboxylation of glutamic acid residues on OCN by osteoblasts (GLA-OCN) is a vitamin K dependent process that increases its affinity for calcium and facilitates mineralization. As the pH decreases within the sealed zone of osteoclasts, there is less carboyxlated OCN (GLU-OCN), which is released into the bloodstream as proteases degrade the bone. Thus, the ratio of GLA-OCN to GLU-OCN can be indicative of bone mineralization or bone turnover.[87] As mineralization continues with the deposition of hydroxyapatite and other minerals, osteoblasts can have several fates including apoptosis, becoming a bone lining cell, or entombment within the mineralized matrix and terminal differentiation into osteocytes.[4]

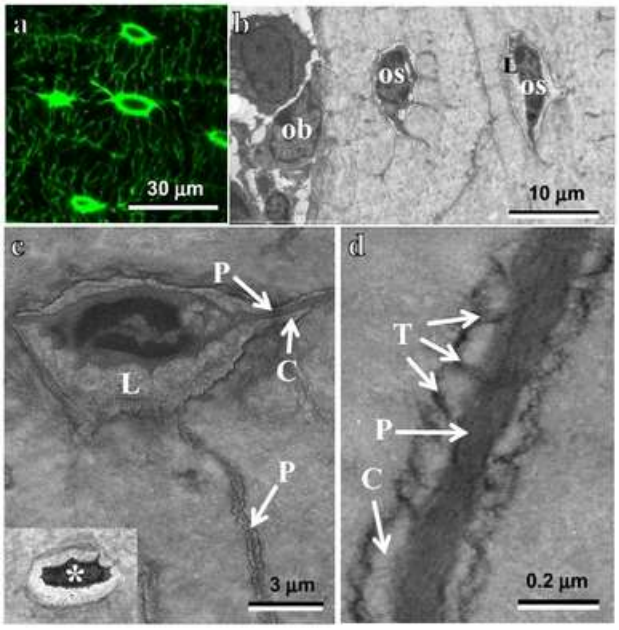

Figure 2. Osteocytes **a)** Confocal image showing an extensive network of osteocytes. **b**–**d** Transmission electron micrographs showing osteocyte lacunae (*L*), osteocyte processes (*P*) and canaliculi (*C*). Osteocyte (*os*) connects to other osteocyte and osteoblast (*ob*) at bone surface. Tethering elements (*T*) bridges osteocyte process to the canalicular wall. *Asterisk* indicate apoptotic osteocyte[4]

Osteocytes

Osteocytes reside within fluid-filled cavities, known as lacunae, in the mineralized bone matrix. Their dendritic projections form a network through canals, or canaliculi, allowing communication with other osteocytes as well as cells on the bone surface (**Figure 2**).[93] At rest, osteocytes produce

sclerostin, a cytokine that binds low density lipoprotein receptor-related protein receptors-5 and 6 (LRP5/6) on osteoblasts, effectively blocking the binding of Wnt ligands to the LRP5/6 associated frizzled receptors (Fzd). The downstream targets of Wnt signaling cascades include osteoblast genes that induce bone formation such as BAP.[94,95] Osteocytes are also a significant source of TGF-β which both impedes osteoclastogenesis and attracts pre-osteoblasts to sites of resorption to initiate formation.[90,96] Just as osteocytes can inhibit bone formation, they can also inhibit resorption with the production of TGF-β under resting conditions.[96] TGF- β production by osteocytes is shown to be upregulated by estrogen and upregulates the productions of OPG which binds RANKL, thus inhibiting osteoclastogenesis. Likewise, inflammatory chemoattractant molecules released by osteocytes and surrounding these cells during apoptosis (i.e., HMGB1 and RANKL) can stimulate osteoclast activity and resorption.[97] Osteocyte apoptosis is induced as a result of changes in skeletal loading, inflammation, and endocrine factors (e.g., estrogen deficiency).[98,99] Although osteocytes are major regulators of bone remodeling, interactions directly with osteoclasts and osteoblasts by increased inflammatory molecules, free radicals, and reactive oxygen species (ROS) plays a major role in changes of bone turnover seen with aging.

Cellular mechanisms in age-related bone loss

A number of factors associated with aging, including increased inflammation and oxidative stress, can alter the bone remodeling process.[100] Aside from the direct interaction of gonadotropic hormones such as estrogen that decrease with aging with bone cells, inflammation and oxidative stress are identified as the main drivers of disruption to normal bone metabolism that occur with age-related bone loss.[101] Inflammatory cytokines and ROS directly modulate osteoblasts and osteoclasts, disrupting signaling cascades that drive activation of transcription factors integral to these bone cells' differentiation, proliferation, survival, and activity.[102] Specific cytokines identified in this process include IL-6, IL1-β, and TNF-α.[103-105] Further, CRP, an acute phase protein and clinical biomarker of inflammation, may directly impact cellular bone

metabolism by suppressing differentiation of both osteoblasts and osteoclasts effectively reducing bone turnover.[106] Both the bone forming osteoblasts and bone resorbing osteoclasts are susceptible to inflammatory mediators.

Osteoclasts and inflammation

Age-related increase in pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 can indirectly or directly promote the differentiation of myeloid lineage cells into osteoclasts, increase their activity, and inhibit osteoclast apoptosis.[101,107] The indirect effects are mediated primarily by these pro-inflammatory cytokines activating T cells and B cells, which increases their expression of RANKL, thereby stimulation osteoclastogenesis and inhibiting apoptosis.[77,108,109] Additionally, IL-6 can bind to its receptor (IL-6R) on osteoblasts to activate the JAK-STAT pathway that upregulates RANKL and sclerostin. Sclerostin inhibits bone formation by inhibiting the stimulation of osteoblastogenic proteins, thereby uncoupling the activity of osteoclasts with osteoblasts.[110] Likewise, IL-1 binding to its receptor (IL-1R) on osteoblasts induces expression of RANKL.[111,112] Taken together, indirect effects of inflammation on osteoclasts impact multiple pathways.

Cytokines also directly interact with their specific receptors on pre-osteoclasts and osteoclasts. For example, TNF-α binds the TNF receptor (TNFR), also known as p55 receptor, on the surface of osteoclasts activating the nuclear factor-κB (NF-κB) pathway; a downstream target of RANK.[113-115] Activation of this pathway modulates translocation of the transcription factor NFATc1 to the nucleus, controlling transcription of osteoclastogenic genes that initiate fusion of osteoclast precursors to pre-osteoclasts.[116] RANK and TNF-α can also activate the mitogen activated protein kinase (MAPK) pathway and its downstream target the transcription factor protein complex activator protein-1 (AP-1).[117] IL-6 also activates AP-1 by binding the IL-6R, through the JAK-STAT pathway.[118,119] The JAK-STAT pathway is known to activate NF-κB

signaling, essential for production of osteoclastogenic genes that promote differentiation, proliferation, and survival.

The pathways discussed above may provide targets for pharmacological and/or dietary interventions. For example, drugs such as TNF-α antagonists (e.g., infliximab, adalimumab, and etanercept) decrease the differentiation and proliferation of osteoclasts from peripheral blood mononuclear cells in the presence of RANKL and M-CSF.[120] Further, in an hTNF-transgenic mouse model where TNF-α is constitutively expressed, treatment with TNF-α antagonist reduced the number of osteoclast precursor cells (OCPs) expressing c-Fms, the receptor for M-CSF, in the bone marrow.[121] Anti-IL-6R antibodies (e.g., Tocilizumab) are another anti-inflammatory pharmacological agent shown to reduce the number of OCPs in bone marrow of DBA/1J mice exposed to IL-6, significantly reducing bone loss.[122] Due to the effective dosages required and non-specificity of antagonists, use of these medications for the treatment of osteoporosis is not recommended given the effectiveness of current medications. The potent anti-inflammatory effects of polyphenolic compounds in food are less specific than pharmacological treatments; however, they have been shown to have broad effects on aspects of cytokine-mediated regulation of osteoclasts.[123-126] Research of specific polyphenolic compounds with anti-osteoclastogenic effects include, but are not limited to, resveratrol, rosmarinic acid, punicalagin, ellagic acid, hydroxycinnamic acids, and anthocyanins.[123-126] Select polyphenolic fractions from dried plum were shown to decrease osteoclast differentiation by modulating expression of *Nfatc1* through MAPK signaling in primary bone marrow cells.[127] Bu and colleagues[128] showed that an extract of dried plum polyphenols inhibited osteoclast differentiation and activity *in vitro* under normal and inflammatory conditions.[128] Expression of NFATc1, a downstream target of RANK, under normal conditions was suppressed with plum polyphenol treatments even in the presence of RANKL. Taken together, dried plum polyphenols have been shown to reduce inflammation,

inhibiting key signaling pathways that induce osteoclast differentiation and proliferation under normal conditions and under stress.

Osteoblasts and inflammation

In addition to affecting osteoclasts, increases in pro-inflammatory cytokines such as those occurring with cystic fibrosis, celiac disease, and rheumatoid arthritis, can also have negative consequences on osteoblast formation and function.[26] Activation of the JAK/STAT pathway via IL-6 binding to its receptor on the surface of osteoblasts inhibits extracellular signal-regulated kinase (ERK), which in turn suppresses the transcription factor, Runx2 that is essential for osteoblastogenesis.[129] Similarly, TNF-α binding the TNFR on osteoblasts inhibits osteoblastogenic genes (e.g., Runx2, OCN, PTHR, and osterix) by activating Smad ubiquitinoylation regulatory factors (SMURFs) that target SMADs for degradation.[130] TNF-α also activates MAPKs which increase production of sclerostin thereby inhibiting Wnt signaling.[131] Further, TNF-α, IL-6 and IL-1 can inhibit osteoblast activity by upregulating expression of Dickkopf Wnt signaling pathway inhibitor 1 (DKK1), and *Sost*, the gene responsible for encoding the protein sclerostin, in osteocytes.[132,133] Both of these molecules block Wnt signaling and consequently, its downstream transcriptional targets including Runx2 and osterix, as well as structural osteoid proteins.[134]

As with osteoclasts, the effects of inflammatory cytokine antagonist medications on osteoblasts has been considered. Given the regulatory role osteoblasts play in osteoclastogenesis, some of the effects on osteoblasts ultimately regulate osteoclasts as with the reduced production of inflammatory cytokines IL-1β and IL-6 with TNF-α blocking agent, infliximab. Similar to osteoclasts, certain foods and their bioactive components have been studied for their effects on osteoblasts and how those changes might be mediated by reductions in pro-inflammatory cytokines. Tea leaf extracts (e.g., green tea, black tea, and noni) were shown to increase BMD in measures of the tibia and femur accompanied by reduced TNF-α, IL-6, and RANKL and

increased OCN.[135] Further, significant increases in mRNA coding for osteoblast functional and differentiation proteins and decreases in the relative expression of anti-osteoblastogenic genes indicate mineralization may be upregulated. Other studies on the effects of polyphenolic compounds on osteoblasts include hydroxytyrosol extracted from olive, the commercially available product Greens+™, and dried plum polyphenol extracts.[136,137] Shen and colleagues[138] demonstrated the ability of green tea polyphenols to suppress expression of TNF-α and improve bone microarchitecture in female rats under simulated inflammatory conditions (i.e., LPS treatment). Bu and colleagues[139] showed that a dried plum polyphenol extract increased osteoblast activity, function, and differentiation using murine MC3T3-E1 cells, a pre-osteoblastic cell line. These increases in osteoblast function (i.e., calcified nodule formation) were observed under normal and inflammatory (i.e., treatment with TNF-α) conditions. Further, dried plum polyphenols significantly increased gene expression of Runx2, IGF-1, and lysyl oxidase (i.e., enzyme involved in collagen synthesis) under inflammatory conditions. More recently, Graef and colleagues[140] reported fractions of a dried plum polyphenol extract promote bone formation via bone morphogenic protein (BMP) signaling. These results indicate that polyphenols may be able to protect against the negative effects of pro-inflammatory cytokines, such as TNF-α, on bone metabolism.

Osteoclasts, osteoblasts and oxidative stress

Reactive oxygen species (ROS) are a normal byproduct of cellular energy metabolism however production can be perpetuated by pro-inflammatory cytokines activating immune cells that, in turn, further produce ROS. Specifically, cytokines such as TNF-α and IL-6 can induce production of nitric oxide which is shown to promote osteoclast differentiation and function at low levels.[141] The exact mechanisms for this effect remain unclear however, one pathway through which ROS affects osteoclasts and osteoblasts is through direct activation of the NF-κB. In pre- and mature osteoclasts, downstream targets of NF-κB induce osteoclast differentiation,

proliferation, and survival. In the osteoblast, NF-κB pathway upregulates expression of RANKL and stimulates apoptosis of osteoblasts and osteocytes.[19] Moreover, ROS can interfere with Wnt signaling in osteoblast progenitors cells by diverting transcription factors such as forkhead box subgroup O (FoxO) from Runx2 and osterix; genes that regulate osteoblast differentaiton.[134,141]

It is widely accepted that oxidative stress can be mitigated through dietary interventions including fruits and vegetables high in polyphenolic compounds and antioxidants such as beta carotene and vitamin C. Some specific examples of research demonstrating that polyphenols favorably affect bone cells due to their ability to reduce oxidative stress include compounds such as flavonones, anthocyanins, and dried plum polyphenolic extract.[32] A previously mentioned study by Bu and colleagues[128] showed that dried plum polyphenols reduced nitric oxide and iNOS expression while suppressing osteoclastogenesis. Naringin, a flavanone found in citrus fruits like grapefruit, was shown to rescue human adipose-derived mesenchymal stem cells (hADMSCs) from H_2O_2-inhibited osteoblast differentiation.[142] This effect was associated with significant recovery of superoxide dismutase (SOD) and glutathione (GSH) activity and malondialdehyde (MDA) levels in naringin-H_2O_2 co-treated hADMSCs compared to H_2O_2 alone. Investigating the expression of osteoblastogenic genes revealed that naringin-H_2O_2 co-treatment of hADMSCs recovered expression of Runx2 and osterix to normal levels opposed to the decrease seen in H_2O_2-exposure alone; however, naringin alone only slightly increased expression of these genes. Taken together, these findings suggest that products which mitigate oxidative stress in osteoblasts protect against differentiation inhibition by ROS. Furthermore, studies by Bu et al.[139] and Graef et al.[140] showing increased calcified nodule formation suggest that these phytochemicals may increase osteoblastogenesis on their own.

Based on this review of the scientific evidence, it is apparent that increases in oxidative stress and inflammatory cytokines can drive age-related bone loss. Dietary compounds found predominantly in fruits may play an important role in countering the negative effects of

inflammation and oxidative stress on bone cells. Research into these functional foods and their mechanisms is important and ongoing; however, several pharmacological interventions have been produced and continue to undergo development and approval for the treatment of osteoporosis.

Current pharmacological treatments

Current FDA-approved pharmacological treatments are categorized as either anti-resorptive or anabolic based on their mechanisms of action. They act by inhibiting resorption activity of the osteoclasts to prevent further bone loss or stimulating osteoblast activity, which results in the formation of new bone. Presently in the U.S., the approved agents include bisphosphonates, denosumab, hormone replacement therapy, selective estrogen receptor modulators (SERMs), and teriparatide.

Anti-resorptive therapies

Bisphosphonates target osteoclast lifespan and induce osteoclast apoptosis.[143] These inorganic pyrophosphate analogues have a high affinity for hydroxyapatite.[144] Bisphosphonates are endocytosed at sites of resorption by osteoclasts.[145] First generation, non-nitrogen containing bisphosphonates (e.g., clodronate, etidronate, and tiludronate) bind non-hydrolyzed ATP analogues interrupting ATP synthesis and leading to cell death.[146] Second and third generation bisphosphonates (e.g., alendronate, risedronate, ibandronate, and zolendronate) have nitrogen side-chains, which allow tighter binding to hydroxyapatite. These bisphosphonates inhibit farnesyl pyrophosphate synthase (FPPS), required for post-translational lipid modification of guanosine triphosphatase (GTPase) proteins (e.g., Ras, Rab, Rho, and Rac).[147] GTPases modulate osteoclast cellular processes related to cytoskeletal arrangement, membrane ruffling, trafficking of vesicles, and apoptosis. Thus, inhibition of FPPS leads to osteoclast apoptosis, or cell death.[148] Decreased activity and survival of osteoclasts allows osteoblasts to temporarily dominate the remodeling cycle and temporarily increase BMD. Bisphosphonates (e.g., alendronate, risedronate,

ibandronate, and zolendronate) are effective in reducing the incidence of vertebral fracture in postmenopausal women by 41-70% over three years.[26,144] Incidence of non-vertebral fractures in post-menopausal women are reduced by 25-50% and hip fractures specifically are reduced by 41-50%. Bisphosphonates, however, are not appropriate for all patients and compliance can wane due to undesirable side-effects.[143] Side-effects with short-term use include upper gastrointestinal irritation, nausea, and abdominal pain with oral bisphosphonates.[146] In addition, some patients experience severe chronic musculoskeletal pain, hypocalcemia, and ocular inflammation. Long-term administration of bisphosphonates (i.e., >5yrs) is associated with a slightly increased risk for osteonecrosis of the jaw (ONJ) and atypical femoral fractures; transverse low-trauma fractures occurring in the sub-trochanter region or femoral diaphysis.[149,150] Due to the low cost, safety, and efficacy of bisphosphonates, they are usually the first-line of therapy for the treatment of osteoporosis.

Denosumab is a human monoclonal IgG2 antibody that was FDA-approved in 2010 for the treatment of osteoporosis in postmenopausal women at high risk for fracture.[26,151] The antibody binds RANKL with high affinity and specificity, thereby inhibiting osteoclastogenesis.[152] Denosumab decreases markers of bone resorption by >80% and markers of formation by 55-75%, thereby reducing overall bone turnover.[153] Since resorption is decreased over formation, a temporary increase in BMD is observed. Along with increased BMD, denosumab is shown to decrease relative risk for new vertebral fracture by 68%, hip fracture by 40%, and nonvertebral fracture by 20% after three years of administration.[154] The drug is administered as a single subcutaneous injection once every six months.[154] Side-effects include infections, eczema, hypocalcemia, impaired fracture healing, and increased risk for atypical femoral fracture and ONJ.[152] Denosumab is also contraindicated for patients with renal issues.[155] Due to the lack of evidence related to long-term efficacy, safety concerns, and cost-effectiveness, currently denosumab is not used as a first-line therapy.

Hormone replacement therapy (HRT) and selective estrogen receptor modulators (SERMs) are pharmaceutical options for postmenopausal women to treat symptoms of early menopause and may slow bone loss associated with estrogen deficiency. Estrogen plays a role in the regulation of osteoclasts by suppressing osteoclastogenic cytokines, and of osteoblasts by prolonging their survival.[156,157] Hormone replacement therapy is available as a combination of estrogen and progesterone. It is more commonly used to treat and/or prevent symptoms of early menopause than preventing bone loss.[28] NOF guidelines indicate HRT should be used for the shortest duration at the lowest dose due to the increased risk for invasive breast cancer, cardiovascular events, and dementia in older women.[26]

An alternative to counter the safety concerns with HRT, SERMs (e.g., estrogen receptor agonists/antagonist) are designed to target specific tissues to reduce the risk for endometrial hyperplasia and invasive breast cancer but are not shown to reduce the risk for cardiovascular events. Available in both oral and transdermal forms, these medications can be taken without regard to meals, but undesirable side-effects (e.g., muscle spasm, nausea, diarrhea, dyspepsia, upper abdominal pain, dizziness, and neck pain) can negatively impact compliance. Compared to other treatments, reduction of bone loss from decreased turnover with HRT and SERMs treatments is minimal.[158] The mechanism of action of these treatments may be useful for reducing risk of developing osteoporosis, but they are not generally used for treatment as they are not shown to increase BMD.[159] Further, concerns over the risks as well as suitability for the patient contributes to infrequent use for the prevention and/or treatment of osteoporosis.[160]

Anabolic therapies

Teriparatide or recombinant human parathyroid hormone (PTH), is currently the only FDA-approved anabolic agent for the treatment of osteoporosis. Although administration of PTH initially increases the activity of osteoclasts, activity of osteoblasts is sustained over osteoclasts

and therefore, bone formation exceeds resorption; especially in the initial six to 12 months of treatment.[27] Teriparatide is administered as daily subcutaneous injections and, due to the lack of long-term efficacy and safety data, for a lifetime maximum of 24 months.[25] Treatment with Teriparatide may be most effective when combined with anti-resorptive therapies denosumab or the bisphosphonate zolendronate.[161,162] Although side-effects of this medication are less severe than other options (e.g., leg cramps, nausea, dizziness), it can be cost-prohibitive and evidence of benefit over cancer risk is inconclusive.[26]

Alternative treatments

Clinical trials for alternative pharmacological targets to prevent and treat osteoporosis are currently underway for FDA approval. Some pharmacological targets currently in phase II and III clinical trials include cathepsin K, sclerostin, and PTH-related peptide analogs.[163] Although current FDA-approved pharmacological treatments are effective for increasing BMD and decreasing fracture risk, they can only be effective if patients adhere to treatment. Patient compliance is generally low for most of these medications whether it be related to cost, side-effects, or convenience of administration.[164,165] There is a need for alternative therapies. Treatments with a mechanism of action targeting the etiologies of age-related bone loss, such as inflammation and oxidative stress, may be sage candidates for intervention. Studies examining the capacity of certain foods known to decrease inflammation and oxidative stress have shown promising bone sparing properties.

Functional foods for the prevention of bone loss

Functional foods, also known as nutraceuticals, are foods that provide health benefits beyond basic nutrition. These foods are often plant-based products that contain biologically active compounds, or phytochemicals, which exert a protective effect against diseases and illness. Many functional foods are shown to play a role in bone health by mediating bone metabolism. For

example, soy, which were the first to be studied in conjunction with bone health, contains isoflavones that have a molecular structure similar to estrogens, and exert effects comparable to HRT and SERMs in preventing inflammation-induced bone loss.[29] Olive oil is rich in phenolic compounds (e.g., tyrosol, hydroxytyrosol, oleuropeins, and flavonoids) shown to diminish inflammation and oxidative stress.[166] In animal models of postmenopausal and age-related bone loss, olive oil protects against low BMD and prevents bone loss by modulating osteoclast proliferation and supporting mineralization.[167] Epidemiological studies examining olive oil intake and bone health show positive correlations between olive oil intake and BMD.[167] A limitation of these studies however relates to the high intake of other foods high in polyphenols associated with a high intake of olive oil. For example, certain phenolic compounds responsible for the deep red-purple color pigments in fruits and vegetables such as blueberries, blackberries, dried plums, and tart cherries are known to have potent antioxidant and anti-inflammatory properties.

In particular, our lab and others have shown that dietary supplementation with dried plum has the potential to both prevent and reverse bone loss in pre-clinical and clinical trials.[37,39,168-170] In osteopenic ovariectomized animals, dietary supplementation with dried plum restored whole body and femoral BMD as well as trabecular microarchitectural parameters within the vertebrae to that of controls.[37,170] These findings have translated to clinical trials as well. Hooshmand and colleagues[168] showed that one year of daily dried plum supplementation (100 g) in early postmenopausal osteopenic women increased BMD at the ulna and spine. In a subsequent study, this same group of investigators reported that osteopenic, postmenopausal women (65-79 years of age) who consumed 50 or 100 g of dried plum per day for six months were protected from the age-related decrease in BMD.[39] These findings were associated with a reduction in serum TRAP-5b, a marker of bone resorption.[39] In these clinical trials, the proposed mechanism of action of dried plum and its bioactive components was attributed to the reduction of RANKL production, induction of OPG, and inhibition of sclerostin.[127,168] This is supported by animal studies showing

decreases in mRNA coding proteins for the osteoclastogenic gene *Nfatc1* as well as attenuation of changes in MAPK phosphorylation under inflammatory conditions.[37,170] Osteoblast biomarkers and local regulators of osteogenesis were unchanged in models utilizing estrogen deficient animals. Taken together with the modest increase in endocortical mineralization and decrease in regulators of osteoclasts suggests dried plum exerts its effects on bone by decreasing turnover.[170] Recent *in vitro* work in our lab utilizing polyphenolic fractions rich in neochlorogenic acid and cryptochlorogenic acid suggest increased osteoblast activity through bone morphogenic protein (BMP)-2, a member of the TGF-β superfamily, signaling under normal conditions.[140] The increased antioxidant activity of glutathione peroxidase (GPx) and capacity for dried plum to decrease markers of inflammation (e.g., IL-1b, TNF-α, MCP-1) may explain, in part, the mechanism through which these changes are mediated.[37,38,169] The specific bioactive compounds which exert these effects are yet to be determined.

Dried plum is rich in polyphenolic and other compounds shown to decrease inflammation and oxidative stress. Additionally, dried plum is a good source of non-digestible carbohydrates, such as fructooligosaccharides, whose byproducts of digestion by gut bacteria include the anti-inflammatory short-chain fatty acid butyric acid.[171] Dried plums are a particularly rich source of potassium and vitamin K that can interfere with bone resorption and enhance bone mineralization.[171] The dried plum polyphenolic profile includes known anti-inflammatory compounds such as hydroxycinnamic acids (chlorogenic acid and isomers), flavan-3-ols (gallic acid and protanthocyanidins), and anthocyanins.[171] *In vitro* studies have shown that dried plum's polyphenols inhibit osteoclastogenesis by down-regulating *Nfatc1* and enhance bone formation and mineralization by up-regulating Runx2 under inflammatory conditions.[128,139] In an aged, osteopenic animal model of estrogen deficiency, dried plum's polyphenols reversed bone loss by suppressing bone resorption and up-regulating formation via bone morphogenetic proteins.[172] However, recent preliminary evidence from our lab has shown that the carbohydrate component

of dried plum may also play a role (*unpublished data*). Due to the unique effects of dried plum, it is possible that other fruits with a similar polyphenolic profile and oligosaccharide content, as with the tart cherry (*Prunus cerasus L.*), may provide some of the same benefits.[173]

Tart cherry

Tart cherries are of the *Prunus* genus and member of the *Rosaceae* family.[174] They are native to Canada and the U.S. More than 94% of tart cherries consumed in the U.S. are also grown here with about 75% from Michigan alone.[175] Other states producing tart cherry include Utah, Washington, New York, Wisconsin, and Pennsylvania. Tart cherries grow on trees and are harvested in July. Different preparations such as dried, juice, lyophilized powder, and canned, allow tart cherries to be available year-round.[175] Processing tart cherries into a concentrated juice provides a portable and practical approach for daily consumption. Further, tart cherry juice concentrate preserves more total phenolics and total proanthocyanidins than dried, frozen, or canned cherries per gram (**Table 1**).[176] The juice concentrate is also shown to preserve total anthocyanins more than canning or drying. Tart cherries are a novel functional food studied for a number of health benefits.[177] They are believed to exert most of their benefit by their capacity to reduce markers of inflammation and oxidative stress demonstrated in cellular and animal models as well as clinical trials.[178]

Table 1. Proanthocyanidins and total phenolics in processed tart cherry products[176]

	Cherry juice concentrate	Dried cherries	Frozen cherries	Canned cherries
Monomers (μg/g)	137.3 ± 1.1	13.7 ± 2.4	15.3 ± 1.1	20.0 ± 0.0
Dimers (μg/g)	218.3 ± 4.4	11.7 ± 2.4	26.3 ± 1.8	41.3 ± 1.1
Trimers (μg/g)	138.3 ± 4.2	10.0 ± 1.3	32.7 ± 1.6	22.7 ± 0.9
4–6 mers (μg/g)	133.7 ± 6.2	15.7 ± 3.1	39.3 ± 4.0	18.7 ± 5.0
7–10 mers (μg/g)	309.7 ± 57.1	37.7 ± 9.0	30.7 ± 11.6	134.7 ± 12.0
>10 mers (μg/g)	1078.0 ± 66.7	428.7 ± 62.4	253.7 ± 26.4	133.3 ± 58.4
Total in ug/g	2015.3 ± 34.4	518.0 ± 78.7	398.0 ± 42.7	370.7 ± 52.9
Total in mg/serving	57.0 ± 3.4	23.3 ± 1.8	26.7 ± 2.1	44.9 ± 2.7
Total Phenolics (GAE/g)	9.36 ± 0.7	7.45 ± 0.5	4.18 ± 0.6	3.57 ± 0.2
Total phenolics/ (GAE/serving)	264.9 ± 16.7	335.3 ± 21.2	280.1 ± 17.4	431.9 ± 27.5

Mean value per gram of fresh weight ± SEM, $n = 3$ per analysis. GAE = gallic acid equivalents.

Health benefits of tart cherry in clinical trials

Perhaps one of the most studied health benefits of tart cherry in clinical trials is its ability to improve muscle recovery and how this effect coincided with decreases in markers of inflammation and oxidative stress. In trials including strenuous prolonged physical activity (e.g., full marathons and simulated 3-day road race cycling) by trained athletes, daily supplementation with tart cherry juice in the days leading up to and days following the activity significantly reduced elevations in serum markers of inflammation immediately post-race (e.g., IL-6 and uric acid) as well as 24 h (e.g., uric acid and CRP), and 48 h (e.g., CRP, lipid hydroperoxide (LOOH), and TBARS) post-activity compared to placebo.[40,53,54] Further, these studies showed increases in total anti-oxidant status (TAS). Similar results are seen with acute endurance activities (e.g., half marathon and adaptation of Loughborough Intermittent Shuttle Test [LIST]) in trained athletes as demonstrated by decreased IL-6 and increased TAS.[48,179] These findings were accompanied by improved performance, muscle recovery, and upper respiratory symptoms, suggesting that the decrease in inflammation and oxidative stress may be the mechanism by which tart cherry produces some of these benefits. Given its ability to also reduce self-reported muscle soreness, it stands to reason that it may also reduce pain resulting from other inflammatory pathogenesis as with osteoarthritis.

The effectiveness of tart cherry for treating symptoms of osteoarthritis was studied in non-diabetic patients.[57] A randomized double-blind crossover trial supplemented participants suffering from osteoarthritis grade 2-3 on the Kellgren scale, with 8 fl. oz. of tart cherry juice or placebo twice per day for six weeks. Western Ontario McMaster Osteoarthritis Index (WOMAC) scores, measuring pain stiffness, and function, were significantly lower than baseline values after tart cherry juice supplementation, but the change was not significant between groups. The decline in WOMAC scores was associated with the significant decrease in hsCRP. Although further

studies are warranted, these findings suggest that tart cherry juice improves symptoms of osteoarthritis by reducing inflammation.

In addition to studies examining the effects of tart cherry juice on inflammatory mediators, the antioxidant effects have been evaluated in ischemia-reperfusion studies of localized oxidative stress. In a double-blind, placebo-controlled, crossover trial, 12 men and women (61-75 yrs) consumed 8 fl. oz. of tart cherry juice twice per day for 14 days with a 4-week washout period.[180] Response of F_2-isoprostane, an indicator of oxidative damage, to forearm ischemia-reperfusion was significantly reduced with tart cherry juice supplementation compared to placebo. Basal urinary excretion of oxidized nucleic acids, another indicator of oxidative stress, was also reduced. These results indicate that tart cherry supplementation in older adults reduces oxidative stress.

Health benefits of tart cherry in pre-clinical trials

In animal models of genetic and diet-induced obesity, a particularly pro-inflammatory condition, short- and long-term (i.e., 90-days and 8-weeks) tart cherry supplementation was shown to have potent anti-inflammatory and antioxidant effects.[44,181] Specifically, in rats genetically prone to obesity, the abundance of TNF-α and IL-6 mRNA in retroperitoneal fat were significantly reduced, 40% and 44% respectively, compared to controls. Expression of inflammatory mediators were decreased as indicated by reduction in expression of NF-κB, IKBα, IL-6, and TNF-α. These responses were associated with decreased nuclear NF-κB activity.[44] In diet-induced obesity models, activity of the antioxidants SOD and GPx in the liver were significantly increased by tart cherry treatment compared to high-fat diet controls. Furthermore, expression of inflammatory markers IL-6, TNF-α, NF-κB, inducible nitric oxide synthase (iNOS), and interferon gamma (IFN-γ) were significantly reduced, also in the liver. Taken with the lower

body weight of tart cherry treated animals, tart cherry may hinder genetic and diet-induced obesity, which coincided with reductions in systemic inflammation and oxidative stress.

In animal models of aging and chronic inflammation, supplementation with tart cherry (i.e., 14-days and seven weeks respectively), was protective against inflammation and oxidative stress.[182] To induce inflammation and oxidative stress, Saric and colleagues[182] administered incomplete Freund's adjuvant intravenously 12 days before testing. Animals treated with tart cherry juice showed a dose-dependent decrease in COX-2 activity, decreased TBARS, a dose-dependent increase of SOD in erythrocyte lysates, an increase in total liver SOD activity, and increased liver GPx activity. Similarly, in aged animals, expression of inflammatory and oxidative stress markers (NADPH oxidase-2 [NOX-2], and COX-2) were significantly reduced in the hippocampus.[47] While it is clear from these *in vivo* studies that tart cherry has potent antioxidant and anti-inflammatory capabilities, *in vitro* studies have provided insight into mechanisms.

Various cell lines have been used to demonstrate the ability of tart cherry to reduce production of compounds and expression of genes related to inflammation and oxidative stress. The inhibition of COX-1 and -2, responsible for the production of prostaglandins and important for the activation of immune cells, by tart cherry has been demonstrated in pre-clinical trials. In a study with HAPI rat microglial cells, induction of COX-2 and production of TNF-α by LPS was decreased with tart cherry powder pretreatment.[41] Additionally, free radical nitric oxide (NO) release was significantly reduced. These results were mirrored in studies demonstrating the ability of extracts from whole tart cherry to inhibit activity of COX-1 and COX-2 in models using phenol treated hemoglobin.[41,176,183] These effects may be related to the capacity for tart cherry compounds to scavenge free radicals. Tart cherry juice concentrate was shown to have radical scavenging capacity comparable to pure gallic acid, ascorbic acid, and chlorogenic acid treatments (all of which are compounds found in tart cherry) against 2,2-diphenyl-1-

picrylhydrazyl (DPPH) radicals in solution.[51] Treatment with lyophilized tart cherry juice was shown to increase survivability of brine shrimp challenged with hydrogen peroxide at 24 (90% survival) and 72 h (10-20% survival).[51] The work of Ou and colleagues[176] supported these findings by demonstrating the effective antioxidant scavenging capacity of different preparations (e.g., juice concentrate, dried, canned, and frozen) of tart cherries on peroxyl, hydroxyl and peroxynitrite radicals, and the free radical superoxide.[176] Further, this group demonstrated that the concentrate form had the greatest antioxidant capacity compared to other preparations and suggested juice preparations may increase antioxidant capacity.[176] Lending possible identification of specific bioactive components of the fruit, flavonol and anthocyanin purified polyphenolic extractions from tart cherry were also shown to exert these inhibitory effects of inflammation and oxidative stress in similar cellular models.[184,185]

Aside from polyphenolic compounds, tart cherry is a rich source of fructooligosaccharides, especially compared to many other fruits.[186] Fructooligosaccharides are shown to enhance absorption of calcium and magnesium in the colon as well as increase the bioavailability of isoflavones. This occurs with the increased solubility of these minerals due to the acidic environment created by the production of short chain fatty acids as fructooligosaccharides are fermented in the intestines.[187] Given tart cherry's particularly abundant polyphenolic profile and capacity to reduce markers of inflammation and oxidative stress known to modulate bone metabolism, it is reasonable to suggest that tart cherry may influence bone health.[188]

Tart cherry for bone health

Our lab has demonstrated the ability for tart cherry treatment to maintain bone health in mouse models of age-related bone loss.[58] In this study, whole body bone density was increased in five-month-old female C57BL/6 mice fed AIN-93M diets with tart cherry powder (five and 10%

w/w) for 90-days. These mice, predisposed to age-related trabecular bone loss, also showed improvements in femoral cortical thickness and trabecular bone volume at the distal femur metaphysis and lumbar spine compared to younger baseline and control groups thus demonstrating the ability of tart cherry to prevent age-related bone loss. Examination of gene expression in the bone revealed up-regulated bone mineralization evidenced by increased expression of phosphate regulating endopeptidase homolog, X-linked (*Phex*) and decreased peroxisome proliferation activated receptor (*Ppar*)-*y* in mice supplemented with tart cherry. Taken together, the bone protective effects of tart cherry demonstrate promising potential to provide osteoprotective effects in aging.

Bone loss with aging is a major public health issue. It is widely recognized that age-related bone loss is mediated by inflammation and oxidative stress and few approved treatments exist targeting these main factors. Bioactive food components are shown to decrease inflammation and oxidative stress in varying capacities with some showing positive impacts on bone health. Tart cherry has demonstrated potent antioxidant and anti-inflammatory properties in clinical and pre-clinical trials. Further, the health effects of the prebiotic compounds, or non-digestible carbohydrates, in tart cherry specifically have yet to be explored. The health effects of tart cherry on bone have been largely uncharted; however, compelling animal data from our lab show promising results for the ability of tart cherry supplementation to diminish age-related bone loss. Based on these results, we conducted a study designed to determine the dose-dependent effects of supplementation with tart cherry juice on markers of bone formation and resorption in women aged 65-80 years. Moreover, we investigated the effects on inflammation and oxidative stress markers and explored correlations in these markers with biomarkers of bone metabolism.

CHAPTER III

METHODS

Participants

One hundred and eight women were screened, and thirty-three women aged 65-80 years qualified to participate in the study. Recruitment occurred through email flyer distribution to Oklahoma State University (OSU) campus email, an OSU Emeriti Faculty Association, the Oklahoma Home Community Education (OHCE) women's extension group, the Oklahoma Master Gardeners group, as well as posting at university and area community wellness programs, senior centers, and local clinics. Aside from recruiting women in this age group, additional inclusion criteria were the participants had to have the ability to walk without assistance and have the capacity to give informed consent. Exclusion criteria included current smokers, individuals with a body mass index (BMI) <18.5 or >40 kg/m^2 based on self-reported height and weight, and individuals with a previous diagnosis of osteoporosis or any other metabolic bone disease, renal disease, cancer, cardiovascular disease, diabetes mellitus, pulmonary disease, gastrointestinal diseases, liver disease, or other chronic conditions that could affect bone metabolism. Additionally, subjects were excluded who had taken hormone replacement therapy or other medications or supplements known to alter bone or calcium metabolism (e.g., bisphosphonates, denosumab, raloxifene, intermittent parathyroid hormone, growth hormone, steroids, natural estrogens) within three months of starting treatment. All procedures performed were approved by

the Oklahoma State University Institutional Review Board in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards (**APPENDIX A**).

Study design

Participants who qualified for the study visited the Nutritional Sciences Clinical Research Laboratory three times (i.e., baseline, 45-days, and 90-days). At the baseline visit, participants reviewed and signed the informed consent with a member of the research team, completed a fasted blood draw, underwent anthropometric measures and a dual x-ray absorptiometry (DXA) scan, took hand grip strength measurements, and completed a series of questionnaires collecting data about her medical history, physical activity, sun exposure, and calcium intake. Participants were randomized to one of two treatment arms (TC1X=8 fl. oz. tart cherry juice once per day; TC2X=8 fl. oz. tart cherry juice twice per day) at their initial baseline visit. These doses of tart cherry juice have been shown to decrease markers of inflammation (e.g., CRP and interlukin-6) and increase antioxidant capacity in previous clinical trials.[54,57] Participants sampled Montmorency tart cherry juice concentrate (King Orchard's, Central Lake, MI) at the baseline visit to confirm no adverse allergic reactions and were given a minimum 45-day supply of the study product. Instructions were given on how to reconstitute the drink (i.e., 1 fl. oz. concentrate to 7 fl. oz. water) with disposable measuring cups provide. Participants were also informed that the concentrate could be consumed as is (i.e., a syrup). Participants were instructed to consume the tart cherry juice at the same time every day with approximately eight hours between doses for the TC2X group (i.e., morning and evening at least three hours before sleep to prevent reflux). Participants were also instructed to record the day and time they consumed the tart cherry juice on a provided calendar to assess compliance. A form to record food intake for three days was also provided to be mailed back before the participant's second visit. At the second visit, participants

reported any changes in their health status or medications, height and weight were recorded, compliance calendar returned, and the remainder of the test product was provided. The third or final visit was similar to the initial visit; measuring anthropometrics, completing questionnaires and a fasted blood draw. The DXA scan provided context as to the current bone health of participants but was not repeated at the final visit since the 90-day study duration was not expected to induce detectable changes in bone density.

Questionnaires

Health and medical history relevant to the study was collected at baseline by research personnel using a medical history questionnaire. Changes in medical status were reported at the second and final visits. Information provided included basic demographics (e.g., age, ethnicity, education), lifestyle factors (e.g., tobacco and alcohol use) as well as other current and historical information pertinent to bone health (e.g., prior use of HRT, pregnancies and breastfeeding, menopause onset, fractures, surgeries, and previous medical diagnoses). Also included in the questionnaire was a list of prescription and over-the-counter medications and supplements taken in the past three months with respective dosages.

Physical activity was assessed using the Yale Physical Activity Survey (YPAS).[189] The YPAS is specifically designed for administration to older adults and assesses recent (i.e., previous seven days) physical activity and physical activity patterns over the previous month. The survey provides estimated activity and total calorie expenditure for the previous week as well as monthly index scores for total activity of the previous month and individual categories (i.e., vigorous activity, leisurely walking, sitting, and standing). The monthly index scores are a product of weighted values of frequency and duration for given activities. Higher scores indicate more intense activities. Energy expenditures were used to categorize participant activity. Light activity was characterized as participation in daily exercise that expends ~150 kcal (e.g., walk 30 minutes at four mph) in excess of those expended during activities of daily living. Alternatively, active

individuals expend ~250 kcal (e.g., run 20 minutes at six mph) daily in excess of those expended during activities of daily living. At the baseline visit, study participants were asked to maintain their current level of physical activity over the 90-day study period.

Dietary intake of macronutrients and vitamin D was assessed using three-day food records. Usual supplemental and dietary intake of calcium was assessed with the National Institutes of Health (NIH) Short Calcium Questionnaire (version SCQ 2002).[190] Participants were instructed on how to record food intake by trained research personnel using portion size references. The food records were returned by mail within two weeks of the initial visit. A second three-day food record was mailed to participants two to three weeks before their final visits to complete and return at their final visit. Information from the three-day food records were entered into Food Processor II (ESHA Research, Salem, OR) to assess dietary intake. The NIH Short Calcium Questionnaire is a separate assessment used to approximate habitual daily calcium intake from food and supplements.

Sun exposure was determined to predict circulating vitamin D using a sun exposure questionnaire.[191] The questionnaire assigns a sun exposure score (0-56) of the previous week based on duration in the sun and amount of unprotected skin during the exposure, to include time in a tanning bed. Further, participants classified their skin type from six descriptions. The highest index score is indicative of an individual that has spent more than 30 minutes per day outside with the amount of skin exposed similar to that of wearing a swim suit. Scores and skin types correlate with vitamin D status and can be used, as with calcium, to qualify changes in bone metabolism.

Anthropometric measurements

Height, weight, and waist and hip circumference were recorded at the initial and final visits. Only height and weight were measured at the 45-day visit. Body mass index (BMI, kg/m^2) was calculated using height and weight measurements. Waist and hip circumference were measured

using the protocol outlined by the NHANES III to calculate waist-to-hip ratio.[192] Total body soft tissue analysis using a dual-energy x-ray absorptiometry (DXA) system (QDR 4500 Discovery A, Hologic, Waltham, MA) provided body composition, total fat-free mass less mineralized bone, and fat mass at baseline to support hand-grip strength and physical activity assessments as well as qualify BMD measures.

Bone density

Bone density was assessed with a DXA scan at the baseline visit by a certified bone densitometrist. Specific measurements included hip (total, neck, trochanter, and intertrochanter) and lumbar spine (L1-L4) bone mineral density (BMD), bone mineral content (BMC), and bone mineral area (BMA). Hologic software analysis calculated T- and Z-scores. Although the 90-day intervention is not adequate to establish tangible changes in BMD, assessment of bone density provided insight into the participants' bone health at baseline.

Assessment of muscle strength

Muscle strength was assessed at the baseline and final visits with a handheld hand grip dynamometer (Jamar, Lafayette, IN) using a previously published protocol.[193] Briefly, the participant was seated with their feet flat on the floor and arms on the arm-rests of a chair, and the weight of the dynamometer supported by a member of the research staff. Participants performed the hand grip test three times with both their dominant and non-dominant hand, exerting maximal effort each time. The best of three hand grip strength tests for both dominant and non-dominant hand was used as the indicator for muscle strength and function.

Serum biomarkers

Fasting blood samples were collected by a licensed phlebotomist or nurse at the OSU University Health Services Clinic during baseline and final visits. Blood samples were collected in 8.5 mL vacutainer tubes (Serum Separator Tube), allowed to coagulate at room temperature

(10-30 min.) and kept on ice until processing. Samples were centrifuged at 4,000 x g for 20 minutes and serum was separated and aliquoted for storage at -80°C.

Indicators of bone metabolism and mineralization were assessed. Serum bone alkaline phosphatase (BAP), an early indicator of bone formation and osteoblast activity, was assessed using EIA kits from Immunodiagnostic Systems Inc. (IDS, Gaithersberg, MD). Serum N-terminal-mid-fragment and intact osteocalcin (OCN) were measured using an ELISA kits from IDS as an indicator of late formation, or mineralization. Finally, serum tartrate-resistant acid phosphatase type 5b (TRAcP-5b), an indicator of bone resorption, was assessed using ELISA kits, also from IDS.

Biomarkers of inflammation, oxidative stress, and vitamin D status were assessed. High sensitivity C-reactive protein (hsCRP), a biomarker of inflammation, was measured using a commercially available kit (Carolina Liquid Chemistries Corp, Winston Salem, NC) on a Biolis 24i Clinical Chemistry Analyzer. Serum thiobarbituric acid reactive species (TBARS) for oxidative stress was assessed using a Cayman Chemical kit (Ann Arbor, Michigan). Finally, a serum marker of vitamin D status (i.e., 25-hydroxy vitamin D [25-OH-VitD]) was assessed with IDS EIA kits.

Statistical analysis

All data were analyzed using PC SAS version 9.4 (SAS Institute, Cary, NC). Descriptive statistics (e.g., means, standard errors, medians, minima, and maxima) were calculated for all continuous variables. Normality of continuous variables was assessed using Shapiro-Wilk's test for normality. Change in serum bone biomarkers, the primary outcomes, and change from baseline in indicators of oxidative stress and inflammation, the secondary outcomes, were assessed using paired T-test. Categorical data for baseline characteristics, T-scores, BMI, and vitamin D status were assessed using Chi square analyses followed by confirmation with Fisher's exact test. Finally, Pearson r correlation was used to determine the relationship between changes

in hsCRP and TBARS to BAP, OCN, and TRAcP 5b. The alpha was set to 0.05 for all analyses. Data are presented as means ± standard error (SE) unless otherwise specified.

CHAPTER IV

RESULTS

Baseline characteristics

A total of 33 participants were enrolled in the study. Three participants withdrew before the second visit due to complaints of dizziness, exacerbation of an undisclosed ulcer, and interference with sleep. Of the 30 participants that completed the study, three were excluded from the analyses due to unanticipated knee replacement surgery, poisonous spider bite, and serious GI illness with antibiotic use. Compliance was >90% and did not differ between groups. The study population was primarily Caucasian (96%), married (70%), and highly educated (44% reported post graduate education) with a mean age of 70.9 ± 0.9 years (**Table 2**). Self-reported years post menopause was 22.2 ± 1.1 years for the study population overall with no difference between the TC1X and TC2X groups. The TC1X and TC2X groups were similar in age and distribution of ethnicity, marital status, and education.

With regard to lifestyle factors, although 74% of participants reported that they consume alcohol, the mean number of drinks per week was 1.8 ± 0.5, which is well below the recommended limit for women (i.e., no more than one drink/d)[194] (**Table 2**). At baseline, there were no differences between the two treatment groups for the proportion of participants who consumed alcohol, the number of drinks consumed, and those who ever smoked. Post-

menopausal years on hormone replacement therapy was significantly greater in the TC1X group compared to the TC2X group (**Table 2**). Reproductive history, including gravidity, parity, and years of oral contraceptive use, were similar between treatment groups at baseline (**Table 2**).

Dual x-ray absorptiometry

Baseline BMD T-scores obtained from DXA scans revealed 82% of the women were osteopenic in at least one site (**Table 3**). The frequency of osteopenia and osteoporosis in the femur neck for the study population was 20 and three, respectively. The distribution of osteoporosis categories between TC1X and TC2X treatment groups tended to be different at the femur neck but was similar between groups at all other sites. No significant differences were observed in the distribution of T-scores considered normal, osteopenia, or osteoporosis between TC1X and TC2X groups at the hip, hip subregions, or lumbar spine. Specific bone parameters (i.e., BMD, BMC, and BMA) were not different between groups at baseline (**APPENDIX B**).

Anthropometrics

In characterizing baseline anthropometrics of participants, the overall study population distribution of BMI by category was 44% normal, 37% overweight, and 19% obese (*data not shown*), with no differences in distributions between the TC1X and TC2X groups (**Table 4**). There were no differences in weight, height, BMI, percent body fat, waist and hip circumferences, and waist to hip ratio between groups at baseline or final time points for both treatments (**Table 4**). The mean BMI at baseline and final time point for both the TC1X and TC2X groups were within the same category (i.e., overweight). At the final visit, weight and BMI were significantly greater than baseline for the TC1X group however were still within the same category (i.e., overweight) and circumference measurements were not different from baseline. There were no significant differences in percent change for any anthropometric measures between groups (**Table 4**).

Nutritional analysis

Average macronutrient and vitamin D intake were calculated from three-day food records and calcium intake determined from the calcium questionnaire. The mean intake of total kcal and percent of kcal from carbohydrates, fat, and protein were not different at baseline or final time points between TC1X and TC2X treatment groups (**Table 5)**. On average, participants in both groups consumed 15-20% of their calories from protein, 40-50% from carbohydrates, and 30-40% from fat at baseline and final visits. The percent change in total kcal intake and contribution of protein, carbohydrates, and fats in the diet were not significantly different within and between TC1X and TC2X treatment groups over the 90-day study period. The tart cherry juice provided an additional 70 kcal and 19 g of carbohydrates per day to the TC2X group over the TC1X group (**Figure 3**).

Figure 3. King Orchards Montmorency Tart Cherry Juice

Nutrition Facts	
32 servings per container	
Serving size	**30 ml**
Amount Per Serving	
Calories	**70**
	% Daily Value*
Total Fat 0g	**0%**
Saturated Fat 0g	**0%**
Trans Fat 0g	
Sodium 15mg	**1%**
Total Carbohydrate 19g	**7%**
Dietary Fiber 0g	**0%**
Total Sugars 15g	
Includes 0g Added Sugars	**0%**
Protein 0g	**0%**
Not a significant source of cholesterol, vitamin D, calcium, iron, and potassium	
*The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice.	

Calcium intake was examined at baseline and final visits using the calcium questionnaire. In this cohort, supplements accounted for ~600 mg of daily calcium intake at baseline on average and ~1000 mg was consumed from food sources, thus meeting the 1200 mg/d total intake recommendation for women over 50 years (**Table 5**). Total calcium intake, as well as intake from supplements or food, was not different between TC1X and TC2X treatment groups at baseline and the proportion of participants not meeting the RDA for calcium was similar between groups. Following 90-days of treatment, there was no difference in calcium intake between the treatment groups resulting in no alterations in percent change in calcium intake (**Table 5**). The proportion of participants meeting the RDA for calcium intake did not differ between groups, despite the fact that the number of participants who were not meeting the RDA in the TC2X group went from three to six during the course of the study.

Dietary intake of vitamin D from food and from dietary supplements was similar between groups at baseline (**Table 5**). Forty-three percent of participants reported supplementing with vitamin D and the overall mean intake from supplements was ~1300 IU/d for this cohort (*data not shown*). Vitamin D intake remained similar between TC1X and TC2X treatment groups at the final visit and was not different from baseline values within each group (**Table 5**). Moreover, change in vitamin D intake from food, supplements, and total intake were similar, despite a large mean percent change (~39%) for the TC2X group.

Sun exposure

The most important source of vitamin D is sun exposure. Each participant was assigned a sun exposure score based on their responses to a validated questionnaire. A score $\geq$7 indicates a participant spent 5-30 minutes outside daily with at least hands and face exposed over the previous week and a score $\geq$14 indicates a participant spent >30 min outside with at least hands and face exposed daily over the previous week. No differences in sun exposure score were revealed between groups at baseline (TC1X=18.4 ± 2.9; TC2X=21.1 ± 3.3). However, at the final timepoint the TC2X mean sun score was lower than the baseline visit score (TC2X=13.4 ± 3.3; p=0.0455), but were not different between groups (TC1X=17.6 ± 2.4) (*data not shown*).

Grip strength and physical activity

Hand grip strength was used as the functional indicator of muscle strength and function. Overall, mean grip strength was statistically similar between groups at the baseline visit (*data not shown*) and fell within normal ranges for women 65-69 years of age (15.4-27.2 kg) and women 70-99 years (14.7-24.5 kg). Evaluation of hand grip strength (kg) normalized to kg of lean mass in left and right arms obtained by DXA at baseline were also similar between groups (**Table 6)**. Grip strength was not altered over the course of the study when comparisons were made within and between treatment groups and when percent change was considered (**Table 6**). Baseline lean

arm mass was used to normalize final grip strength although DXA was not performed at the final visit as it would be unlikely to see a large change in the 90-day study period.

Physical activity was assessed using the YPAS which provides index scores and estimates of energy expenditure (EE). The overall study population was categorized as lightly active, given a mean daily exercise EE of ~200 kcal (i.e., ~1500 kcal/wk; *data not shown*) and neither exercise nor total EE differed between TC1X and TC2X treatment groups (**Table 6**). Exercise and total EE remained similar between groups at the final visit however total EE tended to be lower at the final visit for the TC2X group however there were no differences in percent or absolute change in these estimates of EE between groups throughout the study. In terms of the index scores for physical activity, there were no differences in sub-scores or summary scores between the treatment groups at baseline, but at the final visit the vigorous activity score was significantly lower in the TC2X group compared to the TC1X group and the moving index score tended to be lower (**Table 6**). Although the final moving index score was significantly lower than baseline for the TC1X group, the summary score was not different. The TC2X group saw significantly lower vigorous activity and leisurely walking index scores at the final visit and thus significantly lower summary index score. All other index scores were not different within groups at the final visit.

Serum indicators of bone formation and resorption

Analysis of serum samples from baseline and final visits provided indication of changes in bone formation and resorption over the course of the study. Mean serum BAP (**Figure 4A**), an early indicator of bone formation, and OCN (**Figure 4B**), an indicator of bone turnover and mineralization, was not statistically different between groups at baseline. Similarly, mean serum TRAcP-5b (**Figure 4C**), an indicator of resorption, was not statistically different between groups at baseline. Similarity between groups was maintained at the final visit for all biochemical markers of bone metabolism and there were no differences between groups or percent change in

response to the tart cherry treatment (**Figure 4D**). Although there was no change from baseline to final visit within the TC1X treatment group for any bone biomarker parameters, serum TRAcP-5b was significantly lower at the final visit compared to baseline within the TC2X treatment group (**Table 7**). Other parameters (i.e., BAP and OCN) were not different from baseline for the TC2X group.

Serum vitamin D

Vitamin D status was determined based on analysis of serum 25-OH-VitD. According to the IOM, Food and Nutrition Board guidelines for adequacy (>20 ng/mL) and at risk for deficiency (12 to 30 ng/mL), this cohort had mean 25-OH-VitD within the reference range at baseline (34.0 ± 1.98 ng/mL) (**Table 7)**. Nine out of the 27 participants in this cohort were considered at risk for vitamin D deficiency. There were no differences between the TC1X and TC2X groups 25-OH VitD at baseline nor were there differences within or between groups after the 90-day treatment period (**Table 7**). Likewise, when considering the distribution of participants who were at risk for vitamin D deficiency using the IOM guidelines, there were no differences between groups at either time point.

Serum indicators of inflammation and oxidative stress

Inflammation and oxidative stress were determined by assessing serum hsCRP and TBARS, respectively. Overall baseline hsCRP for the cohort (2.2 ± 0.5 mg/L) was within the normal reference range (<3 mg/L) and similar between the TC1X and TC2X treatment groups (**Table 7**). There were no differences within groups, between groups, and therefore percent change at the end of the treatment period. TBARS, an indicator of lipid peroxidation, was similar between groups at baseline with means higher than the normal human serum reference range (1.86-3.94 μM).[195] There was no significant percent change in serum TBARS or between and within groups at the final time point.

Correlations between hsCRP and TBARS with biomarkers of bone metabolism were explored. hsCRP was negatively correlated with TRAcP-5b (r = *-0.32*) and tended to be negatively correlated with OCN (*r=-0.26)* (**Table 8**). Further, there was a significant negative correlation for OCN (*r* = *-0.30*) with TBARS and BAP tended to be negatively correlated (*r=-0.25*). Other relationships that were explored (i.e., hsCRP with BAP; TBARS with TRAcP-5b) showed no statistically significant correlations (**Table 8**).

Table 2
Baseline characteristics

Characteristic	Total (n=27)	TC1X[a] (n=14)	TC2X[a] (n=13)	*P*-value[b]
Age (years)	70.9 ± 0.9	70.6 ± 1.1	71.3 ± 1.4	0.6776
Post menopause (years)	22.2 ± 1.1	22.5 ± 1.8	22.0 ± 1.3	0.8348
Ethnicity				1.0000
Caucasian (%)	26 (96.3)	13 (92.9)	13 (100)	
American Indian (%)	1 (3.7)	1 (7.1)	0.0 (0.0)	
Marital status				0.4323
Married (%)	19 (70.4)	10 (71.4)	9 (69.2)	
Widowed (%)	4 (14.8)	3 (21.4)	1 (7.7)	
Divorced (%)	4 (14.8)	1 (7.1)	3 (23.1)	
Education				
High school diploma (%)	2 (7.4)	0 (0.0)	2 (15.4)	0.4094
Some college (%)	5 (18.5)	2 (14.3)	3 (23.1)	
College degree (%)	8 (29.6)	4 (28.6)	4 (30.8)	
Post graduate (%)	12 (44.4)	8 (57.1)	4 (30.8)	
Lifestyle				
Ever smoked (%)	8 (29.6)	4 (28.6)	4 (30.8)	1.0000
Consumes any alcohol (%)	20 (74.1)	10 (71.4)	10 (76.9)	1.0000
Frequency (drinks/week)	1.8 ± 0.5	2.4 ± 0.8	1.2 ± 0.4	0.2244
Reproductive history				
Gravidity	2.6 ± 0.3	2.9 ± 0.4	2.3 ± 0.4	0.9800
Parity	2.1 ± 0.2	2.2 ± 0.3	2.0 ± 0.3	0.5616
Oral contraceptive use (years)	8.1 ± 1.9	10.4 ± 3.2	5.7 ± 2.0	0.2244
Post-menopause HRT (years)	5.1 ± 1.8	9.0 ± 3.1	0.9 ± 0.5	0.0198

Data are presented as mean ± standard error (SE) unless specified otherwise.
[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d
[b]*P*-values represent comparison paired t-test for continuous data and Chi Squared confirmed by Fisher's Exact test for categorical data between TC1X and TC2X.
Hormone replacement therapy (HRT)

Table 3
Categorization of T-scores by site obtained by dual x-ray absorptiometry at baseline

Characteristic	Total (n=27)	TC1X[a] (n=14)	TC2X[a] (n=13)	*P*-value[b]
Any site T-score[c]				0.4900
Normal (%)	2 (7.41)	2 (14.3)	0 (0)	
Osteopenia (%)	22 (81.5)	10 (71.4)	12 (92.3)	
Osteoporosis (%)	3 (11.1)	2 (14.3)	1 (7.7)	
Total hip T-score				0.3179
Normal (%)	9 (33.3)	6 (42.9)	3 (23.1)	
Osteopenia (%)	17 (63.0)	7 (50.0)	10 (37.0)	
Osteoporosis (%)	1 (3.7)	1 (7.1)	0 (0.0)	
Femur neck T-score				0.0881
Normal (%)	4 (14.8)	4 (28.6)	0 (0.0)	
Osteopenia (%)	20 (74.1)	8 (57.1)	12 (92.3)	
Osteoporosis (%)	3 (11.1)	2 (14.3)	1 (7.7)	
Trochanter T-score				1.0000
Normal (%)	10 (37.0)	5 (35.7)	5 (38.5)	
Osteopenia (%)	16 (59.3)	8 (57.1)	8 (61.5)	
Osteoporosis (%)	1 (3.7)	1 (7.1)	0 (0.0)	
Intertrochanter T-score				0.3342
Normal (%)	15 (55.6)	9 (64.3)	6 (46.2)	
Osteopenia (%)	11 (40.7)	4 (28.6)	7 (53.9))	
Osteoporosis (%)	1 (3.7)	1 (7.1)	0 (0.0)	
Lumber spine T-score				0.2650
Normal (%)	14 (51.9)	8 (57.1)	6 (46.2)	
Osteopenia (%)	11 (40.7)	4 (58.6)	7 (53.9)	
Osteoporosis (%)	2 (7.4)	2 (14.3)	0 (0.0)	

[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d
[b]*P*-values represent Fisher's Exact test and Chi Square between TC1X and TC2X.
[c]Considers the lowest T-score at any site and classifies into normal, osteopenia, or osteoporosis T-score ranges.

Table 4
Anthropometrics at baseline and final visit

Parameter	TC1X[a] (n=14)				TC2X[a] (n=13)				*P*-value[c]		
	Baseline	Final	% Change	*P*-value[b]	Baseline	Final	% Change	*P*-value[b]	Base-line	Final	% Change
Weight (kg)	66.7 ± 4.1	67.6 ± 4.3	1.3 ± 0.5	0.0203	74.3 ± 3.5	75.4 ± 3.4	1.6 ± 1.0	0.1752	0.1759	0.1746	0.7975
Height (cm)	161.8 ± 1.8	161.8 ± 1.7	-0.03± 0.2	0.8549	163.9 ± 2.3	463.6 ± 2.2	-0.2 ± 0.2	0.3312	0.4755	0.5211	0.5603
BMI (kg/m^2)	25.4 ± 1.4	25.7 ± 1.4	1.4 ± 0.5	0.0158	27.5 ± 0.9	28.0 ± 0.8	2.0 ± 1.1	0.1287	0.2042	0.1769	0.5826
Normal (%)	8 (57.1)	7 (50.0)	-12.5	1.0000	4 (30.8)	3 (23.1)	-25.0	1.0000	0.5218	0.3719	-
Overwt (%)	4 (28.6)	5 (35.7)	25.0	-	6 (46.2)	6 (46.2)	0.0	-	-	-	-
Obese (%)	2 (14.3)	2 (14.3)	0.0	-	3 (23.1)	4 (30.8)	33.3	-	-	-	-
Waist circ (cm)	84.9 ± 3.3	84.5 ± 3.4	-0.5 ± 0.9	0.5982	88.7 ± 2.2	89.8 ± 2.2	1.4 ± 1.1	0.2644	0.3639	0.2034	0.2153
Hip circ (cm)	102.7 ± 2.8	103.7 ± 3.2	0.9 ± 0.6	0.1384	106.5 ± 2.3	107.5 ± 2.3	1.0 ± 0.9	0.2932	0.3060	0.3384	0.8884
Waist/Hip ratio	0.82 ± 0.01	0.81 ± 0.02	-1.3 ± 0.9	0.1336	0.83 ± 0.02	0.84 ± 0.02	0.4 ± 1.2	0.7896	0.6701	0.2955	0.2365
Body fat (%)	36.3 ± 1.6	-	-	-	40.1 ± 1.1	-	-	-	0.1628	-	-

Data are presented as mean ± standard error (SE).

[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d

[b]*P*-values represent comparison within groups between baseline and corresponding final values.

[c]*P*-values represent comparison of baseline, final, and % change values between TC1X and TC2X groups.

Body mass index (BMI), Overwt (Overweight), circ (circumference)

Table 5
Average daily nutrient intake at baseline and final visit

Parameter	TC1X[a] (n=14)				TC2X[a] (n=14)				*P*-value[c]		
	Baseline	Final	% Change	*P*-value[b]	Baseline	Final	% Change	*P*-value[b]	Base-line	Final	% Change
Macronutrients											
Total energy (kcal)	1729.1 ± 116.8	1931.2 ± 119.3	17.2 ± 2.4	0.2259	1641.8 ± 96.6	1787.9 ± 136.2	12.6 ± 9.4	0.3438	0.5733	0.4345	0.7290
Protein (%kcal)	18.0 ± 1.4	15.7 ± 1.0	-6.9 ± 9.0	0.1780	17.7 ± 1.6	17.2 ± 1.2	1.6 ± 7.9	0.7572	0.8804	0.3133	0.4834
Carbohydrates (%kcal)	46.2 ± 3.1	43.9 ± 2.2	0.5 ± 8.2	0.3901	50.1 ± 2.1	48.6 ± 2.0	-1.2 ± 5.4	0.5847	0.3131	0.1272	0.8617
Total Fat (%kcal)	36.9 ± 3.0	40.6 ± 2.2	35.6 ± 13.5	0.1439	34.1 ± 1.4	35.8 ± 1.6	6.6 ± 5.9	0.3916	0.4251	0.1031	0.4019
Daily Calcium Intake[d]											
Supplements (mg)	557.3 ± 110.0	563.9 ± 107.0	5.9 ± 16.8	0.9533	616.7 ± 176.0	523.8 ± 129.7	-4.0 ± 5.9	0.4048	0.7736	0.8124	0.5932
Dietary Intake (mg)	942.8 ± 71.2	888.1 ± 84.7	-3.5 ± 6.6	0.4246	1091.2 ± 121.9	1079.7 ± 125.6	6.1 ± 11.7	0.9186	0.2950	0.2120	0.4735
Total (mg)	1500.1 ± 141.6	1452.1 ± 130.0	1.8 ± 8.0	0.6964	1707.9 ± 191.1	1603.5 ± 199.6	-3.7 ± 6.9	0.5015	0.3861	0.5248	0.6146
Below RDA (%)	4 (28.6)	4 (28.6)	-	1.0000	4 (23.1)	6 (46.2)	-	0.4110	1.0000	0.4401	-
Vitamin D Intake											
Supplements (IU/d)	1585.7 ± 515.1	1585.7 ± 515.1	0.0 ± 0.0	1.0000	1076.9 ± 338.0	1076.9 ± 338.0	0.0 ± 0.0	1.0000	0.4243	0.4243	1.0000
Dietary Intake (IU/d)[e]	141.9 ± 25.2	100.7 ± 26.0	-11.8 ± 16.4	0.1233	143.2 ± 22.8	115.6 ± 18.1	29.4 ± 48.6	0.2881	0.9698	0.6464	0.4159
Total (IU)	1727.6 ± 509.3	1686.37 ± 510.1	-6.1 ± 6.5	0.1233	1220.1 ± 345.3	1192.5 ± 341.1	39.2 ± 47.2	0.2881	0.4244	0.1381	0.3324

Data are presented as mean ± standard error (SE).
[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d
[b]*P*-values represent comparison within groups between baseline and corresponding final values.
[c]*P*-values represent comparison between TC1X and TC2X at baseline and final values.
[d]Values calculated from responses to National Institute of Health Short Calcium Questionnaire (version SCQ 2002).
[e]Average daily intake calculated from 3-day food record.

Table 6
Hand grip strength and physical activity at baseline and final visit

Parameter	TC1X[a] (n=14)				TC2X[a] (n=13)				*P*-value[c]		
	Baseline	Final	Change	*P*-value[b]	Baseline	Final	Change	*P*-value[b]	Base-line	Final	Change
Hand grip strength [d]											
Dominant hand	11.5 ± 0.6	11.5 ± 0.6	-0.1 ± 0.2	0.9042	12.3 ± 0.6	12.3 ± 0.7	-0.01 ± 0.4	0.9756	0.3971	0.4076	0.9721
Non-Dominant hand	11.8 ± 0.7	12.4 ± 0.7	0.6 ± 0.4	0.1232	11.6 ± 0.6	11.9 ± 0.7	0.4 ± 0.5	0.4795	0.8265	0.6471	0.7052
Weekly EE (kcal/week)											
Exercise (1 x10^3)	1.4 ± 0.3	1.6 ± 0.3	0.2 ± 0.2	0.2161	1.8 ± 0.5	1.5 ± 0.4	-0.3 ± 0.5	0.5178	0.4920	0.7773	0.2679
Total (1 x 10^3)	6.4 ± 0.6	5.7 ± 0.6	-0.7 ± 0.6	0.2571	8.3 ± 1.6	6.2 ± 0.9	-0.2 ± 1.0	0.0575	0.2799	0.6452	0.2255
Month index score (unit/month)											
Vigorous activity	27.1 ± 4.9	28.2 ± 4.9	1.1 ± 5.2	0.8413	26.2 ± 4.0	13.8 ± 4.4	-12.3 ± 4.0	0.0100	0.8775	0.0391	0.0565
Leisurely walking	14.5 ± 2.9	16.3 ± 3.6	1.8 ± 2.6	0.5066	21.2 ± 3.1	12.9 ± 2.7	-8.3 ± 2.0	0.0016	0.1231	0.4659	0.0059
Moving	9.9 ± 0.9	8.1 ± 0.7	-1.7 ± 0.7	0.0261	9.9 ± 1.2	9.9 ± 0.7	0.0 ± 0.9	1.0000	0.9642	0.0944	0.1379
Standing	9.4 ± 1.1	9.9 ± 0.8	0.4 ± 0.9	0.6349	8.5 ± 1.1	8.8 ± 0.9	0.2 ± 0.7	0.7533	0.5727	0.3803	0.8645
Sitting	1.7 ± 0.2	1.8 ± 0.2	0.1 ± 0.4	0.7929	2.0 ± 0.3	2.0 ± 0.3	0.0 ± 0.3	1.0000	0.4808	0.4979	0.8480
Summary	62.6 ± 6.7	64.3 ± 7.2	1.7 ± 4.2	0.7016	67.8 ± 7.4	47.5 ± 7.5	-20.4 ± 4.3	0.0005	0.6042	0.1194	0.0012

Data are presented as mean ± standard error (SE).
[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d
[b]*P*-values represent comparison within groups between baseline and corresponding final values.
[c]*P*-values represent comparison between TC1X and TC2X at baseline and final values.
[d]Grip strength assessed using Jamar hand dynamometer and reported as the best of three attempts in kg of pressure exerted for each hand. Results are normalized to baseline kg lean mass in the corresponding arm (i.e., kg pressure/kg lean mass).

Table 7
Effect of 90-d supplementing with tart cherry juice on biomarkers of inflammation and oxidative stress

	TC1X[a] (n=14)				TC2X[a] (n=13)				*P*-value[c]		
Parameter	**Baseline**	**Final**	**Change**	***P*-value[b]**	**Baseline**	**Final**	**Change**	***P*-value[b]**	**Base-line**	**Final**	**Change**
Vitamin D Status											
25(OH)D (ng/mL)	36.7 ± 3.1	35.6 ± 3.2	-1.0 ± 1.9	0.5949	31.2 ± 2.3	30.8 ± 2.4	-0.4 ± 1.3	0.7831	0.1734	0.2501	0.7829
At risk (%)[d]	4 (28.6)	5 (35.7)	25.0	1.0000	4 (30.8)	6 (46.2)	50.0	0.6882	1.0000	0.7036	-
Inflammation											
hsCRP (mg/L)	2.2 ± 0.5	2.4 ± 0.6	0.2 ± 0.3	0.5565	2.5 ± 0.7	4.4 ± 0.2	0.3 ± 0.6	0.5925	0.7388	0.6423	0.9980
Oxidative Stress											
TBARS (μM)	4.6 ± 0.5	3.9 ± 0.5	-0.7 ± 0.5	0.1799	5.1 ± 0.6	2.8 ± 0.6	-0.7 ± 0.5	0.2349	0.5448	0.3877	0.8480

Data are presented as mean ± standard error (SE).
[a]TC1X = 1 fl. oz. tart cherry juice concentrate/d; TC2X = 2 fl. oz. tart cherry juice concentrate/d
[b]*P*-values represent comparison within groups between baseline and corresponding final values.
[c]*P*-values represent comparison between TC1X and TC2X at baseline and final values.
[d]At risk for 25-hydroxy vitamin D deficiency=12-30ng/mL; no participants were deficient (25(OH)D<12ng/mL)
Bone specific alkaline phosphatase (BAP), osteocalcin (OCN), isoform 5b of tartrate resistant acid phosphatase (TRAcP-5b), high sensitivity C-reactive protein (hsCRP), thiobarbituric acid reactive substances (TBARS)

Table 8
Correlations of hsCRP and TBARS with bone biochemical markers

Parameter	*r*	*P*-value
Correlations with hsCRP		
BAP	-0.06	0.6702
OCN	-0.26	0.0586
TRAcP-5b	-0.32	0.0177
Correlations with TBARS		
BAP	-0.25	0.0662
OCN	-0.30	0.0285
TRAcP-5b	-0.15	0.2836

All correlations consider all values from baseline and final visits.

Bone specific alkaline phosphatase (BAP), osteocalcin (OCN), isoform 5b of tartrate resistant acid phosphatase (TRAcP-5b), high sensitivity C-reactive protein (hsCRP), thiobarbituric acid reactive substances (TBARS)

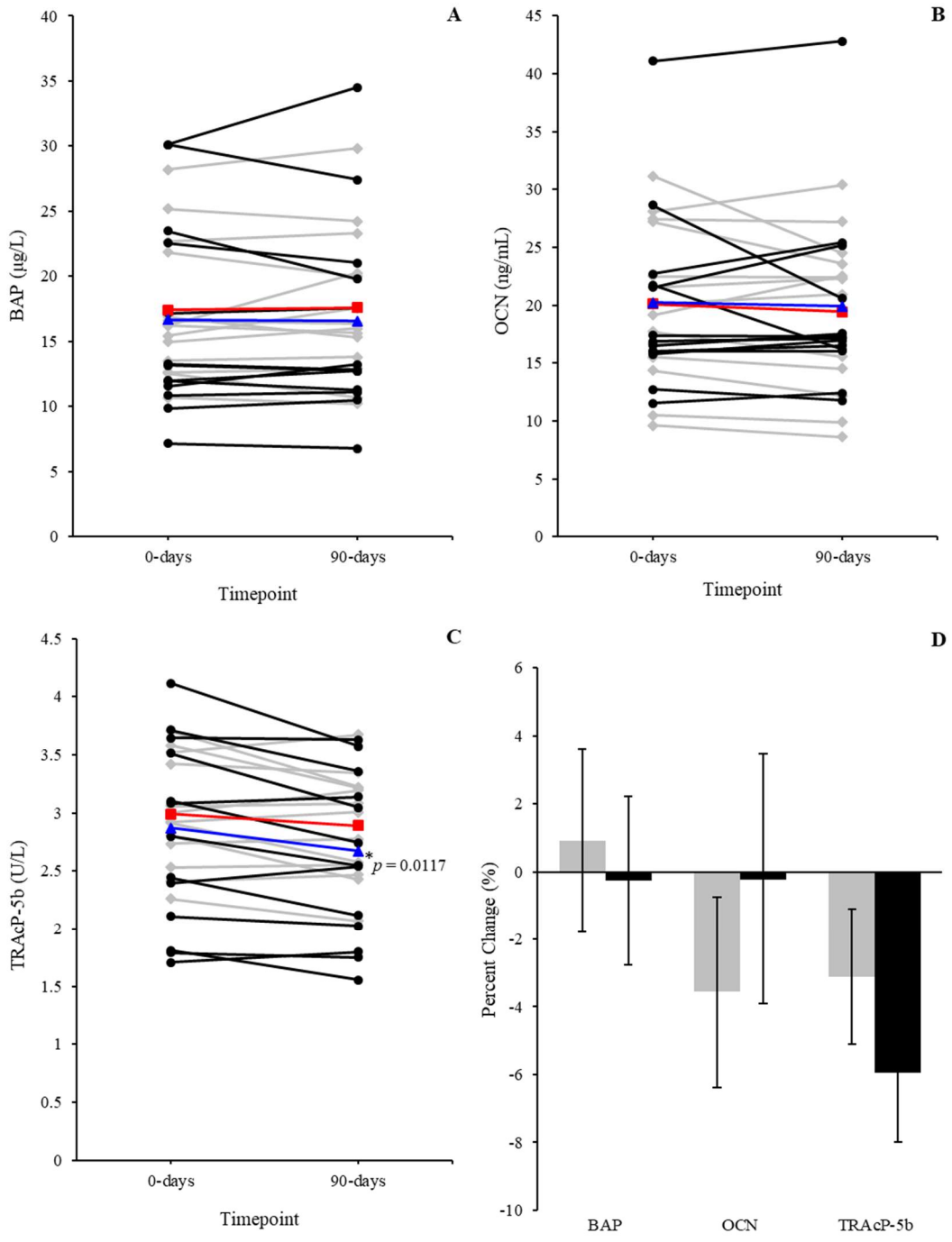

Figure 4.
Effect of 90-d supplementing with tart cherry juice concentrate on serum bone biomarkers. Individual participant and group mean biomarker data are shown for bone specific alkaline phosphatase (BAP), osteocalcin (OCN) and tartrate-resistant acid phosphatase (TRAcP)-5b. Participants consuming TC1X are shown with the grey lines (◆) and the group mean (red line,■) and participants consuming the TC2X are shown with the black lines (●) and the group mean (blue line,▲). **A)** Mean serum BAP and **B)** OCN, markers of bone formation and turnover, were unchanged from baseline to final visit for both the TC1X and TC2X groups. **C)** Mean serum TRAcP-5b, a marker of bone resorption, significantly decreased from baseline to final visit (*) within the TC2X group, but not the TC1X group. **D)** Percent change from baseline was not different between TC1X (gray bars) and TC2X (black bars) groups for any of the biomarkers of bone metabolism that were assessed.

CHAPTER V

DISCUSSION

This is the first study to investigate the extent to which tart cherry juice supplementation, over 90-days, alters serum biomarkers of bone formation and resorption in older women. Two doses of tart cherry juice were investigated due to the exploratory nature of this study. In this cohort of women 65-80 years of age, only the higher dose of tart cherry juice decreased TRAcP-5b from baseline and neither dose of tart cherry juice altered serum BAP or OCN, which are some of the classic systemic indicators of bone formation and turnover. Interestingly, serum biomarkers of bone metabolism were unaltered in pre-clinical studies with tart cherry powder although significant improvements were seen in BMD and microarchitectural bone parameters attributed to local changes indicating increased mineralization.[58] Clinical trials with dried plum, a fruit in the same genus and with similar phytochemical profile to tart cherry, showed decreases in TRAcP-5b, among other changes to systemic markers of bone metabolism and increased BMD.[39,168,196,197] Taken together, these results indicate that tart cherry juice supplementation may have the capacity to attenuate bone loss with aging through its ability to decrease bone resorption; however, supplementation with whole fruit, perhaps a dried preparation, may yield more significant changes in markers of bone metabolism and therefore significant alterations in BMD, a clinical outcome. A larger and longer clinical trial is needed to examine the effects of dried tart cherry preparation on BMD.

In our previous study utilizing an animal model of age-related bone loss, a diet supplemented with 5% and 10% w/w tart cherry powder increased bone mineral density, significantly improved trabecular bone microarchitecture in the distal femur metaphysis and lumbar spine, and enhanced cortical thickness in the mid-diaphysis of the femur.[58] These improvements in bone density and microstructure coincided with alterations in gene expression within the bone characterized by an increase in phosphate regulating endopeptidase homolog, X-linked (*Phex*) and decrease in peroxisome proliferator activated receptor (*Ppar*)-γ, which are consistent with improved mineralization. However, systemic indicators of bone formation and local OCN were not significantly affected in the animals receiving the diets supplemented with tart cherry. In the current study, serum OCN was not altered by either dose of tart cherry juice. Although the local expression of OCN is involved in regulating bone mineralization within the tissue, it should be noted that, in the serum, OCN is considered an indicator of bone turnover.[198]

With regard to bone resorption biomarkers in our previous study utilizing an animal model of age-related bone loss, there were no effects of tart cherry on systemic or local indicators of osteoclast activity.[58] The finding of the current clinical study, as it relates to classic serum biomarkers of bone metabolism, suggest that the tart cherry juice preparation may affect bone metabolism through inhibiting bone resorption. This conflicts with finding of our pre-clinical study utilizing tart cherry powder which may be related to timing and/or use of lyophilized powder versus the juice preparation. Regardless, the lack of alterations to systemic biomarkers of bone metabolism in the animal model is perplexing given the phenotypical changes to bone and local changes in gene expression. Current studies are underway in our laboratory to enhance our understanding of this phenomenon utilizing animal models.

Considering the different tart cherry products used in the animal and clinical studies, the test diets in the animal study were supplemented with tart cherry powder and intake closely monitored. Administering treatments through the drinking water to mice is more complicated to

monitor and dosing is more difficult to control.[138] Previous clinical studies with tart cherries that examined and showed changes to indicators of inflammation and oxidative stress have used tart cherry juice and the product was easily accessible,[199-201] therefore tart cherry juice was utilized in this clinical study. The loss of bioactive components of the tart cherry that may be responsible for its osteoprotective effects such as the polyphenolic compounds is minimal during heat treatment used in preparing juice concentrates.[202] However, other bioactive components within the fruit, such as non-digestible carbohydrates, that may contribute to the bone protective effects of the tart cherry due to their effects on mineral absorption and the microbiota, may not be preserved in the juice preparation to the same level as the powder.[202,203] A subsequent, larger and longer study utilizing dried tart cherries may be warranted.

Tart cherry juice has been shown to have potent antioxidant and anti-inflammatory properties in adult athletes and adults with osteoarthritis that are otherwise healthy.[48,57,179] Since increased oxidative stress and pro-inflammatory mediators can increase osteoclast activity resulting in an increase in bone resorption, we also investigated whether tart cherry juice supplementation altered indicators of inflammation and oxidative stress during this 90-day study. Although TRAcP-5b was decreased by the larger dose of tart cherry juice in this study compared to baseline, there were no significant differences in serum hsCRP or TBARS, systemic indicators of inflammation and oxidative stress. Although inflammation and oxidative stress generally increase with age, other studies reporting improvements in indicators of inflammation and oxidative stress have included otherwise healthy participants who were experiencing acute increases in these biomarkers such as those associated with running a marathon, competing in a triathlon, or high intensity interval training.[40,54,204] Thus, tart cherry juice supplementation may have more pronounced effects in acute scenarios. Studies examining pharmacokinetics of tart cherry phytochemicals report peak concentration of phenolic acid degradation products in plasma one to two hours post-consumption of eight to 16 fl. oz. of tart cherry juice that return to baseline

levels six to eight hours from initial dose consumption under fasted conditions.[205,206] It is not without reason to consider that regular doses of tart cherry juice, as with twice per day, may provide transient protection against inflammation and oxidative stress to reduce osteoclast activity however not significantly affect the markers examined in this study. Furthermore, this study may have been underpowered to detect a significant difference in these markers given biological variability. However it is also possible that tart cherry juice influences osteoclast activity directly through other mechanisms as has been seen in cellular dried plum studies.

Although significant changes in the serum indicators of inflammation and oxidative stress were not observed in this study, we did examine the relationship between hsCRP and TBARS with biochemical markers of bone metabolism. Interestingly, a negative correlation was observed between hsCRP and TRAcP-5b. Pro-inflammatory mediators promote osteoclast differentiation and activity, especially the major regulator of osteoclast differentiation, RANKL, which is a member of the TNF superfamily.[207] Thus, the negative correlation of TRAcP-5b, a phosphatase whose expression is induced by inflammatory cytokines, would not be expected. Overall, there was no significant change in hsCRP from baseline to final visits for either dose. Therefore, as TRAcP-5b is decreased with no change in hsCRP, a negative correlation can emerge although hsCRP was unchanged. The trend for a negative correlation between OCN and hsCRP would be expected. Further investigation into other inflammatory cytokines (e.g., TNF-α and IL-6) are warranted to better understand the relationship between inflammation and bone resorption in this study. With regard to oxidative stress and bone biochemical markers, a negative correlation was observed between TBARS and OCN. There also tended to be a negative correlation between TBARS and BAP. This is a response that would be anticipated as oxidative stress is known to suppress bone formation and mineralization.[134] However, one should interpret this finding with caution given that serum levels of OCN are considered an indicator of bone turnover.[208] Further

investigation with other biomarkers of oxidative stress are warranted to better understand this relationship.

At this time, there are limitations of the study that need to be acknowledged. First, our previous animal study showed positive effects on bone parameters utilized a lyophilized tart cherry powder where this study used tart cherry juice. The decision to use tart cherry juice in this clinical trial was based on changes shown in other clinical trials for markers of inflammation and oxidative stress as well as ease of administration and access. It is possible that the juice preparation has a reduced concentration of bioactive components per serving responsible for the osteoprotective effects of tart cherry seen in the animal study. Second, it is possible that this study, although considered pilot in nature, may have been underpowered to detect changes in markers of inflammation and oxidative stress and perhaps more subtle differences or changes at lower doses. Because this is the first clinical trial of tart cherry juice on bone biomarkers, the power calculation was based on previous studies of this duration to detect differences in bone biomarkers with dried plum, which has a similar polyphenolic and non-digestible carbohydrate profile as the tart cherry.[171,205] A larger study population may be required to achieve adequate power to show changes in inflammation and oxidative stress or subtle changes in markers of bone metabolism at lower doses.

In conclusion, supplementation with 8 fl. oz. of tart cherry juice for 90 days decreased TRAcP-5b, a biomarker of bone resorption. This occurred when the cherry juice was consumed twice per day and there were no changes on biomarkers of bone formation or turnover in this cohort of older women. It is clear, between the clinical and pre-clinical trial in our lab examining the effect of tart cherry on bone, that tart cherry positively influences bone metabolism in age-related bone loss, but the evidence supporting the mechanism differed. The use of different tart cherry preparations must be considered when comparing effects of tart cherry on bone metabolism to that of the pre-clinical trial. Furthermore, although this study was not able to

demonstrate the ability of tart cherry juice to reduce indicators of inflammation and oxidative stress, other studies have shown tart cherry juice has the capacity to do so in healthy adult populations under conditions of stress. Although this study was powered to show changes in biomarkers of bone metabolism, it is unclear if a larger study may yield significant changes in markers of inflammation and oxidative stress. Taken together, a larger, longer study examining BMD as the primary outcome is needed utilizing a dried preparation of tart cherry to account for both difference in product and biological variability, especially for markers of inflammation and oxidative stress.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This study was designed to examine the effects of tart cherry juice supplementation on biomarkers of bone metabolism in older women and how these changes coincide with alterations in markers of inflammation and oxidative stress. Women 65-80 years (n=33) of age were randomized to consume 8 fl. oz. of tart cherry juice once per day (TC1X) or twice per day (TC2X) for 90 days. Blood was drawn at baseline and final visits to determine serum biomarkers of bone formation (i.e., bone-specific alkaline phosphate [BAP] and osteocalcin [OCN]) and bone resorption (i.e., tartrate resistant phosphates [TRAP-5b]). Further, serum indicators of oxidative stress (i.e., thiobarbituric acid reactive species [TBARS]) and inflammation (i.e., high sensitivity C-reactive protein [hsCRP]) were assessed. There were 33 participants enrolled in the study with three withdrawals before completion. All participants were >90% compliant throughout the study with a mean compliance >98%. Results indicated biomarkers of bone resorption were decreased from baseline visit in the TC2X group. There was no change in biomarkers of bone formation, inflammation or oxidative stress observed in response to either dose of tart cherry juice compared to baseline values. Furthermore, there were no differences in these serum measures when comparisons were made between the two treatment groups at the end of the study.

Conclusions

The purpose of this study was to determine the dose-dependent effect of 90-day tart cherry juice supplementation on biochemical markers of bone formation and resorption in women aged 65-80 years as well as influence on markers of inflammation and oxidative stress. The following is a list of aims and working hypotheses for this study:

***Aim 1*:** Determine the change in serum biomarkers of bone metabolism (i.e., BAP, TRAP-5b, and OCN) after 90 days of consuming one of two doses of tart cherry juice (i.e., 8 fl oz once [TC1X] or twice [TC2X] per day) in a population of healthy women aged 65-80 years.

Aim 1 Working Hypothesis: Tart cherry juice supplementation will increase serum biomarkers of bone formations (i.e., BAP and OCN) and decrease markers of bone resorption (i.e., TRAcP-5b) from baseline in a dose-dependent manner.

Markers of bone formation (BAP) and mineralization (OCN) were unchanged in TC1X or TC2X groups. This was the case when comparisons were made between groups at final visits and from baseline to final visit within either group. Serum TRAcP-5b marker of bone resorption was significantly decreased from baseline to final visit only for the TC2X group (p=0.0117) however final values did not differ from the TC1X treatment group. We partially reject this hypothesis as findings showed no change in biomarkers of bone formation within or between groups however the marker of resorption was decreased by the TC2X treatment when compared to baseline.

***Aim 2*:** Assess the change in serum indicators of inflammation (i.e., hsCRP) and oxidative stress (i.e., TBARS) after 90 days of consuming one of two doses of tart cherry juice (i.e., 8 fl oz once or twice per day) in a population of healthy women aged 65-80 years.

Aim 2 Working Hypothesis: Tart cherry juice supplementation will decrease serum biomarkers of inflammation and oxidative stress from baseline in a dose-dependent manner.

There were no changes in serum hsCRP or TBARS after 90 days of consuming 8 fl. oz. of tart cherry juice once or twice per day when compared to baseline and compared between groups. We reject this hypothesis as the findings showed no change in biomarkers of inflammation or oxidative stress within or between TC1X and TC2X treatment groups.

***Aim 3*:** To correlate the effects of tart cherry juice treatment on indicators of oxidative stress and inflammation with changes in biochemical markers of bone metabolism.

Aim 3 Working Hypothesis: As indicators of inflammation and oxidative stress decrease with tart cherry juice supplementation, markers of bone resorption will also decrease, and markers of bone formation will increase.

A significant negative correlation was observed for hsCRP with TRAcP-5b (r=-0.32; p=0.0177) and OCN tended to be negatively correlated (r=-0.26; p=0.0586). Further, there was a negative correlation for TBARS with OCN (r=-0.30; p=0.0285) and BAP tended to be negatively correlated (r=-0.25; p=0.0662).

We partially reject this hypothesis. Although a negative relationship was observed between TBARS and OCN, there was no significant decrease in TBARS with tart cherry juice treatments within or between TC1X and TC2X groups. Further, the negative relationship shown between hsCRP and TRAcP-5b is not the expected relationship between these markers of inflammation and bone resorption, respectively.

Recommendations:

This pilot study showed decreases in a marker of bone resorption with the higher dose of tart cherry juice. Although indicators of formation and turnover did not increase as hypothesized, the results of this trial along with those of the pre-clinical study, suggest that tart cherry supplementation positively alters bone metabolism in age-related bone loss. A larger, longer clinical trial is needed to determine if tart cherry is able to influence the clinical outcome of bone

loss, BMD. This future study should be a minimum year in length utilizing a tart cherry powder, as in the pre-clinical trial, to account for the discrepancy in evidence of mechanism (i.e., increased mineralization versus decreased osteoclast). Moreover, tart cherry supplementation in a larger cohort of women over a longer duration may allow for detection of more subtle changes to biomarkers of bone metabolism in the lower dosage and account for markers with a wider range of biological variation, as with inflammation and oxidative stress which were unchanged in this trial. Serum biomarkers could lend to mechanistic explanations laying the groundwork to pursue a full understanding through cellular and animal study models.

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APPENDICES

APPENDIX A. IRB Approval

Oklahoma State University Institutional Review Board

Date:	Tuesday, March 28, 2017
IRB Application No	HE179
Proposal Title:	The Influence of Tart Cherry Juice on Bone Biomarkers in Women Aged 65-80 Years
Reviewed and Processed as:	Expedited

Status Recommended by Reviewer(s): Approved Protocol Expires: 3/27/2018

Principal Investigator(s):

Brenda Smith 420 HES Stillwater, OK 74078	Tiffany Dodier Stillwater, OK 74078	Janice R. Hermann 315 HES Stillwater, OK 74078
Edralin A. Lucas 422 HES Stillwater, OK 74078	Mark Payton Stillwater, OK 74078	Sandra Peterson Stillwater, OK 74078

The IRB application referenced above has been approved. It is the judgment of the reviewers that the rights and welfare of individuals who may be asked to participate in this study will be respected, and that the research will be conducted in a manner consistent with the IRB requirements as outlined in section 45 CFR 46.

☐ The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

As Principal Investigator, it is your responsibility to do the following:

1. Conduct this study exactly as it has been approved. Any modifications to the research protocol must be submitted with the appropriate signatures for IRB approval. Protocol modifications requiring approval may include changes to the title, PI advisor, funding status or sponsor, subject population composition or size, recruitment, inclusion/exclusion criteria, research site, research procedures and consent/assent process or forms.
2. Submit a request for continuation if the study extends beyond the approval period. This continuation must receive IRB review and approval before the research can continue.
3. Report any adverse events to the IRB Chair promptly. Adverse events are those which are unanticipated and impact the subjects during the course of the research; and
4. Notify the IRB office in writing when your research project is complete.

Please note that approved protocols are subject to monitoring by the IRB and that the IRB office has the authority to inspect research records associated with this protocol at any time. If you have questions about the IRB procedures or need any assistance from the Board, please contact Dawnett Watkins 219 Scott Hall (phone: 405-744-5700, dawnett.watkins@okstate.edu).

Sincerely,

Hugh Crethar, Chair
Institutional Review Board

APPENDIX B

Dual-energy x-ray absorptiometry at baseline

Measure	TC1X [a] (n=14)	TC2X [a] (n=13)	*P* value
Lumber spine			
BMA (cm^2)	56.3 ± 1.6	59.0 ± 2.1	0.3186
BMC (g)	54.1 ± 2.6	55.0 ± 2.7	0.7958
BMD (g/cm^2)	1.0 ± 0.04	0.9 ± 0.02	0.4918
T score	-0.8 ± 0.4	-1.1 ± 0.2	0.4925
Total hip			
BMA (cm^2)	35.6 ± 0.9	35.8 ± 1.4	0.8917
BMC (g)	29.1 ± 1.5	28.6 ± 1.9	0.8205
BMD (g/cm^2)	0.8 ± 0.03	0.8 ± 0.02	0.5158
T score	-1.0 ± 0.3	-1.2 ± 0.2	0.4278
Femur Neck			
BMA (cm^2)	5.1 ± 0.07	5.2 ± 0.1	0.5350
BMC (g)	3.5 ± 0.1	3.5 ± 0.1	0.9313
BMD (g/cm^2)	0.7 ± 0.02	0.7 ± 0.02	0.4469
T score	-1.6 ± 0.2	-1.7 ± 0.2	0.7757
Trochanter			
BMA (cm^2)	11.4 ± 0.3	10.6 ± 0.3	0.0506
BMC (g)	6.8 ± 0.4	6.4 ± 0.2	0.3126
BMD (g/cm^2)	0.6 ± 0.02	0.6 ± 0.01	0.0813
T score	-1.1 ± 0.2	-1.0 ± 0.1	0.8653
Intertrochanter			
BMA (cm^2)	19.0 ± 0.9	20.0 ± 1.2	0.5130
BMC (g)	18.8 ± 1.2	18.7 ± 1.7	0.9646
BMD (g/cm^2)	1.0 ± 0.04	0.9 ± 0.02	0.1888
T score	-0.7 ± 0.2	-1.2 ± 0.1	0.1828

Data are presented as mean ± standard error (SE).

[a] TC1X = 1 fl. oz. tart cherry juice concentrate consumed once per day; TC2X = 1 fl. oz. tart cherry juice concentrate consumed twice per day at least 8 hours apart.

Bone mineral area (BMA), bone mineral content (BMC), bone mineral density (BMD)

VITA

Tiffany M Dodier

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF TART CHERRY JUICE SUPPLEMENTATION ON BIOCHEMICAL MARKERS OF BONE METABOLISM IN WOMEN AGED 65-80 YEARS

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2019.

Completed the requirements for the Bachelor of Science in Dietetics at University of New Hampshire, Durham, New Hampshire in May 2016.

Experience:

Dietetic Intern, Oklahoma State University – Oklahoma
Nutritional Sciences Department (March 2019-present)

Information Technology Specialist, Oklahoma Air National Guard – Tulsa, OK
138 FW Communications Flight (August 2016-present)

Information Technology Helpdesk, US Air Force – Al Udeid AB, Qatar
379 Communications Squadron (July 2018-February 2019)

Graduate Research Assistant, Oklahoma State University – Stillwater, OK
Nutritional Sciences Department (January 2017-July 2018)
Public Health (May 2016-January 2017)

Professional Memberships:

National Guard Association of Oklahoma (2016-present)
Oklahoma Academy of Nutrition and Dietetics (2016-present)