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The Role of Antioxidant Rich Berries in the Prevention of Postmenopausal Bone Loss

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Citation

THE ROLE OF ANTIOXIDANT RICH BERRIES
IN THE PREVENTION OF POSTMENOPAUSAL BONE LOSS

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IN THE PREVENTION OF POSTMENOPAUSAL BONE LOSS

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ABSTRACT

Postmenopausal osteoporosis is the most prevalent form of osteoporosis and results in fragility fractures. Smoking is one of the major risk factors for osteoporosis and is known to aggravate bone loss in postmenopausal women due to increased oxidative stress and inflammation. Diet-based interventions using berries have shown bone protective affects in animal studies partially due to antioxidant and anti-inflammatory properties of their phenolic compounds. The goal of this research was to determine the effects of antioxidant-rich fruits in the prevention of postmenopausal bone loss. Our first study examined the dose dependent effects of blackberries in preventing bone loss in an ovariectomized rat model of postmenopausal osteoporosis. Findings from the animal study indicated that blackberries consumed at the level of 5% (w/w) and not 10% (w/w) may modestly prevent ovariectomy-induced bone loss. Next, we conducted a clinical study to determine the effects of berries on bone loss. To explore this objective, postmenopausal smokers were required to consume 45g of blackberries or blueberries for a 9 month period. Bone mineral density of total body, and sites were determined at baseline and after 9 months. In addition, biomarkers of bone metabolism and biomarkers of oxidative stress were measured. This study found that blackberries and not blueberries modestly protected against smoking-induced bone loss of the total body bone mineral density. No significant changes were noted on biomarkers and other bone indices. The results of this research are inconclusive. Future studies are necessary to confirm these findings and explore mechanisms by which berries may prevent bone loss and effective doses in both postmenopausal smokers and nonsmokers.

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to the Graduate Council.

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Chapter 4

Reproduced with permission from: Kaume L.; Howard L.R.; Devareddy L.; Blackberry fruit:

review of its composition and chemistry, metabolism and bioavailability, and health benefits

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I.   INTRODUCTION

Osteoporosis is one of the most prevalent and debilitating bone diseases in the United States (US) and worldwide [1]. The disease impacts the quality of life of individuals affected and results in enormous direct and indirect socioeconomic costs. Current annual cost for the treatment of osteoporosis related fractures in the United States is estimated at $18 billion and will increase to $25.3 billion by 2025[1].

Postmenopausal osteoporosis is the most prevalent form of osteoporosis [1]. In the United States, nearly half of all women over the age of 50 will suffer an osteoporosis-related fracture, resulting in more than 1.5 million fractures a year [2]. In addition to hormone deficiency, smoking is considered a major risk factor in the pathogenesis of osteoporosis [3]. According to 2010 Centers for Disease Control and Prevention statistics, 21% of women aged 45-64 in the US are cigarette smokers [4]. Cigarette smoke contains over 10^{15} free radicals/g in what is known as the tar phase; the smoke that is drawn into the mouth of an active smoker [5]. These radicals have a life span of few hours to months [5], and result in increased oxidative stress in smokers compared to non-smokers. This is because long-term smoke exposure results in systemic oxidants-antioxidants imbalance hence oxidative stress [6].

Oxidative stress in biological systems occurs due to an imbalance between the free radicals and the body's antioxidant mechanism [7]. Higher levels of oxidative stress are evidenced by elevated levels of products of lipid peroxidation such as isoprostanes and thiobarbituric acid reactive substances (TBARS) [6]. Also, depleted levels of plasma antioxidants including vitamins A and C and reduced glutathione have been reported in smokers [6].

In women, heavy smoking throughout adult life has been reported to result in 5-10% less bone by the age of 45-50 years, compared to non-smokers [8]. Studies have demonstrated that

oxidants from cigarette smoke down regulate the expression of three important genes involved in bone metabolism, osteocalcin, type 1 collagen and alkaline phosphatase. These genes are important mediators of bone formation and essential for bone structure [9].

Although pharmacological options for prevention and treatment of postmenopausal osteoporosis exist, the incidence of postmenopausal osteoporosis remains high. This is mainly due to poor adherence to treatment, side effects associated with treatments, and poor access to safe products [10]. Intervention studies on postmenopausal osteoporosis are necessary to develop alternative feasible nutritional strategies that women can embrace to reduce fracture risk. Natural alternative therapies such as diet modification and phytotherapy are perceived to be safe and have little to no side effects [11].

Consumption of a diet high in fruits and vegetables is known to improve serum oxidant states *in vivo,* and reduce the incidence of cancer, vascular diseases, and other degenerative diseases [12]. Fruits and vegetables are rich sources of bioactive components including, vitamins, minerals, fiber, and phytochemicals including phenolic compounds [13]. Phenolic compounds are widely studied for their strong superoxide scavenging capacity [12]. In fact, these compounds account for 80% of total antioxidant capacity in most fruits and vegetables [14]. The antioxidant capacities of certain phenolic compounds (e.g. flavonoids) may be stronger than vitamins C and E based on molar concentrations [14].

Berries are a rich source of phenolic compounds plus several other nutrients including, B vitamins, ascorbic acid (vitamin C), carotenoids (β carotene) and tocopherols, calcium, selenium, and dietary fiber [15]. Blueberries primarily contain the class of phenolic compounds known as flavonoids, which includes: anthocyanins, catechin, epicatechin, quercetin, kaempferol, myrecetin, and phenolic acids (e.g., gallic acid, p-hydroxybenzoic acid, chlorogenic, p-coumaric,

caffeic, ferulic, and ellagic acids) [16, 17]. Blackberries on the other hand contain chiefly anthocyanins, flavonols, and hydrolyzable tannins or ellagitannins [18]. Considering the abundance of literature on blueberries compared to blackberries, a thorough review of blackberries was written as part of this thesis paper.

Recently, the effects of berries and their extracts on bone health have been explored. Animal studies have demonstrated that phenolic compounds from berries scavenge ROS formed by phagocytes (i.e. monocytes, macrophages and neutrophils) [19] resulting in bone resorption [20]. Polyphenols such as chlorogenic acid, caffeic acid, and rutin in dried plum have prevented bone loss in animal models by reducing oxidative stress biomarkers [21]. Recently, blueberries and blackberries have shown bone protective effects in an ovariectomized (OVX) rat model of postmenopausal osteoporosis [22, 23]. These animal studies provide some evidence of a positive impact of antioxidant-rich berries on bone metabolism, paving way for clinical trials that fully explore their effects in humans.

The *overall goal* of this research was to determine the effects of antioxidant-rich fruits in the prevention of postmenopausal bone loss. To achieve this goal our objectives were as follows:

-1) to examine the dose-dependent effects of blackberries in the prevention of bone loss in an ovariectomized rat model of postmenopausal osteoporosis, and

2) to determine the role of antioxidant-rich blueberries and blackberries in prevention of smoking-induced bone loss in postmenopausal women.

Hypothesis for the animal study was that antioxidant-rich blackberries will dose-dependently prevent bone loss in an ovariectomized (OVX) rat model of postmenopausal osteoporosis. For the clinical study, the hypothesis was that consumption of antioxidant rich

blackberries or blueberries for nine months will prevent smoking-induced bone loss in

postmenopausal women.

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II. LITERATURE REVIEW

OSTEOPOROSIS

Osteoporosis is a metabolic bone disease that affects the bone quality and density and is characterized by low bone mass and microarchitectural deterioration of bone tissue. This leads to porous bone, fragility and increased fracture risk [1]. Osteoporosis progresses silently, and unless a fracture occurs, the disease is mostly asymptomatic. The main areas of the body at risk of fracture are the lumbar spine, hip, and wrist although fractures can occur on any bone in this condition [2]. The most common fractures on the lumbar spine are known as vertebral fractures. Low grade vertebral fractures occur on disks and when they eventually compress, they become compression fractures. Compression fractures are responsible for loss of height and deformities such as kyphosis, or stooped posture, associated with osteoporosis [2]. The lifetime risk of vertebral fracture has been estimated to be 15.4% after age 45 years, although true risk is likely to be higher. Hip fractures are the most debilitating, and contribute significantly to age-related deaths. In fact, even with rehabilitation, only 30% of hip-fracture patients regain pre-fracture function [3]. Measurements of the bone mineral density at these three regions lumbar spine, hip, and wrist are used as predictors of fracture risk. The FDA has approved some techniques for measurement of bone mass and density based on their ability to predict fracture risk [1]. These include: central computed tomography (CT), peripheral CT which focuses on the forearm or tibia and dual energy x-ray absorptiometry (DXA) which is used to measure BMD and BMC of total body and necessary body regions. DXA is considered the gold standard technique for measuring bone mass due to its accuracy and precision [1]. The World Health Organization defines bone loss according to stages of severity: osteoporosis as BMD greater than -2.5 standard deviations below the young adult reference mean BMD and osteopenia as BMD between -1.5 and -2.5 standard

deviations below the young adult reference mean BMD. Estimates from the National Osteoporosis Foundation state that 10 million Americans are already afflicted with osteoporosis and another 42 million individuals are osteopenic and at risk for osteoporosis-related fractures [2].

POSTMENOPAUSAL OSTEOPOROSIS

Postmenopausal osteoporosis, also known as primary osteoporosis, is the most common form of osteoporosis. In the United States, a conservative estimate is that nearly half of all women over the age of 50 will suffer an osteoporosis-related fracture, resulting in more than 1.5 million fractures a year[2]. Current annual cost for treatment of osteoporosis related fractures in the United States is estimated at 18 billion [2]. Due to the large population of aging baby boomers and the fact that people are living longer, experts estimate that these costs will increase to $25.3 billion by 2025[2].

A woman is defined as postmenopausal if 12 consecutive months of amenorrhea is confirmed without medical reasons. Before menopause sets in, women experience a pre-menopausal period known as the perimenopause stage. During perimenopause, ovarian production of estrogen gradually declines [4]. This decline eventually leads to a 90% reduction of serum estradiol by the time a woman is confirmed to be menopausal [5]. This loss of estrogen causes a bone loss of about 5% for cancellous bone and 1 to 3% for cortical bone per year in early postmenopausal women (1-5 years after menopause) [5], [6]. In addition, during early menopause, women loose approximately 3% of bone in the spine per year [7] accounting for 50% of lifetime vertebral bone loss [7]. In fact, 20 years into menopause, women lose up to 50% of trabecular bone and 30% of cortical bone [6]. It is now well understood that estrogen is essential for bone resorption and formation process to remain coupled [7]. The 90% reduction in serum

estrogen in the early menopause rapidly increases bone turnover and the rate of resorption without a matched increase in bone formation, hence irreversible bone loss [7].

There are other hormonal and environmental factors that affect skeletal health. Decreased secretion of the growth hormone and inadequate or lack of physical activity may lead to impaired osteoblast function and less bone formation resulting in bone loss [5]. Also, environmental factors such as a diet low in calcium intake in early years of life, low body weight at 1 year or at maturity, delayed puberty, and early menopause may control an individual's bone mass [7]. Furthermore, after menopause, bone loss may be accelerated by certain drugs such as corticosteroids. Corticosteroids are medications that relieve inflammation and their actions resemble certain hormones made by your own body. When taken long-term (more than 6 months) or at high doses, these drugs can cause bone loss that leads to osteoporosis and fractures [7]. These medications, also known as steroids and glucocorticoids may be used in the treatment of many conditions including arthritis and asthma. Tumors of the pituitary gland or diseases such as metabolic syndrome, diabetes and cancer have also been known to cause bone loss [7].

In summary, although there are many factors that may affect skeletal health, estrogen deficiency plays a key role in postmenopausal bone loss, increasing bone turnover and the rate of resorption without a matched increase in bone formation. This change results in an imbalance in remodeling and irreversible bone loss [7]. Major mechanisms by which estrogen deficiency leads to bone loss are discussed later in this chapter.

THE OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

The FDA requires that potential agents proposed for any human therapy are evaluated in animal models [8]. Previous and current research assures the suitability of the ovariectomized

(OVX) rat in demonstrating the efficacy and safety of postmenopausal-related therapies before use in humans [8]. Ovariectomy in young but skeletally mature rats makes the rat's sex-hormone deficient, and stimulates accelerated loss of bone that is similar to what occurs in women following menopause [9]. Investigations have found the OVX rat model mimics postmenopausal cancellous bone loss when studies are conducted in less than 12 months and optimally within 6 months of life [8]. OVX rat model is reported to undergo rapid loss of trabecular bone mass and strength after ovariectomy [10, 11]. A study by Laib and others reported a 40% rapid decrease in bone volume and a two-fold decrease in tibia cancellous bone volume after ovariectomy [11]. Other reported similarities in OVX rat model and postmenopausal women include: increased resorption rate compared to formation rate and an initial rapid phase of bone loss followed by a slower phase. Both also report more cancellous than cortical bone loss, and decreased intestinal absorption of calcium. Another similarity is that in both, obesity provides protection against bone loss. Finally, they exhibit similar treatment effects after administration of exercise, hormones such as estrogen, parathyroid hormone and calcitonin, and drug therapies tamoxifen, and bisphosphonates [9].

There are disadvantages linked to the use of the OVX-rat as a model of postmenopausal osteoporosis. These include the fact that long bone of the rat grows longitudinally briefly after ovariectomy which can slightly affect results, however the use of 9-12 month old rats lessens the effects of this disadvantage [12]. Secondly, haversian systems in rats are either lacking or poorly developed unlike the well-developed haversian system in humans. [12]. Finally, the OVX rat model does not experience fragility fractures as seen in humans [13]. Despite these limitations, the OVX rat model is a suitable rat model to study postmenopausal osteoporosis [8].

SKELETAL SYSTEM

The adult human skeleton is a highly specialized organ comprised of 206 bones, each formed by a process known as modeling, and every bone constantly being renewed in a process known as remodeling [14]. The skeleton serves three main functions although depending on position each bone may serve more than one specific function including, 1) mechanical support and levers for muscles, 2) protection of vital organs, and 3) repository for calcium and phosphorous [15]. Bones are also rich sources of growth factors and cytokines that may have both inhibitory and stimulatory effects on bone functions [14]. Bone may be classified according to process of formation into flat bones and long bones. Flat bones are such as the skull, mandible, and scapula while the long bones include the femur, tibia, and radius [14]. Structurally, bone tissue is divided into trabecular bone and compact bone [16]. Trabecular bone is also known as spongy or cancellous bone has 75%-95% porosity and is found in the cuboidal bones such as flat bones and the ends of long bones. The pores are interconnected and filled with marrow. Compact bone is also called cortical bone and its porosity is only 5%-10%. Cortical bone forms a cortex or shell around vertebral bodies and other spongy bones and also is found in the shafts of long bones. The pores in the cortical bone consist of three categories of spaces: 1) Haversian canals that are about 50μm in diameter and contain capillaries and nerves, 2) Volkmann's canals are transverse canals that connect Haversian canals to each other and also contain blood vessels, and 3) Resorption cavities which are more temporal in nature and are created by osteoclasts in the process of bone remodeling [16]. In terms of function, although there may be differences due to species or situation, cancellous bone is considered to be more metabolically active than cortical bone, which largely serves a mechanical role [14].

Mature bone has an outer fibrous sheath known as the periosteum, and the inner surface which is in direct contact with the marrow known as the endosteum. Periosteum is made of connective tissue and it covers all bone surfaces except at joints where the articular cartilage lines the joints. The periosteum contains bone cells, nerves and blood vessels to ensure nourishment, and is held strongly by collagenous fibers known as Sharpeys' fibers that penetrate the bone tissue. The endosteum is a membranous sheath well served with blood vessels and bone cells and covers the surface of the cancellous bone and lines Volkmann's canals [14].

BONE TISSUE

Bone tissue is composed of three primary components- water, organic matrix (largely collagen), and minerals [17]. These components are commonly categorized into: collagen, hydroxyapatite mineral, small amounts of proteoglycans, noncollagenous proteins, and water. Collagen is a rigid, rod-like protein molecule composed of two alpha chains consisting of repeating amino acids with glycine in every third position and a high content of proline and lysine. These chains form a triple helix that is stabilized by the hydroxylation of proline and lysine residues by ascorbic acid [18]. Although several types of collagen are identified in animals, the predominant collagen in bone is type I. Collagen is found in tendons, skin, and ligaments, and has great structural properties. Collagen can spontaneously organize itself into strong fibers which give bone flexibility and tensile strength. In addition, collagen provides the nucleation of bone mineral crystals, which account for the rigidity and compressive strength in bone [16]. The inorganic mineral of bone almost entirely consists of hydroxyapatite crystals that contain carbonate, citrate, sodium, and magnesium [17]. The crystals are rods or hexagonal plates. Proteoglycans are proteins that carry sulfated carbohydrate component known as glycosaminoglycan attached to them [17]. Glycosaminoglycans are important binding sites for

fibroblast, endothelial growth factors and extracellular matrix proteins such as fibronectin and interstitial collagens [17]. Osteocalcin which is important in new bone mineralization is the most abundant noncollagenous protein [16]. Bone marrow is found in the spaces in the trabeculae of all bones. It contains red blood cells, white blood cells, and platelets providing a continuous supply of oxygen and nourishment ensuring the tissue's demands for immunity and coagulation are adequately met [16, 19].

BONE CELLULAR COMPONENTS

The formation and maintenance of bone extracellular matrix is carried out by four major cellular components- osteoclasts, osteoblasts, and osteocytes and bone lining cells [16]. According to Riasz, osteoclasts and osteoblasts are derived from multipotent hematopoietic cells and not monocyte/macrophage lineage as previously thought [20]. Osteoclasts differentiate as mononucleated cells that express tartrate resistant acid phosphatase (TRAP) and calcitonin receptors even before fusion occurs [20]. Mature multinucleated osteoclasts erode their way through bone by demineralizing adjacent bone with acids and then dissolving its collagen with enzymes [16]. On the other hand osteoblasts are mononucleated bone forming cells akin to the fibroblasts, which are cells that produce structural molecules in other tissues. Osteoblasts are responsible for the production of osteoid, the organic portion of the bone matrix. The boundary that separates osteoid and calcified bone is known as the calcification or mineralization front [16]. Osteocytes are former osteoblasts that become buried in bone and sits in the cavities called lacunae. These cells communicate among themselves and to active osteoblasts via tunnels known as canaliculi or little canals [16]. Bone lining cells are retired osteoblasts that escaped being buried in newly formed bone and are found on the surface after bone formation. These cells are flattened against the bone surface with gaps in between them and via these gap-junctions

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maintain communication with osteocytes [16]. Both lining cells and osteocytes work to transfer minerals into and out of the bone [21] and are important for sensing mechanical strain [16].

The development and differentiation of cellular components is controlled by growth factors, cytokines, and systemic hormones [22]. These growth hormones and cytokines are produced in the bone marrow microenvironment. It is proposed that these processes occur through positive and negative feedback loops, cascades formed by growth hormones and cytokines, and via hormonal control and/ or action of local mediators [6].

Several cytokines and local regulatory factors influence hematopoiesis and affect osteoclast development [22]. These cytokines include interleukin 1(IL-1), interleukin 11 (IL-11), interleukin 3 (IL-3), interleukin 6 (IL-6), tumor necrosis factor –alpha (TNF-α) and granulocyte macrophage-colony stimulating factor (M-CSF). The cytokines that inhibit osteoclast development and in turn bone resorption include interferon-γ, IL13, IL-4, IL-10, and IL-18 [20].

Mesenchymal stem cells (MSCs) differentiate into functional osteoblasts capable of bone formation through a complex multistep process [20]. Commitment of MSCs to form osteoblasts, osteoblastogenesis, is orchestrated by bone morphogenetic proteins (BMPs) [23]. Osteoblast differentiation is also influenced by several factors, including transforming growth factor β (TGFβ), platelet-derived growth-factor (PDGF), insulin-like growth factors (IGFs), and members of the fibroblast growth factor (FGF) family and peptide hormones (e.g. parathyroid hormone PTH) [20].

MODELING AND REMODELING

Modeling is the process responsible for skeletal change in size and shape during growth and development of bones. Modeling occurs as a result of independent action of osteoblasts and osteoclasts while remodeling is defined as resorption and formation that occur at the same site [20].

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According to Martin et al. modeling and remodeling are distinguished from one another in four main ways: 1) modeling at a particular site is continuous and prolonged, while remodeling is more episodic with a precise beginning and end, 2) remodeling occurs throughout life although the process is slower after growth stops, 3) modeling involves independent actions of osteoclasts and osteoblasts while remodeling involves a series of coupled actions of osteoclastic and osteoblastic cells, and 4) modeling results in altered bone size, shape, or both while remodeling has no effect on shape or size [16]. Bone formation dominates during periods of skeletal growth such as during childhood and adolescence, but once the skeleton has reached maturity, new bone tissue is formed through remodeling [14]. The rate of remodeling is age dependent although there is evidence that remodeling results in complete regeneration of the skeletal tissue every 10 years [22]. Remodeling is considered a vital process because longitudinal growth does not give rise to bone of correct geometry. Moreover, during growth each child loads on his or her skeleton differently, thus requiring customization. On the other hand, remodeling repairs microscopic damages to the skeleton and prevents fatigue fracture and maintains calcium homeostasis [16]. At the beginning of the third decade of life, there is a steady decrease in bone mass due to the higher rate of resorption. Bone remodeling becomes uncoupled and the osteoclast activity becomes greater than the osteoblast activity resulting in bone loss [20].

SYSTEMIC HORMONES REGULATING BONE METABOLISM

A large number of local and systemic factors activate and regulate functions of the skeleton. Two major calcium-regulating hormones, the parathyroid hormone (PTH) secreted by the parathyroid gland and dihydroxycholecalciferol (1, 25 $(OH)_2$ D) control bone remodeling [24]. Parathyroid hormone regulates bone remodeling by enhancing mobilization of calcium from the skeleton [19]. The active hormonal vitamin D, 1, 25 $(OH)_2$ D or calcitriol, is critical for optimal

intestinal absorption of calcium and phosphorus and maintaining skeletal growth and development [25]. Calcitriol also exerts a tonic inhibitory effect on PTH synthesis [26]. Calcitonin, a hormone produced by cells in the thyroid gland to oppose effects of PTH, appears to play a lesser role in regulating bone turnover even although it inhibits bone resorption by acting directly on the osteoclasts [27]. The growth hormone is another systemic hormone which increases both circulating and local levels of insulin-like growth factor–I (IGF-I). Growth hormone (GH) plays a role in bone modeling and remodeling, and directly stimulates cartilage cell proliferation. GH and IGF stimulate both formation and resorption and determine skeletal size before epiphyseal closure [24].

Sex hormones are critical in the regulation of skeletal development and bone remodeling [24]. Both estrogen and androgen hormone receptors are found in bone cells [27]. Estrogens acts on the skeleton through direct effects on cartilage and by its effects on other hematopoietic cell lineages. Estrogen deficiency is believed to be the single most important factor in pathogenesis of postmenopausal osteoporosis [24].

ESTROGEN AND BONE

Estrogen regulates the cytokine receptor activator of nuclear factor kappa B ligand (RANKL) which acts directly on the hematopoietic lineage of osteoclasts resulting in osteoclast recruitment and activity [24]. RANKL is expressed on the surfaces of bone marrow stromal or osteoblast precursor cells, T-cells, and B-cells [28]. Receptor activator of nuclear factor for kappa B (RANK) is its cognate receptor found on osteoclast lineage cells [29]. The binding of RANKL to RANK is essential in the formation, development, and function of osteoclasts, and suppresses their apoptosis [24]. RANKL action is opposed by osteoprotegerin (OPG), a neutralizing soluble antagonistic receptor, produced by osteoblasts to bind to RANKL and ensure a balance between

bone loss and formation. The RANKL/RANK/OPG regulatory system is vital in osteoclast formation and function and is responsible for the antiresorptive role of estrogen [28]. After postmenopausal stage, RANKL production is increased and/or OPG expression is decreased resulting in bone loss [30].

Expression of RANKL and OPG is also influenced by PTH, vitamins, and other molecules. For instance, RANKL production is increased while OPG is inhibited by PTH and glucocorticoid [31]. Vitamin K is also reported to modulate osteoblastogenesis and osteoclastogenesis in bone marrow cells via the RANKL pathway [32]. Hormonal 1, 25-(OH)$_2$ D enhances RANKL expression without any effect on OPG [33]. Other factors include tumor growth factor β (TGF β) that amplifies OPG production while IFN-γ suppresses RANKL induced osteoclastogenesis [34].

In addition, estrogen affects bone formation by prolonging the lifespan of the osteoblast through inhibition of osteoblast apoptosis at the cellular level [35]. Estrogen prefers the development of osteoblasts to adipocytes from their common precursor cells [36]. Kousteni and colleagues demonstrated that estrogen inhibits apoptosis of osteocytes in the OVX rat model [35]. Since osteocytes play a critical role in sensing mechanical loading, a reduction in the number of osteocytes may result in bone loss similar to that found in weightless conditions [37].

INFLAMMATION AND BONE LOSS

Inflammation is a normal immune response stimulated by pro-inflammatory cytokines and is a protective mechanism against invading microorganisms and infectious agents [38]. However, excessive and prolonged inflammation may cause extensive tissue and organ damage and even death due to septic shock [38]. The link between bone loss and inflammation is that pro-inflammatory factors TNF-α, IL-1, IL-6, and ROS also function as bone resorbing cytokines.

Nuclear factor kappa B (NF-kappa B) is a transcription factor that is important in modifying the intracellular regulation of the immune response, inflammation, and cell cycle coordination [39]. NF-kB also functions as a regulator for the expression of TNF-α, interleukin (IL)-1, and interleukin (IL)-6 among other important pro-inflammatory cytokines [39]. Conditions associated with prolonged inflammation result in chronic elevation of these factors, TNF-α, IL-1, IL-6, and increased ROS resulting in bone loss. Examples of conditions associated with bone loss include periodontal disease [40], autoimmune diseases, rheumatoid arthritis multiple sclerosis, and inflammatory bowel disease [41].

ROLE OF ESTROGEN IN INFLAMMATION

There are functional estrogen receptors on osteoblasts and osteoclasts which when activated block the synthesis of pro-inflammatory and bone resorbing cytokines IL-6, IL-1, and TNF-α [42] Estrogen deficiency, therefore, creates an environment for increased inflammatory mediators and results in accelerated bone turnover characterized by increased bone resorption compared to bone formation[42], [43]. Reactive oxygen species (ROS) (e.g.O_2-, H_2O_2, NOO-) produced in states of inflammation, further enhance inflammation through the activation and phosphorylation of stress kinases. c-Jun N terminal kinase [44], and transcription factors such as NF-κB and activator protein-1 (AP-1) [45].

ESTROGEN, OXIDATIVE STATUS AND BONE LOSS

Estrogen is an efficient scavenger of free radicals generated in both the aqueous and the lipophilic phases, and its antioxidant activity has been found to be up to 2.5 times that of vitamin C and vitamin E [46]. Estrogen's antioxidant effects are attributed to the aromatic hydroxyphenol structure of the A ring although estrogen receptors may mediate some antioxidant action as well [47]. Recently, it also has been suggested that estrogen augments intracellular oxidant defenses

lowering the concentration of ROS within cells [48]. Estrogen inhibits bone resorption by suppressing the production of cytokines IL-1, IL-6, M-CSF, prostaglandin (PG) E2, and TNF-α, which are stimulators of ROS production [6].

Experiments using ovariectomized animal models have demonstrated that in states of depleted intracellular antioxidants, mainly glutathione and thioredoxin, osteoclast activity increases via up-regulation of TNF-α [49]. TNF-α is a pro-inflammatory cytokine associated with activation of inflammation and osteoclastic activity [49]. In estrogen-deficient animal models, administration of antioxidants (e.g. *N*-acetyl cysteine or ascorbic acid) has shown to increase levels of glutathione and thioredoxin, and decrease TNF-α production [48] Cell culture experiments have also shown that administration of glutathione and thioredoxin in osteoclasts protects against bone loss during estrogen deficiency [48]. For example, to investigate the effects of ovariectomy and estrogen on oxidant defenses in bone, Lean and colleagues found that administering a single dose of 17-β estradiol (10μg/kg) subcutaneously- the standard replacement dose for rodents normalized previously low levels of thiol antioxidant system within 24 hours [48]. This experiment demonstrates that estrogen increases oxidant defenses and its deficiency may directly or indirectly promote bone resorption [48].

ESTROGEN, CALCIUM ABSORPTION AND PTH IN BONE HEALTH

Estrogen plays an important role in calcium absorption in the gut [50] and its reabsorption in the kidney [51]. Reduced estrogen production leads to both decreased intestinal calcium absorption and high loses of calcium in urine [51]. This provides evidence that estrogen regulates renal tubular calcium absorption [51]. Intestinal mucosal cells contain estrogen receptors that have been shown to respond to 17-β-estradiol by enhancing calcium transportation [52]. The effect of

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low estrogen on calcium absorption in the intestines and the increased loss through the kidneys can each account for late phase bone loss in postmenopausal women [53].

Estrogen indirectly effects bone resorption through its effect on PTH secretions. The slow phase of bone loss in postmenopausal women is linked to both an increase in PTH levels and actions, and to senescence of osteoblasts. These factors are similar in both men and women age 60 and above and are attributed to calcium deficiency. Calcium deficiency is as a result of reduced absorption in the intestines and less reabsorption in the kidneys due to organ atrophy. Bone loss may also be attributed to a vitamin D deficiency due to decreased 1-alpha-hydroxylase activity in the kidneys in women who do not get enough sunlight[54, 55]

SMOKING AND BONE LOSS

Tobacco use is the number one preventable cause of mortality and morbidity in the United States (CDC 2009). Cigarette smoking is a risk factor for cardiovascular diseases [56], several neoplasmic conditions including cancer of the throat, tongue, lung, and bladder [57]. Smoking is also a major risk factor for osteoporosis [58]. As mentioned earlier, radicals inhaled from cigarette smoke down regulate the expression of three important genes: osteocalcin, type 1 collagen, and alkaline phosphatase which are key mediators of bone formation and structure [59]. Moreover, there are over 150 known toxic compounds that have been found in cigarettes, although nicotine is the main chemical substance and one that leads to addiction [59]. Nicotine is known to alter bone metabolism, increase fracture risk, and compromise healing after a fracture by delaying bone callous formation and remodeling [60]. Tobacco use is also implicated in poor spinal fusion [61, 62]. Chronic administration of nicotine has been linked to low bone mineral density, low bone mineral content, and shorter bones [63].

The following are summaries of human studies that report the negative effects of cigarette smoking on bone.

A population-based cohort study of subjects aged 60 years and older reported that smoking levels recorded 16–18 years earlier were accurately indicative of lower BMD at the hip in both men and women even after adjustment for multiple confounders including age, exercise, alcohol use, body mass index and hormone use [64]. Krall and Dawson-Hughes [65] also found that bone density at the radius of the wrist was inversely related to pack-years of smoking exposure in postmenopausal women, after controlling for confounders such as BMI and years after menopause [65]. A large Danish study of 2015 postmenopausal women aged 45–58 years revealed significant negative associations between smoking and bone mass in the lumbar spine, femoral neck, and total body. This study reported a 3% deficit at the femoral neck in current smokers when their BMD was compared with non-smokers [66]. Additionally, smoking was correlated with a 4% lower BMD at the spine compared with non-smokers in a three year study of 153 early postmenopausal women [67]. Similarly, a Southern Californian longitudinal study of 8600 postmenopausal women found that current smokers had an increased hip fracture risk compared to those who had never smoked [68].

Effects of smoking on bone health are best summarized using three meta-analyses published over the past 10 years [69]. The first by Wong and colleagues gathered 29 published cross-sectional studies examining 2156 smokers and 9705 non-smokers, and 19 cohort and case-control studies representing 3889 hip fractures and reported the following: [69]: (i) in women, smoking accounts for one in eight hip fractures; (ii) the relative risk of hip fracture in smokers compared with non-smokers is strongly associated with age, thus the longer one smokes the higher the risk for fracture; (iii) the difference in BMD at the femoral neck, radius, and heel bone

(calcaneus) in smokers compared to non-smokers increased with age and BMD of smokers fell 2% more than that of non-smokers every 10 years; (iv) smoking has a direct effect on BMD that cannot be attributed to variations in body mass, menopausal history, exercise levels, or hormone therapy [69].

The second meta-analyses by Ward and Klesges pooled data from 86 studies representing 40753 subjects [70]. The results were as follows: (i) smokers had less bone mass at hip, lumbar spine, forearm and calcaneus; (ii) smoking cessation had a beneficial effect on bone mass, with BMD being between life-long non-smokers or current smokers; (iii) smoking increased the lifetime risk of hip fracture by 31% in women and 40% in men, and of lumbar spine by 13% in females and 32% in males; and (iv) smoking accounted for up to 10% of all hip fractures. These results mean that an estimated 34000 hip fractures per year in the United States alone are attributable to smoking [70].

The third and most recent meta-analysis of 10 prospective cohort studies in five continents examined 59232 subjects and reported that, smoking increased fracture risk by 25% when risk in smokers was compared with the risk in never-smokers. The highest risk reported was for hip fracture. Secondly, smoking-associated fracture risk was related to body mass index but the effects were not significant [69].

MECHANISMS BY WHICH SMOKING CAUSES BONE LOSS

Smoking and bone loss has been investigated and some mechanisms by which it causes bone loss have been identified as: 1) reduced active vitamin D in plasma, 2) malabsorption of calcium, and 3) oxidative stress and 4) inflammation.

Reduced Active Vitamin D in Plasma

There is evidence that high concentration of nicotine in plasma is associated with low 1, 25 (OH) 2D levels in animal models [71]. Fung and colleagues reported a 25% decrease in serum levels of 1, 25 (OH) 2D when seven month old female rats were treated with 4.5mg/kg/day of nicotine. The dose was administered via subcutaneous osmotic pumps for 90 days and results compared to both control group and low nicotine group (3mg/kg/day) [71]. The role of vitamin D and calcium in bone health is discussed earlier and cannot be over emphasized.

Malabsorption of calcium

In humans, tobacco use is associated with decreased calcium absorption [65]. Since restoration of systemic plasma calcium balance is extremely crucial, the body increases retention or withdraws calcium from the skeleton [72]. Long periods of calcium insufficiency thus cause bone loss since bone resorption will be higher than formation. The importance of calcium in bone metabolism is also discussed earlier in this paper.

Oxidative stress

Studies have shown that long-term smoke exposure can result in systemic oxidants-antioxidants imbalance evidenced by increased products of lipid peroxidation such as isoprostanes and thiobarbituric acid reactive substances (TBARS) (indices of lipid peroxidation and oxidative stress), and depleted levels of plasma antioxidants including vitamin A and C and GSH related antioxidants in plasma of smokers [73]. The major free oxygen radicals or reactive oxygen species (ROS) responsible for oxidative stress in biological systems are hydroxyl radicals (OH·), peroxide anions ($O·_2-$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) [74]; smoking is an additional source of radicals [75]. These radicals account for oxidation of specific enzymes,

proteins, lipid peroxidation, and DNA causing cell and tissue damage [74]. Damaging effects of

ROS are mitigated by endogenous antioxidant enzymes such as superoxide dismutase (SOD),

glutathione peroxidase (GPx), and catalase [49] as well as by dietary antioxidants [76]. Aged

osteoporotic women are reported to have decreased plasma antioxidants and tend to benefit from

dietary antioxidants such as vitamin C. Vitamin C supplementation is known to improve BMD in

postmenopausal women [77].

Free radicals such as NO from cigarette smoke activate NF-kappa B which is oxidative

stress-responsive transcription factor that induces bone resorption [78]. Additionally, H_2O_2 is

known to inhibit differentiation markers in osteoblastic cells in marrow stromal cells modifying

differentiation of vascular and bone cells [79]. Rothem et al reported lowered osteoblastic

proliferation and induce cell death when osteoblast-like cells (MG63) are incubated for 72 h in

levels of nicotine similar to that of habitual cigarette smokers [59]. Furthermore, smokeless tobacco

extracts and nicotine in isolation have also been reported to suppress the growth of osteoblast-

like cells *in vitro* [80] and decrease collagen synthesis [81].

Inflammation

In addition to oxidative stress, cigarette smoke causes systemic inflammation. Serum

levels of pro-inflammatory and bone resorbing cytokines IL-1 and IL-6 were reported to be

elevated in 21 three-month old male Sprague-Dawley rats treated with 7mg/kg/ body weight of

nicotine. The study also reported decreased trabecular bone volume and thickness at metaphyseal

region of left femurs, and decreased mineral appositional rate and bone formation rate [82].

Population-based studies have consistently reported a low-grade systemic inflammatory state in

smokers [83, 84]. This inflammatory state in humans is characterized by increased circulatory

inflammatory mediators such as acute phase proteins and pro-inflammatory cytokines, C-reactive

protein (CRP), fibrinogen, and interleukin-6, as well as increased white blood cell count (WBC) [73]. Studies such as the large-scale NHANES III study using 7,685 subjects, confirmed systemic inflammation and revealed a strong independent dose-response relationship between cigarette smoking and elevated levels of CRP, fibrinogen, and blood leukocytes [85]. Another study that analyzed data on 4,187 smokers, 4,791 former smokers, and 8,375 never-smokers and adjusted for cofactors reported a dose-effect relationship between the number of cigarette smoked per day and plasma fibrinogen concentrations [86, 87]. Biomarkers such as urinary 8-iso-$PGF_{2\alpha}$, a major F_2-isoprostane, plasma GPx, and enzymatic SOD are reliable *in vivo* biomarkers of oxidative stress and inflammation [77]. A Swedish study involving 48 women and 53 men aged between 22-85 years reported that high urinary concentration of 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) were significantly correlated with low BMD of total body, lumbar spine, and heel [88].

TREATMENT OPTIONS FOR POSTMENOPAUSAL OSTEOPOROSIS

Osteoporosis medications fall into two categories: the antiresorptive medications that slow the breakdown of bone and the anabolic treatments that help build bone. These medications work with the body systems to protect bone mineral density and reduce the risk of fractures. Currently, teriparatide is the only Food and Drug Administration (FDA) approved anabolic medication available. Several FDA approved antiresorptive drugs are available and include bisphosphonates, calcitonin, estrogen therapy/hormone therapy, and selective estrogen receptor modulators (SERMs). These treatments have risks associated with their use, but all are geared towards elimination or reduction of fracture risk [1]. Selected approved therapies are discussed below.

Raloxifene

Raloxifene was approved for women only and is taken as an oral tablet daily for treatment of osteoporosis. It is a drug derived from benzothiophene and is a SERM that comes in a brand name Evista ®. SERMs represent a class of drugs that act as either estrogen receptor agonists or antagonists in a tissue-selective manner [89]. This pharmacologic profile of raloxifene enables it to act by demonstrating estrogen-like effects on the skeleton and cardiovascular system, and anti-estrogen effects on the breast and endometrium [90]. In study a large human study, 7,705 postmenopausal women aged 31–80 years with osteoporosis; raloxifene was successful in increasing lumbar spine and femoral neck BMD by 2–3%, while reducing the risk of vertebral fractures by 30%–50%. Additionally, the drug decreased the incidence of breast cancer [91]. Side effects associated with the use of raloxifene include: increased risk for blood clots in the leg veins and/or the lungs, increased hot flashes, leg cramps, and fluid retention [89].

Estrogen therapy

Estrogen therapy (ET) is administration of estrogen hormone alone as opposed to hormone therapy (HT) which refers to estrogen with progesterone hormone therapy. Estrogen used in many clinical trials may be conjugated equine estrogen, estradiol, estrone, esterified estrogen, ethinyl-estradiol, and mestranol. Estrogen replacement is used in the treatment of bone loss prevention of fractures risks [53] and increase in bone mass of the spine, hip, and total body[1]. Therapies that use estrogen either target estrogen receptors directly or increase the diminishing hormone level. As discussed earlier, estrogen favors bone formation by up-regulating osteoblast activity and suppressing osteoclast activity. Estrogen increases synthesis of growth factors in osteoblasts if administered in high doses and may increase mean wall thickness and other histomorphometric indices of osteoblast activity in humans [92]. In a Framingham cohort study that

evaluated effects of hypogonadism and estradiol levels on BMD in men and women, estradiol levels were strongly correlated with BMD at forearm, spine, and hip [93]. However, while estrogen can prevent osteoporosis in postmenopausal women, it also increases the risk of other health problems. Estrogen therapy use is associated with possible estrogen-dependent tumors such as breast cancer, stroke, ischemic attack, pulmonary embolus, deep vein thrombosis, or complex hyperplasia [94].

Parathyroid hormone (PTH)

Parathyroid hormone is approved for the treatment of osteoporosis in both women and men who are at particularly high risk for fractures, including subjects who are younger than age 65 and have low bone mineral density measurements (T scores ≤3.5) [95]. Teriparatide recombinant human PTH 1–34 (contains the first 34 amino acids of human PTH) [96] and is currently the only FDA approved in the first in a class of osteoporosis medications called anabolics. Teriparatide is available in a brand name Forteo® and taken as a daily injection. The action of PTH on bone requires the presence and contact with a combination of several specialized cells including, bone marrow stromal cells, and hematopoietic precursors of osteoclasts, mature osteoclasts and osteoblasts and works to form new bone [97]. PTH enhances the formation of trabecular bone when administered intermittently, but not chronically [98]. In fact certain dosages and continuous administration of PTH are associated with bone resorption. The results of large study in which, 1637 postmenopausal osteoporotic women were receiving PTH indicated for two years demonstrated that PTH reduced fracture risk by 65% and increased vertebral lumbar spine bone mineral density by 9%–13% [99]. Teriparatide therapy is not recommended for more than 2 years since longer use may induce osteosarcoma as observed in a

rat model of carcinogenicity [95] Side effects associated with PTH use include: headache, hypercalcemia, dizziness, leg cramps, marrow fibrosis, tunneling resorption, and nausea [100].

Bisphosphonates

Bisphosphonates are compounds derived from pyrophosphate. The drugs in this class are antiresorptive and act by slowing down the breakdown of bone by inhibiting hydroxyapatite formation [3]. Other mechanisms by which bisphosphonates slow bone loss include initiation of osteoclast apoptosis [101], suppression of protein tyrosine phosphatases [102] and disruption of the formation of cytoskeletal actin-ring in polarized resorbing osteoclasts [103].Osteoporosis drugs available in this class are alendronate (Fosamax®), ibandronate (boniva®), risedronate (Actonel®)and zoledronic acid (Reclast®). Despite proven efficacy, regular administration of bisphosphonates is known to cause gastrointestinal problems such as upset stomach, diarrhea, pain in arms and legs, dysphagia, esophageal and stomach ulcers [104].

Calcitonin

Calcitonin is a polypeptide hormone that has been used to treat osteoporosis and other diseases involving accelerated bone turnover for over 25 years [105]. In our bodies, calcitonin is produced in the C cells of the thyroid [27] and salmon calcitonin is clinically administered for treatment of osteoporosis [105]. This hormone acts on the osteoclasts by inhibiting the differentiation of osteoclast precursors [106] and suppressing the proliferation of progenitor cells [106]. Several controlled clinical studies have demonstrated that calcitonin is an antiresorptive hormone that preserves or increases bone mineral density (BMD) and reduce the risk of vertebral fractures. An added advantage of calcitonin is its direct analgesic effect. Calcitonin has been demonstrated to be clinically useful in improving pain, from the acute vertebral fractures of

osteoporosis and other sources of musculoskeletal pain unlike any other therapy [105]. Calcitonin is available in two brand names, Fortical® and Miacalcin®).

NUTRIENTS AND BONE METABOLISM

Protein

Protein is important in bone formation although there is a possibility that animal protein has deleterious bone resorbing effects when consumed in excess [107]. The main functions of protein are growth; tissue repair, and muscle function and a spare source of energy when carbohydrates are lacking in the diet [107]. The current Dietary Reference Intake (DRI) is 0.8mg/kg body weight [108]. Protein is involved in bone metabolism, in the formation of the organic bone matrix as collagen, in mineralization, and in calcium intake and metabolism [109]. Past studies report that diets high in protein increased urinary calcium loss, but it is unknown whether the calcium excreted is purely from bone or from increased calcium absorption [110]. There are mechanisms that explain the possible deleterious effects of foods rich in animal protein. Considering that the extracellular fluid pH is tightly controlled between 7.35 and 7.45 [111], hepatic oxidation of sulfur-containing amino acids (e.g. methionine and cysteine) to sulphuric acid and ammonium ions causes a reduction in blood pH [107]. Also, consumption of protein from meat, dairy, and legumes has potential to cause renal acid loads resulting in diet-induced, low grade metabolic acidosis [112]. Both processes induce bone loss due to the key role played by bone minerals in buffering the net acid load of the diet [107]. A diet low in protein however, is associated with reduced intestinal calcium absorption, which results in increased serum PTH, a situation that leads to bone resorption [110]. Dietary alkali load is achieved through consumption of vegetables and fruits since potassium, sodium; calcium and magnesium generate bicarbonates or citrate salts which lower extracellular pH [112, 113]. However, protein intake requires careful

balance due to its importance in other body processes including growth through regulation of insulin-like growth factor (IGF), a recognized mediator of anabolic processes [107]. Studies on effects protein on bone are inconsistent, although beneficial effects have been reported in high protein intake in the setting of adequate calcium intake [107].

Calcium

Calcium is an essential skeletal component that plays a critical role in many other body functions. Calcium is plays a key role in blood coagulation, enzyme activity, muscle contraction, membrane permeability, and hormones secretion [114]. A constant concentration of calcium is required in extracellular fluids to maintain health in humans. Blood calcium homeostasis is maintained through the endocrine system mainly by regulation of vitamin D, parathyroid hormone and calcitonin [114]. As mentioned earlier, calcium is stored in the bone and when dietary calcium is inadequate or its absorption impaired, the body withdraws calcium from bone, hence loss of bone mineral density. There is clear evidence that an increased level of blood calcium causes low levels of PTH and vice versa [114]. Moreover, high blood levels of phosphorous inhibit the formation of $1, 25 (OH)_2D$ resulting in low blood calcium and PTH stimulation [114]. Calcium supplementation is reported to be beneficial to postmenopausal women by increasing BMD, because calcium deficiency causes an increase in PTH levels and PTH action aggravates bone loss [115]. Calcium can be intravenously infused to bring PTH levels to normal [115].

Vitamin D

Vitamin D is a fat soluble prohormone that is obtained from sun exposure, food, and supplements in a biologically inert form. Vitamin D must undergo two hydroxylation reactions to be activated in the body. The two inert forms of vitamin D are ergocalciferol or vitamin D_2 and

cholecalcificerol or vitamin D_3 which must be converted to the active form, 1,25 (OH) $_2$D [5]. It is believed that the body stores of vitamin D are best reflected in the serum levels of their precursors D_2 and D_3 (25-OH-vitamin D). Several major mechanisms by which vitamin D affects bone have been identified. First, the active form of vitamin D works together with PTH and calcitonin to regulate calcium homeostasis. These hormones regulate calcium homeostasis by increasing and decreasing calcium absorption in the gut and modulating bone remodeling and growth [5]. Secondly, vitamin D reduces fracture risk by improving muscle function [115]. Furthermore, in humans, decreased levels of 25-OH-vitamin D, below 30ng/ml (80 nM) is associated with elevated levels of PTH [55] resulting in bone turnover and ultimately bone loss. Vitamin D has also been reported to stimulate production of OPG in human osteoblast lineage cells which in turn suppresses osteoclastogenesis [116].

Micronutrients

Micronutrients, magnesium (Mg), fluoride (F), and Vitamin K are considered vital in bone health. Fluoride is mainly found in calcified bone tissue and plays key role in dental health [117]. Magnesium deficiency is a recognized risk factor for osteoporosis [118] possibly because approximately two thirds of the mg found in the body is stored in the bone and is an essential cofactor for over 300 enzymes [118].

Vitamins are essential factors for metabolism, growth, reproduction, and immunity [119]. Vitamins, A, K, E, and C have shown bone protective effects in epidemiological studies [77]. Some vitamins are vital in the development and maintenance of bone health. Vitamin A for instance is important for bone formation, but excess intake may lead to toxicity associated with bone loss and a higher risk for hip fracture [120].

Recent work has focused on the role of vitamin K in bone health since its role in blood coagulation is fairly well understood [121]. Evidence suggests that vitamin K is essential for the post-translational conversion of glutamyl residues to carboxyglutamyl (Gla) residues in vitamin K-dependent proteins prothrombin and osteocalcin [121]. Furthermore, vitamin K is an important factor in the modulation of osteoblastogenesis and osteoclastogenesis in bone marrow cells via the RANKL pathway [32].

Vitamin E, contained in small amounts in most fruits and some vegetables, is a strong antioxidant that scavenges reactive oxygen species protecting cells from oxidative damage. Vitamin E supplementation is associated with maintaining BMD [122]. Epidemiological studies propose that low dietary intake of vitamins E and C is linked to increased hip fracture in smokers [123].

Vitamin C, also known as ascorbic acid, is found in most fruits and vegetables. Vitamin C is a key antioxidant vitamin recognized as an elemental component in the formation and maturation of stable collagen in connective tissue [77]. It is also required in hydroxylation of lysine and proline necessary for collagen formation. Overt vitamin C deficiency is characterized by non-organized and thinner growth plate, thinner trabecular network, very low collagen synthesis, and decreased differentiation of osteoblasts from mesenchymal cells[77]. Studies have shown that through vitamin C, TGFβ may act to stimulate osteoblast differentiation resulting in increased bone formation [124]. Low vitamin C intake in growing guinea pigs results in higher than normal bone turnover [125]. Several epidemiological studies have found an association between dietary vitamin C intake and BMD in postmenopausal women [126].

Current evidence suggests that antioxidants both dietary and endogenous and especially vitamin C plasma concentrations are reduced in elderly osteoporotic women [123]. In an

observational study of 533 non-smokers, randomly selected postmenopausal women, Pasco and others [123] studied the associations among the use of antioxidant supplements, vitamins C and E, serum levels of biochemical markers of bone turnover CTX-1 and BSAP, and whole body BMD. This study found that only 22 participants were users of vitamin C and E, and duration of antioxidant supplement use was negatively associated with age-adjusted and weight-adjusted serum CTX-1. The results further showed that mean CTX-1 levels were 0.022 units lower for each year of exposure and differences were not significant for adjusted serum BSAP or whole body BMD [123]. Dietary supplementation with antioxidant vitamin E or C suppresses bone resorption in nonsmoking postmenopausal women [123].

FRUITS AND HEALTH

It is widely accepted that a diet high in plant-based foods decreases the risk for many degenerative diseases [127]. Consumption of fruits and vegetables has been associated with reduced incidence of chronic inflammatory conditions including various cancers [128, 129], cardiovascular disease, [130,131], and osteoporosis [132].Current efforts in human health are focused on preventing diseases that may progress due to oxidative stress. Oxidative stress leads to cell and tissue damage and is the etiology of many chronic diseases including cardiovascular conditions and osteoporosis [133]. There is growing evidence that dietary antioxidants from fruits and vegetables reinforce the endogenous antioxidant defense systems present in the body and prevent chronic diseases [127].

Fruits are a source of bioactive components including, minerals, fiber, vitamins and phytochemicals [134]. Vitamins found in fruits include, C, A, E, K and B1, B2, B6, niacin, folate, and pantothenic acid [134]. Minerals contained in fruits are essential cofactors in formation of many enzymatic processes and metabolic processes hence adequate amounts prevent chronic

conditions [134]. Most fruits contain important bone forming minerals, potassium, calcium, phosphorus, magnesium and sodium [135]. Other important minerals found in varying amounts in fruits include: selenium, manganese, copper, and zinc [134]. Soluble and insoluble fibers found in fruits slow down digestion hence increasing absorption of nutrients. For example, Ohta and others noted increased absorption of magnesium and calcium administered together with dietary fructooligosaccharides [136]

Fruits also contain phenolic compounds with superoxide scavenging capacities [127]. Phenolic compounds function as antioxidants by easily donating hydrogen atoms from their aromatic hydroxyl groups and by supporting unpaired electrons [137]. Phenolic compounds classified as antioxidants must meet two basic conditions when present at low concentrations relative to the substrate: i) delay, retard, or prevent oxidation and ii) the radical formed must be stable [138].

Phenolic compounds account for 80% of total antioxidant capacity in most fruits and vegetables [139]. The antioxidant capacities i.e. oxygen radical absorbance capacity (ORAC), of certain phenolic compounds including flavonoids may be stronger than vitamin E and C based on molar concentrations [139]. Using eight healthy elderly women, Cao et al [119] investigated the effects of consuming either 240g of strawberries, 294g of spinach, 300mls of red wine, or 1250mg of vitamin C on serum antioxidant capacity. The study confirmed increased serum antioxidant capacity from all four foods [119].

EFFECTS OF PHENOLIC COMPOUNDS PRESENT IN BLUEBERRY AND BLACKBERRY ON HEALTH

The major groups of phenolic compounds present in berries are reported to be flavonoids, flavonols, flavanols, ellagitannins, gallotannins, proanthocyanidins, and phenolic acids [140].

Flavonoids include one of the largest and most important groups of the phenolic compounds, the anthocyanins, which are prevalent in berries reaching 10g/kg in some varieties. In the United States the most current estimation of anthocyanin intake is 12mg/day [141]. Blueberries contain mainly the flavonoids: anthocyanins, catechin, epicatechin, quercetin, kaempferol, myrecetin, and phenolic acids (e.g., gallic acid, p-hydroxybenzoic acid, chlorogenic, p-coumaric, caffeic, ferulic, and elagic acids) [142, 143]. Blackberries on the other hand contain primarily anthocyanins, flavonols, and hydrolysable tannins, chiefly ellagitannins [144]. Anthocyanins display biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities [145]. These reports are especially useful in the prevention of obesity-induced inflammation which is a major risk for cardiovascular diseases, type II diabetes, and certain types of cancer [145]. Anthocyanins are reported to improve vision and induce apoptosis of cancer cells [146]. Anthocyanins have also demonstrated positive effects on blood vessels and platelets that may reduce the risk of coronary heart disease [146]. For example, antioxidant activity of anthocyanins from blackberries, red raspberries, sweet cherries, blueberries and strawberries inhibited human LDL oxidation [144]. Animal studies show that a type of anthocyanins, cyanidin-3-glucosides improved hyperlipidemia and hyperglycemia in mice fed a high-fat diet [145].

Anthocyanins elicit anti-inflammatory properties by suppressing the production of pro-inflammatory mediators such as nitric oxide and prostaglandin E2 through nuclear factor- kappa B inactivation in lipopolysaccharide-stimulated macrophages [145]. Recently blueberry and cranberry anthocyanins and hydroxycinnamic acids demonstrated anti-inflammatory properties against H_2O_2 and TNF-α induced damage to human microvascular endothelial cells [147]. Blueberry anthocyanins also inhibited inflammation of neural cells [148] and mitigated the effects of age related neural behavior decrements [149]. Joseph and colleagues reported reversal of age

related dysfunction using blueberry extract. Eight weeks on 1.86% blueberry diet, forty 19-month-old adult male rats had improved motor performance on two motor tests that rely on balance and coordination [149].

Procyanidins have potential health benefits and are reported to reduce the production of a vasoconstrictive peptide endothelin-1 produced by endothelial cells and linked with chronic inflammation of the arterial wall [150]. Moreover, procyanidins known as A-type derived from cranberries are known to be protective against urinary tract bacterial infections [151], cancer, and vascular diseases [152]. Bioactive berry phenolics are exploited in today's market as food supplements.

BLACKBERRIES AND BLUEBERRIES AND BONE HEALTH

Animal studies have demonstrated that phenolic compounds from berries scavenge ROS formed by phagocytes (i.e. monocytes, macrophages and neutrophils) [38] resulting in bone resorption [153]. Polyphenols such as chlorogenic acid, caffeic acid, and rutin in dried plum have prevented bone loss in animal models by reducing oxidative stress biomarkers [154]. Resveratrol the major phenolic compound in grapes has potential bone protective properties as demonstrated by its ability to increase intracellular bone formation markers in experimental studies [155]. Dried plum and blueberries have some structurally similar phenolic and flavonoids compounds [156]. Recently blueberries have shown bone protective effects in ovariectomized (OVX) rat model of postmenopausal osteoporosis. Feeding 5% blueberries (w/w) down regulated mRNA levels of three bone matrix proteins: alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and collagen type 1 (COL) and significantly improved total bone mineral density [157]. Similarly, supplementation with blackberries at the level of 5% (w/w) protected bone mineral density at fourth lumbar vertebra, right tibia, and left femur compared to controls in an

ovariectomized rat model of postmenopausal osteoporosis [158] Although whole berries were administered in the blueberry and black berry animal studies, phenolic compounds are thought to be the major bioactive compounds responsible for bone health effects. The mechanism is still unclear although it is reasonable to speculate that the compounds work by lowering oxidative stress.

METABOLISM AND BIOAVAILABILITY OF ANTHOCYANINS

Although studies have revealed the human health benefits of anthocyanins, low bioavailability has been suggested by the low concentration observed in plasma and urine after ingestion of anthocyanins [159]. Anthocyanins have been detected in human plasma in glycosylated form by HPLC analysis [160]. After 1.5g of anthocyanin intake given as an elderberry extract, Cao and Prior [161] reported a maximal plasma anthocyanin concentration of 100µg/L after 30mins in a human study. The anthocyanins were detected in their glycosidic forms [161].

Jian and colleagues [159] evaluated the impact of chemical structure of anthocyanins on absorption and metabolism. Anthocyanin-rich extracts fed to rats for 3 months demonstrated that anthocyanin diglucosides are better absorbed than their corresponding monoglucosides [159]. The study also showed that intact acylated anthocyanins are absorbed into plasma and excreted through urine [159].

Matsumoto and others [162] fed a single dose of 3.57mg cyanidin-3-glycoside per kg body weight to subjects and detected within 2 hours in plasma (0.120 nM) and urine samples (0.11% of total ingested) [162]. The anthocyanins in this study were from blackcurrant concentrate. Investigating the bioavailability of anthocyanins from red wine, Lapidot and others traced anthocyanins in human urine after consumption of 300mL of red wine containing approximately 218mg of anthocyanins. An estimated 1.5 - 5.1% of anthocyanins were recovered in urine 12

hours after consumption and highest anthocyanin levels were reached within 6 hours after consumption [163]. Consumption of 1.2g anthocyanins from freeze-dried wild blueberry powder led to identification of 11 different anthocyanins in serum [160]. Maximal serum concentration reached 4 hours later revealed the most abundant compound, cyaniding-3-glucoside ranged from 5.43-16.9ng/mL. Others identified include malvidin 3-galactoside, malvin-3-glucoside and delphinidin-3-glucoside accounting for 27, 20 and 10 % of the total respectively [160].

A study by Mazza et al., compared serum antioxidant status of subjects after consumption of blueberry powder plus a high fat meal to serum antioxidant status after a high fat meal without blueberry powder [160]. Subjects consumed a McDonald's meal containing 853 calories, (46.7g of fat-15.5g saturated fat) for the study [160]. Blueberry consumption was associated with a significant increase in serum lipid-soluble antioxidant status and prevented a decrease in serum antioxidant capacity postprandial [160]. Unlike other flavonols, such as quercetin glycosides, the bioavailability of anthocyanins has been found to be consistently low across all studies. The amount appearing in urine is often 0.1% of ingested dose [141]. Low bioavailability of anthocyanins from red grape juice, elderberry extract, boysenberry concentrate, and blackcurrant has been confirmed [141]. The highest anthocyanin recovery reported to date is a urinal excretion of 1.80% after consumption of 200g of strawberry yielding 179 μM of pelargonidin-3-glucoside [164].

Therefore, current evidence suggests that anthocyanins are absorbed rapidly and reach maximal plasma concentration 15-60 minutes after consumption, and they are excreted 6-8 hours later [141]. The quick appearance in the circulatory system has led to the interpretation that anthocyanins are absorbed in the stomach [141]. Although gastric absorption of anthocyanins is proven [165], evidence also suggests that absorption of cyanidin-3-glucoside may occur in the

jejuna and duodenal tissue [166]. There is also evidence that intact anthocyanin glycosides appear to be absorbed, distributed, and then excreted in urine [141].

Anthocyanins may accumulate in tissue, and this may explain the apparent contradiction of observed benefits and low plasma availability of anthocyanins [159]. Gut microflora also play a role involved in anthocyanin bioabsorption. For example, anthocyanin glycosides are degraded by colonic bacteria into aglycones and smaller phenolic compounds [167]. Aura et al used cyanidin-3-glucoside and rutinoside as inoculums in human fecal microbiota and reported that through deconjugating enzymes, rutinoside is metabolized into cyanidin-3- glycoside. Additionally, gut microflora were found to deglycosylate anthocyanins and produce protocatechuic acid as the major metabolite [167]. At present, it is unclear how anthocyanins and their metabolites enter circulation, are distributed to tissues in vivo and exert beneficial health effects [141]. However, cell culture studies have demonstrated that anthocyanins can penetrate mammalian cell walls. These experiments show their potential bioactivity such as protection against oxidative stress, induction of apoptosis, and changes in inflammatory response *in vitro* [141, 168].

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III. THE BLACKBERRY FRUIT: A REVIEW ON ITS COMPOSITION AND CHEMISTRY, METABOLISM AND BIOAVAILABILITY, AND HEALTH BENEFITS

ABSTRACT

Blackberry (*Rubus sp.*) fruit contains high levels of anthocyanins and other phenolic compounds, mainly flavonols and ellagitannins, which contribute to its high antioxidant capacity and other biological activities. Blackberry phenolic composition and concentrations are known to be influenced by genetics, growing conditions, and maturation. Despite our knowledge of their chemistry, research specific to blackberry phenolic compounds' health benefits, metabolism, bioavailability, and mechanism by which they confer health benefits is scarce. Blackberry phenolic compounds have protective effects on age-related neurodegenerative diseases, and bone loss *in vivo*, and can inhibit LDL and liposomal oxidation *in vitro*. Blackberry extracts have also exerted anti-mutagenic effects *in vitro* and *in vivo* by modifying cell signaling pathways and suppressing tumor promotion factors. However, the anti-obesity, anti-diabetic, antimicrobial, and anti-inflammatory properties of blackberry phenolic compounds need investigation. Similarly, studies that elucidate the *in vivo* physiologically effective concentrations of blackberry phenolic compounds are necessary.

KEYWORDS: blackberry, anthocyanins, bioavailability, flavonoids, health benefits.

INTRODUCTION

Worldwide commercial production of blackberry (*Rubus sp.*) is estimated to be approximately 154,578 tons annually *(1)*. North America, Europe, Asia, South America, Oceania Central America and Africa [in descending order of tons cultivated] are the main regions for blackberry production *(2)*. Wild blackberries are also cultivated in considerable amounts, and in some regions may negatively affect the net sales of commercially cultivated fruit *(2)*.

The "berry" weighs from 3-12 g depending on variety, and is best described as an aggregate fruit made up of several drupelets, each containing a seed *(1)*. Blackberries are mostly consumed fresh but can be processed and sold as individually quick frozen packs, bulk frozen, seedless or seed puree, freeze-dried, or as juice or concentrate *(1)*. In industry, blackberries are used for the production of dietary supplements, ice-cream, jam, marmalade and other confectionaries *(3)*.

Blackberry is a fruit of interest because of its high content of anthocyanins and ellagitannins (ETs) as well as other phenolic compounds that contribute to its high antioxidant capacity *(4)*. Several studies document the high antioxidant activity of blackberries based on their oxygen radical absorbance capacity (ORAC) compared to other fruits *(4)*. In fact, blackberries' medicinal qualities have been known since the 16th century in Europe where they were used to treat infections of the mouth and eyes *(5)*.

Epidemiological and clinical studies *(6-10)* suggest that consumption of anthocyanins and other flavonoids found in most fruits and vegetables may decrease the risk of obesity, coronary heart disease, degenerative conditions, and various types of cancer. These health benefits and mechanisms by which anthocyanins confer them have been explored *in vitro (7, 11, 12)* and in

animal models *(13-16)*. This review presents current knowledge on the chemistry, composition, metabolism and bioavailability, and health benefits of blackberries.

NUTRIENT COMPOSITION

Blackberry chemical composition varies based on variety, growing conditions, stage of ripeness, and harvest and storage conditions *(17)*. In addition to valuable polyphenolic compounds, blackberries contain carbohydrates, and several essential vitamins and minerals (Table 1) *(18)*.

The principle sugars in blackberries are glucose, fructose and sucrose and their ratios differ among cultivars *(19)*. The principle sugars are glucose and fructose in the ratio of approximately 3.24 - 2.88g/100g FW and 0.81-1.17 g/100 g FW glucose to fructose. Sucrose content is estimated to be 0.24 g/100 g FW *(19)*. Levels of glucose, fructose, total sugars and total soluble solids increase markedly as the fruit ripens from light red to dark bluish purple stages *(20)*.

Blackberries contain malic acid as the primary organic acid although different acids including ascorbic acid have been detected in the fruit *(3)*. Fan-Chiang *(19)* reported the following averages of nonvolatile organic acids from 52 samples of blackberry: 280 mg/100g FW malic acid; 293 mg/100g FW lactoisocitric acid; 599 mg/100g FW isocitric acid; and 572 mg/100g FW citric acid. Furthermore, trace amounts of shikimic, fumaric and succinic acids were identified *(19)*. These organic acids in blackberries are important for stabilizing anthocyanins and ascorbic acid, and extending the shelf-life of fresh and processed berries *(17)*. The balance in proportion of organic and phenolic acids in berries is evaluated for overall index of fruit quality, whereas low pH is considered an indicator of poor quality *(17)*. Another important quality index for berries is the ratio of total soluble solids to total titratable acidity. This ratio

Table 3.1. Chemical composition of blackberries From the U.S. Department of

Agriculture Nutrient Database *(18)*

Proximates and carbohydrates		vitamin content		mineral content	
		total ascorbic acid			
water (g)	88.20	(mg)	21.00	calcium (mg)	29.00
energy (Kcal)	43.00	thiamin (mg)	0.02	iron (mg)	0.62
protein (g)	1.39	riboflavin (mg)	0.03	magnesium (mg)	20.00
total lipids (g)	0.49	niacin (mg)	0.65	phosphorus (mg)	22.00
ash (g)	0.37	pantothenic acid (mg)	0.28	potassium (mg)	162.00
carbohydrate (g)	9.61	vitamin B6 (mg)	0.03	sodium (mg)	1.00
total fiber (g)	5.30	total folate (µg)	25.00	zinc (mg)	0.53
total sugars (g)	4.88	vitamin B12 (µg)	ND	copper (mg)	0.17
sucrose (g)	0.07	vitamin A (IU)	214.00	manganese (mg)	0.65
glucose (g)	2.31	α-tocopherol (mg)	1.17	selenium (mg)	0.40
fructose (g)	2.40	β- tocopherol (mg)	0.04		
maltose (g)	0.07	γ-tocopherol (mg)	1.34		
galactose (g)	0.03	Δ-tocopherol (mg)	0.90		
starch (g)	ND	vitamin K (µg)	19.80		

ND, not detected

Table 3.2. Total Phenolics and Total Anthocyanin Values of Blackberries across Studies

total phenolics (mg GAE/100)	total anthocyanins (mg/100 g FW)	number of cultivars	source
NA	114-242 [b]	6	*(4)*
292-446	NA	6	*(23)*
248-633	NA	12	*(27)*
NA	70-201 [b]	51	*(28)*
NA	58-219 [a]	12	*(29)*
822-844	154-225 [b]	2	*(30)*
NA	80-230 [b]	27	*(31)*
418-555	111-123 [b]	2	*(32)*
NA	133-172 [b]	3	*(33)*
193-352	67-127 [b]	7	*(34)*
495-583	95-155 [b]	2	*(35)*
221 -342	38-72 [b]	5	*(36)*
114-178	31-118 [b]	4	*(37)*
NA	83-326 [b]	16	*(38)*
682-1056	131-256 [b]	11	(39)

[a]Calculated as cyanidin 3-galactoside equivalents; [b]Calculated as cyanidin 3-glucoside equivalents FW: fresh weight; NA not available

increases in blackberries as the fruit ripens from purple to dark bluish purple stages, reflecting the large increase in sugars and decline in organic acids *(20)*.

Blackberries contain cell wall hydrolase and oxidase enzymes, such as polyphenol oxidase and peroxidase, which contribute to deterioration of quality in fresh, damaged, or pureed fruit *(17)*. Enzyme activity results in loss of texture, brown pigment formation, and destruction of phytochemicals that affects acceptability of particularly fresh fruit *(17)*. Refrigeration, reduced oxygen, addition of enzyme inhibitors, pH modification and addition of reducing agents to control secondary oxidation are some of the techniques currently used to control enzyme activity *(17)*.

PHENOLIC COMPOSITION

Blackberries are a rich source of polyphenolics including, anthocyanins *(21)*, ETs *(22)*, flavonols *(23)*, flavan-3-ols, and procyanidins *(24)*. The berries also contain appreciable levels of phenolic acids *(25)* and low levels of lignans *(26)*. Total phenolics in blackberries have been shown to range from 114 to 1056 mg/100 g fresh weight (FW) (Table 2).Besides genetics, fruit maturity can influence levels of total phenolics in blackberries. For example, analysis of three blackberry cultivars namely 'Chester Thornless', 'Hull Thornless', and 'Triple Crown' berries demonstrated that total phenolic content significantly decreases as the fruit matures from the green to ripe stages *(33)*. However, in another study involving tropical highland blackberries levels of total phenolics fluctuated as the fruit ripened from light red (580 mg/100g FW) to purple (460 mg/100g FW) to dark bluish purple (520 mg/100g FW) stages *(20)*.

Anthocyanins

Anthocyanins are the phenolic compounds responsible for the red to purple to black pigments of fruits and vegetables *(28)* and are recognized for their potential health benefits *(7)*. Anthocyanins are flavonoids by classification and are predominantly found in berries and red grapes *(4)*. They are anthocyandinins glycosylated with one or more sugar moieties at the C3 position of the flavan structure *(25)*. Approximately 94% of blackberry anthocyanins occur in nonacylated form and 90% of these exist as monoglycosides while 10% are found as diglycosides *(21)* (Table 3).

Blackberry anthocyanins are mainly cyanidin derivatives with glucose, rutinose, xylose and arabinose moieties attached at C3 *(21, 25)* (Figure 1). Blackberry anthocyanins identified include: cyanidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-xyloside, cyanidin 3-dioxalyl-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside and cyanidin 3-glucosylrutinoside cyanidin 3-arabinoside, malvidin 3-arabinoside, perlargonidin 3-glucoside, cyanidin 3-(3-malonyl) glucoside and cyanidin 3-(6-malonyl) glucoside *(40, 41)*. Cyanidin 3-dioxaloyl-glucoside, a zwitterionic anthocyanin is unique to blackberries *(42)*. Studies show that anthocyanin content of blackberry varies due to differences in variety, environmental conditions, and cultivation site, degree of ripeness and processing *(43)*. Cho et al. reported total anthocyanin content of 6 blackberry genotypes to range from 114.4 mg/100g fresh weight (FW) to 241.5 mg/100g FW *(4)*. In a similar study, Fan-Chiang and Wrolstad found monomeric anthocyanin content in 51 cultivars ranged from 70 to 201 mg/100 g FW and, the cyanidin derivatives dominated in all samples with a mean peak area of 83% *(28)*.

Table 3.3 A Summary of Anthocyanins, Flavonols, and ETs Identified in Blackberries

anthocyanins	flavonols	ellagitannins
cyanidin 3-galactoside	quercetin 3-galactoside	sanguiin H-6
cyanidin 3-glucoside	quercetin 3-glucoside	lambertianin C
cyanidin 3-arabinoside,	quercetin 3-rutinoside	pedunculagin
cyanidin 3-xyloside	quercetin 3-xylosylglucuronide	lambertianin D
alvidin 3-arabinoside	quercetin 3-glucosylpentoside	galloyl-bis-HHDP glucose
perlargonidin 3-glucoside	kaempferol 3-glucuronide	sanguiin H-10 isomer (2)
cyanidin 3-rutinoside	kaempferol 3-glucoside	sanguiin H-6 minus gallic acid moiety
cyanidin 3-sophoroside	kaempferol 3-galactoside	lambertianin C minus ellagic acid moiety
cyanidin 3-glucosylrutinoside	kaempferol 3-xylosylglucuronide	galloyl-HHDP glucose
cyanidin 3-(3-malonyl) glucoside	quercetin 3-glucuronide	sanguiin H-2
cyanidin 3-(6-malonyl) glucoside	quercetin 3-O-[6-(3-hydroxy-3-methyl-myricetin	sanguiin H-6 plus gallic acid moiety
		castalagin/vescalagin
	quercetin 3-methoxyhexoside	ellagic acid
	quercetin 3-oxalylpentoside	methyl ellagic acid pentose conjugate (2)

Compiled from the following references *(25, 32, 52)*.
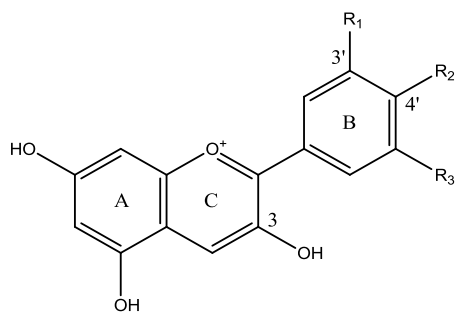
Anthocyanidin	R_1	R_2	R_3
Cyanidin	OH	OH	H
Pelargonidin	H	OH	H
Malvidin	OCH_3	OH	OCH_3

Figure 3.1. Chemical structures of blackberry anthocyanidins
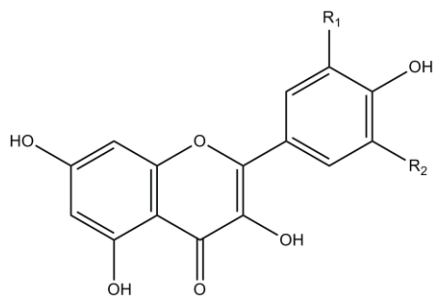
Flavonol	R₁	R₂
Quercetin	OH:	H
Kaempferol	H	H
Myricetin	OH:	OH

Figure 3.2. Chemical structures of blackberry flavonol

Moreover, cyanidin derivatives may vary in blackberries due to genetic differences *(28)*. This concept was demonstrated by Fan-Chiang et al. *(28)* using 51 samples of blackberries as follows: cyanidin 3-glucoside 44% to 95%; cyanidin 3-rutinoside trace amounts to 53%; cyanidin 3-xyloside, undetectable amounts to 11%; cyanidin 3-(malonyl) glucoside, trace amounts to 5%, and for cyanidin 3-dioxaloylglucoside, undetectable amounts to 15% *(25, 28)*.

Similarly, six blackberry genotypes analyzed by Cho et al. *(4)* demonstrated that distribution of cyanidin aglycones in each variety ranged from 75% to 84% for cyanidin 3-glucoside, 1% to 12% for cyanidin 3-rutinoside, 4% to 8% for cyanidin 3-dioxaloyglucoside, 3% to 8% for cyanidin 3-dioloylglucoside and 2% to 3% for cyanidin 3-(malonyl) glucoside *(4, 25)*. Reyes-Carmona et al. *(44)* observed similar differences when comparing three cultivars: Marion, Evergreen and Comanche berries. Unlike Marion and Evergreen, Comache contained higher amounts of cyanidin 3-rutinoside than cyanidin 3-glucosides; however, Comache's content of cyanidin 3-glucoside was still higher than Evergreen and Marion berries. Furthermore, malvidin 3-glucoside was identified in Marion and Evergreen, but not in Comanche berries *(44)*. Anthocyanins are synthesized during blackberry ripening resulting in the development of a dark bluish purple color. Consistent with visual color change total anthocyanins increase markedly as the fruit ripens from pink/light red to dark bluish purple stages *(20, 33)*.

Flavonols

The structure of flavonols entails a double bond at C2 and C3, a hydroxyl group at C3 and a ketone group at C4 of the C ring of the flavan nucleus *(25)* (Figure 2). Quercetin, myricetin and kaempferol are the most commonly identified flavonols in berries; their main difference is the number and position of OH groups at C3 and C5 *(45)*. The sugars commonly attached at the

C3 position are glucose and galactose, but arabinose, rhamnose, rutinose and xylose may also be found *(45)*.

Blackberries contain appreciable amounts of flavonols found exclusively in the fleshy part of the drupelet *(30)* and occur mostly in glycosylated form *(23)*. Reported values for total flavonols calculated as rutin equivalents are as follows: 12 to18 mg/100 g FW, 4 to 9 mg/100 FW, not detectable to 10 mg/100 g FW, 10 to 16 mg/100 FW, 10 to 16 mg/100 FW and 11-30 mg/100 FW *(4, 23, 30, 32, 35, 46)*.

Blackberries have a complex flavonol profile due to the occurrence of nine quercetin and three kaempferol derivatives as well as two acylated quercetin-derived compounds (Table 3). In addition, quercetin 3-xylosylglucuronide, a different flavonol is reported in some blackberry varieties *(43)*. According to Cho et al. *(23)* quercetin 3-galactoside and quercetin 3-glucoside are the main flavonols in five blackberry genotypes, although a few varieties contain high levels of the acylated form of quercetin 3-[6-(3-hydroxy-3-methylglutaroyl)]-β-galactoside *(23)*. The occurrence of kaempferol and myricetin is not consistent for all blackberry genotypes probably due to genetic differences *(4, 39, 47)*. Bilk and Sapers *(48)*, reported that ripe fruits of thorn blackberry (*Rubus* spp.) contain 0.5 to 3.5 mg/100g FW and 0.1 to 0.3 mg/100g FW of quercetin and kaempferol respectively *(48)*, while Sellappan and colleagues *(32)* reported 9.9 mg/100g of myricetin in Georgia-grown blackberries. Levels of total flavonols in tropical highland blackberries are reported to decline as the fruit ripens from light red (30 mg/100g FW) to dark bluish purple (14 mg/100g FW) stages *(20)*.

Phenolic acids

Phenolic acids in blackberry fruit range from 7 to 64 mg/100 g FW and are mainly hydroxybenzoic acid and hydroxycinnamic acids. These acids occur in conjugated forms of

esters and glycosides, but rarely as free acids *(25)*. Esters make up 53.1% of total phenolic acids while glycosides and free acids account for 43.6% and 3.3% respectively *(49)*. Blackberry hydroxybenzoic acids include: *p*-hydrobenzoic, protocatechuic, gallic, vanillic, salicylic and gentisic acid. Glycosidic and ester forms of salicylic acid are the most common *(49)*.

Caffeic, *m*-coumaric, *p*-coumaric and ferulic acid are hydoxycinnamic acids found in blackberries in free, ester and glycosidic forms. The ester forms of *m*-coumaric, 3,4-dimethoxycinnamic and hydroxycaffeic acids are predominant *(49)*. Blackberry hydroxybenzoic and hydroxycinnamic acid derivatives have been classified as follows: chlorogenic acid, neochlorogenic acid, glucose esters of caffeic, *p*-coumaric, ferulic, and gallic acids, as well as protocatechuic acids and the β-*D*-glucosides of *p*-coumaric and *p*-hydroxybenzoic acids *(50)*

Ellagic acid (EA) is a hydroxybenzoic acid, but most of EA found in blackberries is in the form of ETs which is a different class of phenolics. Blackberry EA calculated as "ellagic acid equivalents" following acid hydrolysis has been reported in several studies to range from: 2 to 4 mg/100 g FW *(30)*, 2 to 34 mg/100 g FW *(49)*, 12 to 18 mg/100 g FW *(46)*, and 21 to 25 mg/100 g FW *(37)*, 30 to 34 mg/100 g FW *(32)*, and 59 to 90 mg/100 g FW *(35)*. Ellagic acid derivatives are reported to decline as blackberries ripen from light red (30 mg/100g FW) to dark bluish purple (20 mg/100g FW) stages *(20)*.

Tannins

Tannins are a group of polyphenolic compounds found in many berry fruits and include oligomeric and polymeric constituents *(41)*. Tannins are known to be anti-nutritional because the tannins bind to –NH groups of peptides, precipitate them and prevent their hydrolysis in the stomach *(41)*. For example flavan-3-ols (catechins) and flavan-3, 4-diols (leucoanthocyanins)

interact with proteins, starch and digestive enzymes, to form less-digestible complexes resulting in reduced absorption of these nutrients *(51)*.

Tannins are classified into two groups based on their structure: condensed tannins .i.e. proanthocyanidins and hydrolyzable tannins i.e. ellagitannins (ETs) and gallotannins *(41)*. Proanthocyanidins are oligomers and polymers of flavonoids and in particular flavan-3-ols whereas the hydrolyzable tannins are glycosylated gallic acids *(41)*. Berry fruits contain two major types of proanthocyanidins: procyanidins and propelargonidins. The procyanidins and propelargonidins are composed exclusively of (epi) catechin and (epi) afzelechin units respectively *(25)*. Based on the hydroxylation pattern of A- and B-rings, proanthocyadinins (MW 2000-4000Da or more) are categorized into three groups: procyanidins, prodelphinidins and propelargonidins *(41)*. There are about 50 procyanidins identified from plants *(41)*. Procyanidins in blackberries have been found to be predominantly epicatechin units linked through C4→C8 bond, the so called "B-type" linkage, with an average degree of polymerization of 3.2 *(24)*. Blackberries contain monomers (7.3 mg/100g FW), dimers (6.7 mg/100g FW), trimers (3.6 mg/100g FW), tetramers to hexamers (7.3 mg/100g FW), heptamers to pentamers (4.2 mg/100g FW), and low levels of polymers (1.5 mg/100g FW) resulting in a total procyanidin content of 27.0 mg/100g FW *(24)*. Hydrolysable tannins, specifically ETs of varying molecular weight are found in blackberries in appreciable amounts *(25)*. Ellagitannin and EA derivatives are identified using high performance liquid chromatography mass spectrometry (HPLC-MS), and results are commonly reported as "ellagic acid equivalents" due to the diversity of ETs in berries, and lack of ellagitannin standards *(25)*.

Blackberry ET structures are complex, large, and diverse, which presents a challenge in their characterization *(52)* (Table 3). The basic structure of ETs consists of a glucose core

esterified with hexahydroxydiphenic acid (HHDP) (Figure 3). When hydrolyzed by either an acid or a base, HHDP is changed to the dilactone form, EA *(25)*. An ET bis-HHDP glucopyranose is also known as pedunculagin, and the galloylated form, or galloylbis-HHDP glucopyranose is also known as casuarictin or potentillin *(52)*. Moreover these compounds can be found as α-or β-glucopyranosides *(53)* with the HHDP units R or S configurations relative to C4 and C1 of the glucose *(54)*. ETs found in blackberry fruit and leaves are mostly polymerized forms of the galloyl-bis-HHDP *(52)*.

Blackberry seeds contain the highest amount of ETs and EA compared to other parts of the fruit i.e. the torus, and flesh *(30)*. In fact, 88% of the ETs and EA is found in the seeds compared to only 12% in the pulp. EA derivatives in blackberries range from 1.2 to 3.0 mg/100 g whereas ETs range from 51.1 to 68.2 mg/100 mg *(46)*. The seeds of Marion and Evergreen blackberry cultivars have higher ellagitannin values than most cultivars containing up to 3230 and 2120 mg/100 g seed of total ellagic acid, respectively *(22)*.

Recently, blackberries were investigated by high-performance liquid chromatography-electrospray ionization-mass spectrometry and matrix-assisted laser desorption/ionization-time of-flight mass spectrometry (MALDI-TOF-MS) to identify the ETs *(52)*. The following eleven ETs were identified mainly in the seed and torus of the Apache cultivar of blackberries: isomeric forms of pedunculagin, castalagin/vescalagin, galloyl-HHDP glucose, lambertianin C, and lambertianin D, and galloyl-bis-HHDP glucose, and sanguiin H-6/lambertianin A, EA *(52)*. This study also proposed that blackberry fruit may contain sanguiin H-10 *(52)*. In another recent study, ETs and EA conjugates in six blackberry cultivars were identified and quantified using ultra high pressure liquid chromatography-quadrapole-time of flight mass spectrometry (UPLC-Q-TOF-MS) and HPLC-DAD analysis *(55)*. In this study the authors isolated and purified the

two major ETs found in blackberries, Lambertianin C and Sanguiin H-6 and used them as calibration standards. Besides Lambertianin C and Sanguiin H-6, which account for 67% of the total ETs, they identified twelve additional ETs, as well as EA and two EA conjugates. The levels of total ETs in the six cultivars ranged from 84.7 to 130.4 mg/100g FW, while total EA conjugates ranged from 12.8 to 27.4 mg/100g FW. The ETs, potentillin, pedunculagin and casuaricitin have also been identified in leaves and shoots of blackberries *(56)*. Notably, ET and ellagic content of blackberries is affected by maturation. For instance, in the tropical highland blackberry cultivar, *Rubus adenotrichus* known to have the highest amount of ETs in an edible fruit when light red (380mg ellagic acid equiv/100g
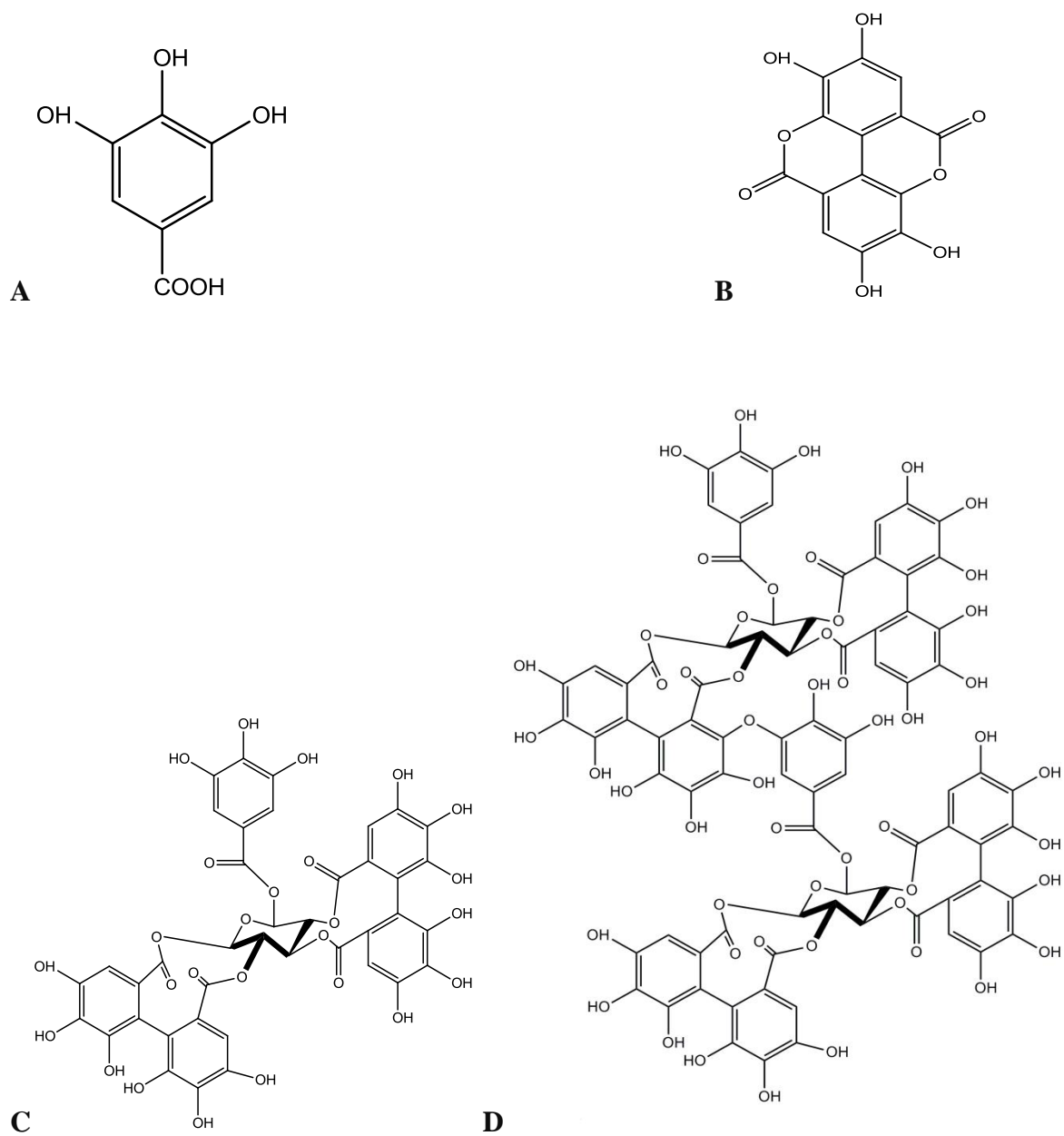
Figure 3.3. Structures of gallic acid (A), Ellagic acid (B) and galloyl-bis-HHDP glucose (C), Sanguiin H-6/lambertianin A (D) Structures adapted from ref 52.
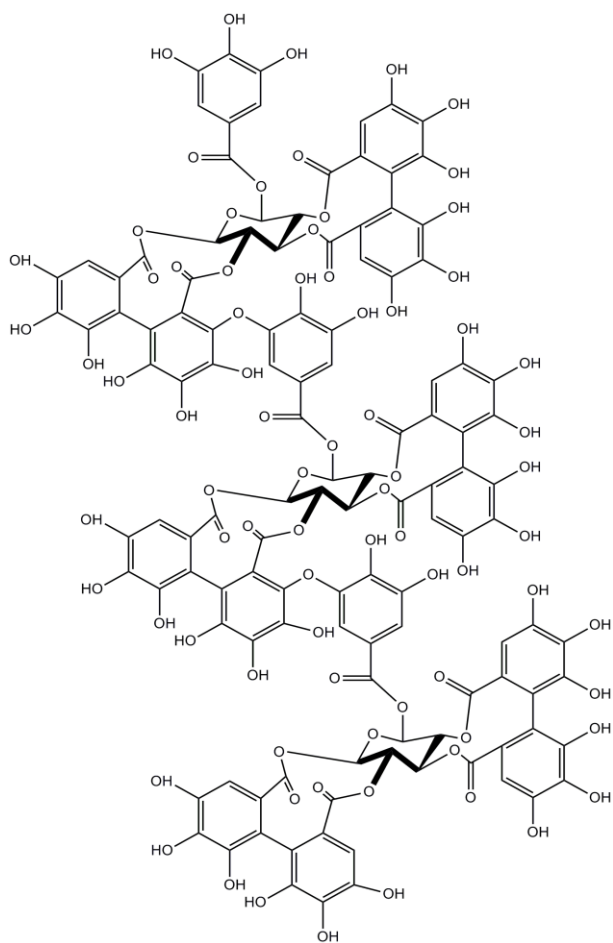
E

Figure 3.3. continued Structure of lambertianin C (E), structure adapted from ref 52

of fresh fruits), the two major ETs Lambertianin C and Sanguiin H-6 decline 33% from light red to dark bluish purple stages *(20)*.

OTHER PHYTOCHEMICALS

Lignans

Lignans are biphenolic compounds recognized as phytoestrogens and play an important role in prevention of hormone-associated cancers such as breast cancer *(57-59)*, and other conditions including cardiovascular disease *(60, 61)* and osteoporosis *(62)*. Blackberries are unique as they contain both secoisolariciresinol (3.72 mg/100 DW) and matairesinol (<0.01 mg/100 g DW) *(26)*. These lignans are known to be converted to mammalian lignans enterolactone and enterodiol by gut microflora *(63)*.

METABOLISM AND BIOAVAILABILITY

Although phytochemicals have been studied extensively, there is insufficient information regarding changes in content and character of polyphenolic compounds once consumed *(64)*. In general, bioabsorption of anthocyanins, which are the most studied and abundant flavonoids, occurs very quickly after consumption appearing in plasma 15-60 min postprandial and excretion is complete within 6-8 h *(65-73)*. Hollman et al. *(74)* noted that glycosylation of flavonols and their derivatives influence their metabolism, absorption and bioavailability *in vivo*. Following absorption, anthocyanins are metabolized differently based on their aglycones via methylation, glucuronidation, and sulfoconjugation *(69, 70, 75-77)*. This pathway uses conjugations with glucuronic acid or with sulfate and is considered important in detoxication pathway of many drugs and xenobiotic substances *(67)*. These processes are catalyzed by UDP-glucuronosyl transferase and sulfotransferase respectively, in the small intestine, liver and or kidney *(67)*. Some animal *(78, 79)* and human studies *(73, 80)* are consistent with these pathways since

glucuronide forms of different subclasses of flavonoids have been found in plasma or urine *(81, 82)*.

Anthocyanins are absorbed in intact glycoside forms from the digestive tract into the blood circulation system in mammals *(83)*. Although some argue that anthocyanins are too hydrophilic to be absorbed into cells of animals and humans *(84)*, there are studies *(85, 86)* that support this pathway for small molecular compounds such as cyanidin-3-glucoside. Other polyphenolic compounds such as free simple phenolic acids, aglycones, can also be metabolized in the gut and absorbed by enterocytes *(87-89)*. Anthocyanins have also been reported to be substrates of bilitranslocase, which is a plasma membrane carrier involved in organic anion uptake and mainly expressed at the sinusoidal domain of the liver plasma membrane and in epithelial cells of the gastric mucosa *(86)*. Using *in situ* exposure to tissue, investigators have found that absorption occurs in the stomach, jejunum and duodenal tissue possibly through interaction with bilitranslocase *(65, 85, 90)*.

Anthocyanins are also thought to be metabolized via intestinal microflora through cleavage of the C-ring to produce a range of phenolic acids which are easily absorbed *(75)*. Previously, scientists thought that the absorption of glycosides was not possible in mammals due to the lack of suitable β-glycosidases *(91)*, but light has been shed on the role of glycosidases from gut microflora in the ileum that aid in metabolism and absorption of glycosides such as quercetin glycosides *(88, 92)*. However, this mechanism is unclear since the gut pH affects the stability of individual flavonoids and especially anthocyanins, which actually results in increased recovery of intact compounds in feces *(68, 79, 93)*. Complex anthocyanins containing di- or triglycosides are reported to remain in the gut longer than simple anthocyanins (e.g.monoglycosides); and consequently plasma total antioxidant capacity and total anthocyanins

remain high longer after feeding *(68)*. It has been suggested that it is by this mechanism that anthocyanins provide significant antioxidant protection in the environment of the gut epithelium *(68)*.

Studies on the metabolism of blackberry cyanidin glycosides in particular are limited. In general, cyanidin glycosides are absorbed intact in the gut or conjugated with glucuronide or methylated in the liver or gut, and either excreted into the bile directly or may enter blood circulation and excreted in urine *(67, 80, 94)*. The conjugated compounds identified so far include, peonidin monoglucuronide, peonidin-3-glucuronide, and cyanidin 3-glucoside monoglucuronide *(67)*. Glucuronidation of cyanidin 3-glucoside has also been established in *vivo* and *in vitro (67, 81)*. It has been suggested that although all four free hydroxyl groups of cyanidin have different glucuronidation capacities, glucuronidation of cyanidin-3-glucoside occurs most readily at the 3-position *(67, 81)*. Quercetin glycosides which are also abundant in blackberries are converted to glucosides resulting in rapid absorption possibly via glucose transporters in the gut *(92, 95-97)*.

Systemic bioavailability of anthocyanins in general is very low across all studies, often between 0.02% and 1.8% of the ingested amounts although as low as 0.004 to 0.11% of administered dose has been reported *(75)*. A human study by Felgines and others *(69)* fed 200 g of blackberries containing 960 μmol of anthocyanins to five healthy volunteers and found that only 0.16% of total amount of anthocyanins ingested were excreted in urine, mainly in the form of methylated and glucuronidated conjugates *(69)*. The study identified cyanidin 3-glucoside, methylated glycosides, glucuronides of anthocyanidins and anthocyanins, and sulfoconjugate of cyanidin, and anthocyanidins. Monoglucuronides of anthocyanidins accounted for > 60% of the total anthocyanins in the urine *(69)*. Findings on the metabolism and bioavailability of

polyphenolic compounds may vary due to differences in animal models and experimental designs. Additionally, these compounds have different chemical structures and solubility can interact with macro and micronutrients, and they also vary in susceptibility to digestion, conjugation, methylation, fermentation, and absorption in the gut *(91)*.

Studies pertinent to the metabolism and bioavailability of blackberry ETs and EA are lacking. However, this section will briefly highlight the metabolism and bioavailability of various other ETs and EA found in literature and hypothesize that the pathways may be similar.

A study on punicalagin, an ET found in both blackberries and pomegranate was detectable in human plasma and urine after consumption of 180 ml of pomegranate juice *(98)*. The juice contained 25 mg and 318 mg of EA and ETs, respectively. The highest concentration of EA in human plasma 1hr postprandial was 31.9 ng/ml and EA was eliminated within 4 h *(98)*. The authors hypothesized that ETs and EA were metabolized by the colon microbiota hence the appearance of free EA in human plasma *(98)*. In rats, ETs are hydrolysable to EA by gut microbiota in the small intestine and cecum *(99)*. EA fed to rats was detected in the form of urolithin A, also known as 3,8-dihydroxy-6*H*-dibenzo[*b*, *d*]pyran-6-one and an unidentified metabolite in urine and feces. Investigators detected as much as 10% of given dose in this study *(100)*. Further work using germ free animals showed that these metabolites resulted from action by gut microorganisms *(100)*. EA can also be metabolized via conjugation possibly in the liver or in the gut as evident by two conjugates of urolithin A found in rats after ingestion of EA *(100)*. A mice study using phenol EA (^{3}H-EA) also detected free EA and conjugates in urine, bile and blood and after gavage and 80% of fecal sample fraction was free EA *(101)*. A study using Iberian pigs, demonstrated that ETs release EA *in vivo* followed by a gradual metabolism of and production of urolithin D, C, A and B in this order from the jejunum to the distal portion of the

intestine *(102)*. Furthermore, absorption of these metabolites was found to increase with their increasing lipophilicity *(102)*. Therefore, ETs and EA are metabolized by the gut microbiota of different mammals *(98, 102, 103)* resulting in dibenzopyranones, urolithin A and its monohydroxylated analog urolithin B, C, and D *(104)*. It is thought that ETs are hydrolyzed to EA due to the intestinal pH and or action by gut microflora, and EA is transformed to urolithins A, B, C and D by gut microflora through lactone-ring cleavage via decarboxylation, and dehydroxylation reactions *(102)*. The Iberian pig study also confirmed enterohepatic circulation activity as glucuronides and methyl glucuronides of EA and urolithin A, C, and D derivatives were detected in bile and urolithins A, and B, and dimethyl-EA glucuronide were found in peripheral plasma *(102)*.

Because it takes 12-56 h to complete excretion of urolithins and their metabolites, their distribution in tissues such as liver, lung kidney, heart, muscle and adipose tissue have been investigated. No such metabolites were detected in these tissues in pigs *(102)*, although traces of punicalagin metabolites were reported in the kidney and liver of rats *(103)*, and in colon, intestinal, and prostate tissues of mice *(105)*.

In summary, gut microbiota are responsible for hydrolysis of ETs to EA and subsequently to glucuronides and methyl glucuronides of EA, and urolithins A, B, C, and D, and urolithin derivatives. It has been suggested that differences exist in the metabolism of ETs and EA based on food sources, and host colonic microflora composition that result in variation in the timing, quantity and types of urolithins absorbed and excreted in urine *(106)*. Therefore, metabolism and bioavailability of particular blackberry ETs and EA require investigation as a step towards exploring their health benefits.

HEALTH BENEFITS

Anthocyanins are reported to exert anti- inflammatory, antiviral, antiproliferant and anticarcinogenic properties *(51)*. The consumption of anthocyanins in the U.S. is generally higher than other flavonoids due to their widespread distribution and occurrence in fruits and vegetables *(65, 107)*, and is estimated to be between 82-215 mg/day. Presently, data on dietary intake of blackberry ETs and EA are lacking. However as a reference, daily dietary intake of ETs from berries in the Finnish and German populations are 12 and 5 mg/day, respectively *(108, 109)*. Health benefits of particular blackberry ETs and EA have also not been explored, although a recently published review of ETs and EA is detailed on the anti-inflammatory, antimicrobial, prebiotic, antioxidant and estrogenic and/or anti-estrogenic of dietary ETs and EA *(110)*.

Oxidative stress

Living organisms have a reduction-oxidation system that is necessary to maintain a balance between free radicals generated and antioxidant system *(75)*. The formation of large amounts of free radicals may cause oxidative stress (OS) leading to many degenerative diseases and aging *(75, 111-113)*. Although some scientists argue that in many instances oxidative stress is not the primary cause of disease and that the formation of radicals is secondary to tissue damage by disease, *(51)* there is some evidence linking OS with several chronic diseases *(114, 115)*.

Wada and Ou *(35)* ranked the antioxidant activity of blackberries third highest after strawberry and black raspberry based on their oxygen radical absorbance capacity (ORAC) *(35)*. This can be attributed to the high amounts of acylated anthocyanins and cyanidin 3-glucoside in blackberries. In fact, cyanidin-3-glucoside is ranked high in ORAC activity, and is reported to be 3.5 times stronger than Trolox (vitamin E analogue), while pelargonidin, also found in

blackberries, was reported to have antioxidant activity equivalent to Trolox *(107)*. Acylated

anthocyanins are highly correlated with antioxidant capacity in fruit *(4)*. Cho et al. observed a

strong linear relationship between oxygen radical absorbance capacity (ORAC) values and total

acylated anthocyanins (r_{xy}= 0.91) in blackberry extracts and this correlation was weaker for total

anthocyanin monoglucosides (r_{xy} =0.69) *(4)*. This correlation is consistent with bioavailability

studies that demonstrate structural differences in anthocyanins such as sugar

Table 3.4. ORAC Values (μmol Trolox Equiv/g) of Blackberries a aFL represents ORAC results using fluorescein probe while PE represents ORAC results using B-PE probe

fresh weight (μmol Trolox equiv/g)	dry weight (μmol Trolox equiv/g)	no. of cultivars	source
63-83 FL		6	*(4)*
49-76 FL		6	*(23)*
34-36 PE		2	*(30)*
27-71 PE		27	*(31)*
20-22 FL	121-146 FL	3	*(33)*
20-25 PE		3	*(33)*
28 PE		2	*(35)*
-	334-560 FL	11	*(44)*
38-76 PE		11	*(39)*
17-67 FL		15	*(117)*
43-62 FL		4	*(118)*

moieties and acylated groups affect bioavailability, stability and other biological effects of anthocyanins *(21)*. It should be noted that biological activities of berry components may vary with species and even cultivars *(75)*.

Anthocyanins work by scavenging free radicals with 3, 4-dihydroxy substituent in the B ring *(75)* being a key criterion for radical scavenging. They react readily with radicals such as hydroxyl (·OH), azide (N3·) and peroxyl (ROO·) to form stable flavonoid radicals *(51)*, hence reducing oxidative stress *(116)*. Flavonoids however are slower at reacting with lipid peroxl (LOO·) radicals and superoxide (O_2-) due to reduced availability of polar flavonoids for reaction with the LOO· radicals *(116)*. They also enhance fatty acid stability by reacting with α-tocopherol radicals to form α-tocopherol *(51, 116)*. Anthocyanins also function as antioxidants by chelating metal ions at moderate pH levels with their ionized hydroxyl groups of the B ring *(75)*.

Several studies have examined the ORAC of blackberries and results are presented in Table 4. When comparing ORAC results by different authors, it should be noted that the use of fluorescein probe results in higher ORAC values, typically 1.5 to 3.5-fold compared to older studies that used the *B*-PE probe *(119)*.

Cardiovascular Disease

Elevated levels of low density lipoproteins in plasma are an independent risk factor for cardiovascular disease *(120)*. Berry phenolic compounds have shown protective effects against cardiovascular disease by inhibiting *in vitro* and *in vivo* oxidation of LDL by quenching free radicals through donation of hydrogen molecules *(41)*. Berry phenolic compounds also protect low density lipoproteins (LDL) from hydrogen peroxide-induced oxidative stress in human endothelial cell *in vitro (75)*.

Blackberries phenolic compounds demonstrated strong inhibitory properties *in vitro* using oxidation assays on human LDL and lecithin liposomes *(121)*. High content of cyanidin glycosides in blackberries are responsible for the high antioxidant activity and protection against LDL oxidation while the hydroxycinnamic acids are most important in liposome oxidation system *(121)*. Anthocyanins, flavan-3-ols and hydroxycinnamic acids have been shown to have inhibitory effects in liposomal oxidation *(121)*. Furthermore, anthocyanins have exerted protective effects *in vitro* on human primary endothelial cells by suppressing the secretion of cytokine-induced chemokine monocyte chemotactic protein 1 (MCP-1) *(72)*. MCP-1 is a protein directly involved in atherogenesis through its role of recruiting macrophages to sites of infection or inflammation *(72, 122)*.

Although not demonstrated directly by blackberry flavonoids, select flavonoids have been shown to play a protective role in platelet function which is critical in the pathogenesis of cardiovascular disease *(123)*. *In vitro* studies have shown that flavonoids reduce platelet aggregation, decrease platelet production of superoxide anions and increase platelet nitric oxide production *(123)*.

Cancer

Evidence from epidemiological and clinical studies suggests that 20% or more of all cancer cases are preventable with a diet consisting of 400 to 800 g of various vegetables and fruits per day *(75)*. Cancer is a complex multistage process that begins with initiation of a cancer cell caused by DNA damage, accumulation of mutations, promotion of cell proliferation and tumor expansion and finally progression to malignancy and metastasis *(124)*. The potential role of berry phenolic compounds to reduce cancer risk has been shown in *in vitro,* animal and clinical studies *(75)*. Berry phytochemicals may act to change the genomic stability of cells at

several points along this sequence of cancer formation *(124)*. The phytochemicals may modulate initiation, promotion and progression of cancer *(124)*. The possible anti-carcinogenesis mechanisms include: antioxidant activity, detoxification activity, induction of apoptosis, anti-proliferation, and anti-angiogenic activity *(75)*. Anthocyanins have been reported to induce phase II enzymes which may inactivate carcinogens activated by phase I enzymes therefore inhibiting possible DNA damage by the carcinogens *(75)*. Tate et al. *(125)* found that blackberry extracts from 8 varieties: Arapaho, Choctaw, Hull, Chicksaw, Triple Crown, Kiowa, Navajo, and Chester suppressed mutagenesis at varying levels. Mutagenesis is a process characterized by uncontrolled cell proliferation and resistance to programmed cell death *(124)*. Furthermore, blackberry extracts prepared from 8 varieties inhibited UV-induced mutagenesis in *Salmonella typhimurium* TA100 by 90% based on the Ames test *(125)*. The Ames test is a good *in vitro* indicator of mutagenic potential *in vivo*, with a 90% correlation between a positive response in assay and carcinogenicity in animal models *(125)*.

Serraino et al. *(126)* reported that blackberry extracts play a protective role against peroxynitrite-induced DNA strand breakage in cultured human vascular endothelial cells. Using blackberry extracts containing 80% cyanidin 3-glucoside the authors investigated the antioxidant activity of the blackberry extract on the endothelial dysfunction in cells and in vascular rings exposed to peroxynitrite. Blackberry extracts at varying dilutions reduced the peroxynitrite-induced suppression of mitochondrial respiration, and DNA damage in human umbilical vein endothelial cells *(126)*.

In addition, *in vitro* studies show that blackberry extract inhibits proliferation of A549 human lung cancer cells and reduced neoplastic transformation in normal epidermal JB6 mouse cells exposed to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), tumor promoter

(124). Blackberry anthocyanins are thought to suppress cancer cell growth by modifying cell signaling pathways such as modulating the expression of activated protein1 (AP-1) and nuclear factor kappaB (NFκ B); important proteins that regulate cell proliferation and cell cycle control *(124)*. Blackberry extracts have also demonstrated inhibitory properties in cancer-induced AP-1 and NFκB and suppressed the expression of the two proteins involved in tumor promotion and progression, vascular endothelial growth factor and COX-2 *(124)*. Moreover, Seeram et al. *(127)* has demonstrated the dose dependent cancer inhibitory properties of blackberry extracts *in vitro* in a study that used human oral, breast, prostate and colon cancer cell lines. Similarly, a study using quercetin extracted from blackberries also demonstrated the anti-carcinogenic properties in animal models and in human carcinoma cell lines (HT29 and Caco-2) *(75, 128)*.

Other health benefits

Studies on blackberries and their effect on body weight are lacking. However, cyanidin 3-glycoside (which is the predominant anthocyanin in blackberries) from purple corn has been shown to prevent obesity in C57BL/6J mice fed a high-fat diet compared to a mice fed high fat diet with no anthocyanins *(16)*. Similarly, purified blueberry anthocyanins have been shown to improve body weights and body composition, and reduce obesity in mice *(129)*. Although matching health benefits may be expected from blackberry anthocyanins, their effects on obesity need to be explored.

Blackberries are reported to have positive effects on age-related changes and may be beneficial for prevention of age-related neurodegenerative diseases such as Alzheimer's disease *(15)*. Shukitt-Hale and others demonstrated that 2% blackberry supplemented in the diet of 19-month old Fischer rats was effective in improving motor performance on three balance and co-ordination tasks, and cognitive performance *(15)*. Cyanidin 3-glucoside from blackberries was

found in the brain of male Wistar rats after 15 days of feeding a 1.5% blackberry diet *(130)* supporting the potential role of blackberry anthocyanins in neuroprotection.

Although the antimicrobial activities of blackberry tannins is not documented, hydrolyzable tannins from red raspberries have shown antimicrobial activities on *Staphylococcus* and *Salmonella* bacteria in animal studies *(75)*. Since blackberries provide a rich source of ETs, its anti-microbial properties should be investigated.

Blackberry anthocyanins in particular have not been studied in relation to diabetes; however, there is evidence that in general anthocyanins can confer protective effects to the vascular system in diabetic patients. Administration of anthocyanins from Bilberry (*Vaccinium myrtillus*) at a dose of 600 mg/day for 6 months demonstrated significant reduction of biosynthesis of polymeric collagen and structural glycoprotein that result in capillary thickness in diabetes *(131)*.

A study by Kaume and others *(132)* has recently demonstrated that supplementation of blackberries at the level of 5% but not 10% in ovariectomized rats modestly increased bone mineral density at the tibia by 2.4%, femur by 4.3%, and fourth lumbar vertebra by 2.7% higher than ovariectomized control animals (P < 0.05). Further, animals fed 5% blackberry (w/w) had a significant decrease in trabecular separation, by 22% less than the ovariectomized control rats. More studies on the health benefits of blackberries on bone are necessary to elucidate on the mechanism by which these berry compounds modulate bone metabolism.

Studies on blackberries reveal that their phenolic composition and concentration are influenced by many factors including variety, location of cultivation, and maturation. The chemistry of blackberry phenolic compounds is well understood. In terms of health benefits,

these phenolic compounds have shown protective effects in age-related neurodegenerative diseases, and bone loss *in vivo*, and inhibited LDL and liposomal oxidation *in vitro*. Blackberry extracts have also exerted anti-mutagenic effects by modifying cell signaling pathways and suppressing tumor promotion factors *in vitro* and *in vivo*. However, the anti-obesity, anti-diabetic, antimicrobial, and anti-inflammatory properties of blackberry phenolic compounds including catabolites from colonic microflora need investigation. In addition, more research is needed to explore their metabolism, bioavailability and the mechanisms by which they confer health benefits. Furthermore, studies that elucidate in vivo physiologically effective concentrations of blackberry phenolic compounds in vivo are necessary.

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I.

IV. CYANIDIN 3-O-B-D-GLUCOSIDE-RICH BLACKBERRIES IMPROVE BONE MASS AND MICROARCHITECTURAL PROPERTIES IN AN OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

ABSTRACT

Postmenopausal osteoporosis is characterized by fragility fractures and afflicts approximately one third of women over 50 years. Oxidative stress promotes bone loss after menopause, and there is evidence that dietary antioxidants may reduce the level of oxidative stress *in vivo*. This study examined the dose dependent effects of blackberries (BB) containing mainly cyanidin-3-O-D-glucoside (C3G) in preventing bone loss in an ovariectomized (Ovx) rat model. Nine-month-old female (N=38) Sprague-Dawley rats were scanned using dual energy X-ray absorptiometry to obtain baseline whole body, bone mineral content (BMC) and bone mineral density (BMD). One group was sham-operated (Sham) and three groups were ovariectomized (Ovx). The groups and corresponding diets were: Sham + control diet (n=12), Ovx + control diet (n=12), Ovx + 5% BB (n=7), and Ovx + 10% BB (n=7). Control diet was AIN-93M rodent diet, and the Ovx + 5% BB and Ovx + 10% BB were a diet modified to contain powdered, freeze dried-BB at levels of 5 and 10% (w/w). Following 100 days of treatment, whole body BMC and BMD were reassessed and bone specimens, and blood and 24h urine samples were collected for analyses. Findings indicate that ovariectomy (Ovx) compromised whole body BMC and trabecular microarchitecture of the proximal tibia and 4th lumbar vertebra. C3G-rich BB at the level of 5% modestly protected BMDs loss of the tibia, lumbar vertebra, and femur by 2.4, 2.7, and 4.3% respectively. BB5% treatment significantly prevented loss of tibial trabecular bone volume and trabecular number by 37% and 21% respectively ($p < 0.05$); and also

significantly prevented tibial trabecular separation by 22%. C3G-rich BB treatments had no effects on both biomarkers of bone metabolism and oxidative stress. We conclude that C3G-rich BB treatment at the level of 5% (w/w) may modestly reduce Ovx-induced bone loss evident by improved tibial vertebral and femoral BMD values, and tibial bone microstructural parameters. Bone protective effects may be as a result of the synergistic effects of phenolic compounds; however, further work is required to determine blackberries' specific mechanisms of action.

INTRODUCTION

Postmenopausal osteoporosis is characterized by fragility fractures and afflicts approximately one out three women over 50 years[1] Postmenopausal women experience increased oxidative stress due to aging and estrogen loss[2,3]. Oxidative stress (OS) plays a major role in activating osteoclastogenesis and promoting bone resorption *in vitro* and in rodent studies [4-7]. As a result of ovarian-hormone-deficiency osteoclasts increase the production of reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide [6, 8, 9]. Heightened OS and diminished levels of thiol antioxidant defenses in bone cells [7,10] induce production of pro-inflammatory factors such as interleukin 1 and 11 (IL-1 and IL-11) and tumor necrosis factor-α that further aggravate the progression of osteoclastic activity [9-11].

The principal goal of osteoporosis therapy is to reduce fracture risk. To this end, drug therapies such as alendronate, risedronate, zoledronic acid, denosumab and strontium ranelate used with calcium and vitamin D have been effective [12]. Nonetheless due to factors such as cost, fear, and side effects associated with these drugs, about 50% of patients discontinue medication or fail to adhere to treatments regimens in the first year of use, which results in increased fracture risk [12, 13]. Hence in addition to drugs and lifestyle modifications, practical diet-based approaches to reduce the level of oxidative stress and improve bone health are necessary. Growing evidence shows that increasing the intake of fruits and vegetables, as part of a nutrient-balanced diet, is beneficial to bone health [14-18]. Additionally, experiments with dietary antioxidants (e.g. *N*-acetyl cysteine, ascorbic acid, alpha-tocopherol, and β-carotene) have demonstrated their potential to promote bone formation [19] and increase levels of enzymatic antioxidants [7]. These antioxidants may stimulate osteoblast differentiation, suppress osteoclast formation, and decrease TNF-α production, hence attenuate the bone resorptive effects of oxidative stress[7, 19-22].

Apart from the traditional dietary antioxidants such as vitamins A, C and E, phenolic compounds from plums and blueberries have demonstrated protective effects against bone resorption due to their strong superoxide scavenging capacity [23, 24,25]. Akin to these fruits, is the blackberry fruit (*Rubus sp.*) that has total phenolics range of 114 to 1056 mg/100 g fresh weight (FW) [26]. The composition of Blackberry (BB) includes anthocyanins [27], ellagitannins [28], flavonols, flavan-3-ols [29], procyanidins [30], phenolic acids [26] and low amounts of lignans [31]. Most berries have several anthocyanins in their phenolic profile; however, BB contains mostly the anthocyanin, cyanidin-3-glucoside (C3G) [32]. The fraction of C3G in the whole blackberries used in this study was 87%. According to studies [33-35] C3G has high anti-inflammatory and antioxidative activity and has potential to play an important role in prevention of lipid peroxidation of cell membranes.

Using an Ovx- rat model of postmenopausal osteoporosis, effects of supplementation with C3G-rich blackberries at the level of 5% (w/w) and 10% (w/w) were assessed on: (i) bone mineral density (BMD), (ii) trabecular microarchitecture (iii) bone biochemical markers, and (iv) plasma enzymatic antioxidants.

MATERIALS AND METHODS

Animal care and diet

Nine-month-old female (N=38) Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in an environmentally controlled animal care facility with standard temperature and light: dark (12:12) cycles. Following a week of acclimatization the animals were randomly assigned to four groups. One group was sham-operated (Sham) and three groups were ovariectomized (Ovx). Baseline bone mineral density (BMD) and bone mineral content (BMC) were obtained using DXA dual X-ray absorptiometry (DXA; GE Lunar DXA, Waukesha, WI) equipped with enCore

software. The groups and corresponding diets were: Sham + control diet (n=12), Ovx + control diet (n=12), Ovx + 5% BB (n=7), and Ovx + 10% BB (n=7). Control diet was AIN-93M rodent diet, and the Ovx + 5% BB and Ovx + 10% BB were control diet modified to contain powdered, freeze dried-BB at levels of 5 and 10% (w/w). Blackberry diets were formulated based on proximate analysis of freeze-dried blackberries to contain the same amounts of carbohydrate, fiber, fat and protein (data not shown). Rats were weighed on a weekly basis, received free access to deionized water, and were match fed to the weekly mean food intake of the sham group.

Animal necropsy

After 100-d treatment period, the animals were placed in metabolic cages for a 24 h period in order to collect a 12-h urine sample. The urine sample was centrifuged at 3000×g for 20 minutes aliquoted and stored at -20 °C for deoxypyridinoline (DPD) and creatinine analyses. After a12-hour fast, animals were anesthetized with a mixture of ketamine and xylazine (70 mg and 3 mg/kg body weight, respectively) to measure final BMD and BMC using DXA. The rats were then sacrificed by exsanguination via cardiac puncture. Blood samples were collected and centrifuged at 3000×g for 20 minutes at 4 °C. Serum was aliquoted and stored at -80 °C until analysis. Bone specimens, the tibiae, the 4th lumbar vertebrae and femur, were collected, cleaned, and stored at -20°C for BMD and micro-architectural analyses. Uteri were collected and weighed for confirmation of ovariectomy.

All animal procedures used in this study were according to the University of Arkansas Institutional Animal Care and Use Committee guidelines.

Assessment of bone mineral area, content, and density

Using procedures previously published [36] and DXA equipped with suitable software for assessing bone indices in small laboratory animals, bone mineral area (BMA), BMC and BMD of the tibiae, the 4th lumbar vertebrae and femur were measured.

Assessment of bone microarchitecture using microcomputed tomography (μCT)

To assess the effect of C3G-rich blackberry treatment on bone trabecular structure, the tibiae, 4th lumbar vertebrae and femur and were scanned using μCT and methods previously reported [37]. After analyzing the volume of interest (VOI), the following bone morphometric parameters were obtained: bone volume over total volume (BV/TV), trabecular number (Tb.N.), separation (Tb.Sp.), thickness (Tb.Th.), connectivity density, and structure model index (SMI).

Assessment of oxidative stress biomarkers

Effects of ovariectomy and C3G-rich blackberry treatments on oxidative stress levels were assessed using four assays. Thiobarbituric Acid Reactive Substances (TBARS) were measured in plasma and were used to determine lipid peroxidation [38], a major indicator of overall oxidative stress. TBARS were determined in duplicate using TBARS assay kit (ZeptoMetrix Corporation Buffalo NY, USA) according to manufacturer's instructions and absorbance of 532 nm read on a Synergy HT (BioTek; Winooski, VT) microplate reader.

The activity of antioxidant enzymes: glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase were assessed in plasma using BioVision's assay kits (BioVision Inc. Mountain View, CA, USA) following manufacturer's instructions. All samples were assayed in duplicate and read using Synergy HT (BioTek; Winooski, VT) microplate reader.

Assessment of systemic biomarkers bone metabolism

Effects of ovariectomy and the two doses of C3G-rich blackberry treatment were evaluated in four systemic biomarkers of bone metabolism. Alkaline phosphatase (ALP) which is required for the hydrolysis of phosphate esters was assessed in serum as an indicator of bone formation using an Alfa Wassermann diagnostic technologies kit and ACE Alera clinical chemistry system (Alfa Wassermann diagnostic technologies, West Caldwell, NJ, USA). Serum osteocalcin (OC), a reliable indicator of bone turnover [39] was measured using Rat-MID™ Osteocalcin EIA (Immunodiagnostic Systems Inc. Fountain Hills, AZ, USA). Serum Insulin-like growth factor-1(IGF-1), a recognized modulator of bone metabolism involved in proliferation of osteoblasts [40]was quantified using an enzyme immunoassay (EIA) kit from ALPCO immunoassays (ALPCO Diagnostics, Salem NH, USA). In addition, a resorption bone biomarker urinary deoxypyridinoline (DPD) was quantified using a competitive enzyme immunoassay kit specific for rats (Quidel, Mountain View, CA, USA). The assay is established in DPD molecule specificity and hence cross-reaction with other collagen crosslinks such as pyridinoline is highly insignificant [41]. Urinary creatinine was measured colorimetrically using an ACE Alera clinical chemistry system and a commercially available kit from Alfa Wassermann diagnostic technologies (Alfa Wassermann diagnostic technologies, West Caldwell, NJ, USA). The final creatinine-corrected DPD results were expressed as nmols DPD/mmols creatinine. All serum and urine samples were assayed in duplicates for the above biomarkers.

Statistical analyses

The data analysis involved estimation of means and SEM using JMP 8 (2009 SAS Institute Inc. Cary, NC). The effects of treatment were analyzed by one-way ANOVA model followed by post hoc analysis using the Fisher's least squares means separation test when F values were significantly different ($P < 0.05$).

RESULTS

Bone mineral area, content, and density

Baseline BMC of the whole body was not different among groups; however, final BMC of the OVX+BB10% and sham group was significantly lower than Ovx+control group. Baseline BMD of the whole body were not different among groups. Effects of Ovx are observed as final BMD values of ovariectomized rats were compared to the sham group (**Table 4.1**).

Table 4.1. Effects of ovariectomy and C3G-rich blackberry treatments on DXA bone parameters of the whole body

Parameter	Sham + Control (n=12)	OVX + Control (n=11)	OVX + BB 5% (n=6)	OVX + BB 10% (n=7)	*P* value
Whole Body					
Baseline BMC (g)	9.142 ± 0.223	9.191 ± 0.233	9.617 ± 0.316	9.186 ± 0.292	0.6424
Baseline BMD (g/cm^2)	0.174 ± 0.002	0.175 ± 0.002	0.179 ± 0.003	0.177 ± 0.003	0.4683
Final BMC (g)	9.975 ± 0.274^a	11.564 ± 0.287^b	11.183 ± 0.379^b	10.557 ± 0.359^b	0.0026
Final BMD (g/cm^2)	0.186 ± 0.002	0.178 ± 0.002	0.180 ± 0.003	0.182 ± 0.003	0.0833

Values are means $\pm$ SEM. Within a row, values that do not share the same superscript letters are significantly different from each other ($P < 0.05$). Abbreviations used: SHAM: Sham operated; OVX-CTRL: ovariectomized control; OVX-BB 5%: Ovariectomized + blackberry 5% (w/w); OVX-BB 10% (w/w): Ovariectomized + blackberry 10% (w/w).

Table 4.2. Effects of ovariectomy and C3G-rich blackberry treatments on DXA bone parameters of the, tibia, 4th

lumbar vertebra and femur

Parameter	Sham + Control (n=12)	OVX + Control (n=11)	OVX + BB 5% (n=6)	OVX + BB 10% (n=7)	*P* value
Tibia					
BMA (cm^2)	1.7425 ± 0.0264	1.6933 ± 0.0264	1.6667 ± 0.0671	1.6429 ± 0.0669	0.2795
BMC (g)	0.368 ± 0.007[b]	0.341 ± 0.007[a]	0.342 ± 0.012[ab]	0.324 ± 0.012[a]	0.0337
BMD (g/cm^2)	0.211 ± 0.002[c]	0.200 ± 0.002[ab]	0.205 ± 0.003[bc]	0.196 ± 0.003[a]	0.0013
4th lumbar vertebra					
BMA (cm^2)	0.6267 ± 0.0120	0.5975 ± 0.0122	0.6100 ± 0.0177	0.5950 ± 0.0177	0.2592
BMC (g)	0.145 ± 0.0041	0.131 ± 0.0042	0.137 ± 0.0063	0.128 ± 0.0063	0.0540
BMD (g/cm^2)	0.230 ± 0.004[a]	0.218 ± 0.004[b]	0.224 ± 0.005[ab]	0.214 ± 0.005[b]	0.0437
Femur					
BMA (cm^2)	1.9642 ± 0.0542	1.83 ± 0.0602	1.9217 ± 0.0435	1.9886 ± 0.0431	0.2588
BMC (g)	0.4460 ± 0.0140	0.410 ± 0.0140	0.452 ± 0.0190	0.430 ± 0.0190	0.2870
BMD (g/cm^2)	0.2390 ± 0.0030[c]	0.224 ± 0.0030[ab]	0.234 ± 0.0043[bc]	0.215 ± 0.0043[a]	0.0004

Values are means ± SEM. Within a row, values that do not share the same superscript letters are significantly different from each other (*P* < 0.05). Abbreviations used: SHAM: Sham operated OVX-CTRL: ovariectomized control OVX-BB 5%: Ovariectomized + blackberry 5% (w/w); OVX-BB 10% (w/w): Ovariectomized + blackberry 10% (w/w).

There were no statistically significant differences in BMA of the tibia, 4th lumbar vertebra and femur among groups but tibial BMC of the Ovx group was lower than the sham group (**Table 4.2**). All three bones had BMA and BMC of the Ovx+control group lower to some degree than the sham group although not to a statistically significant level. Tibial, vertebral and femoral BMDs were significantly reduced by Ovx, 5%, 5% and 6% respectively. C3G-rich BB diet at the level of 5% and not 10% (w/w) increased BMD of the tibia, lumbar vertebra, and femur by 2.4%, 2.7% and 4.3% respectively compared to the Ovx+control, bringing it up to the level of the sham group.

μCT analysis of tibia and fourth lumbar vertebra

Four cortical parameters were assessed at the proximal tibia, including cortical area, porosity, thickness, and medullary area. Tibial cortical bone microarchitecture was markedly compromised by Ovx (**Table 4.3**). Ovx resulted in decreased cortical thickness and cortical area by 7% and 8% respectively, and an increase in cortical percent porosity of 8.5%.

C3G treatment at the level of 5% but not at 10% (w/w) increased cortical thickness by 5% bringing it up to the level of the sham group. C3G treatments had no impact on cortical area and cortical porosity. Neither Ovx nor dietary treatments had any effects on cortical medullary area.

Table 4.3. Effects of ovariectomy and dose-dependent C3G-rich blackberry treatments on tibia cortical parameters

Parameter	Sham + Control (n=9)	OVX + Control (n=8)	OVX + BB 5% (n=6)	OVX + BB10% (n=6)	P value
Cortical bone					
Cort Th (mm)	0.5932 ± 0.0113^b	0.5538 ± 0.0113^a	0.5820 ± 0.0123^{ab}	0.5477 ± 0.0123^a	0.0510
Cort Area (mm^2)	4.5800 ± 0.1000	4.2400 ± 0.1100	4.3800 ± 0.1200	4.3200 ± 0.1200	0.1129
Med Area (mm^2)	0.1849 ± 0.0046	0.1860 ± 0.0046	0.1873 ± 0.0050	0.1960 ± 0.0050	0.3293
Porosity (%)	3.880 ± 0.1300^a	4.21 ± 0.1300^{ab}	4.12 ± 0.1400^{ab}	4.35 ± 0.1400^b	0.1074

Values are means ± SEM. Within a row, values that do not share the same superscript letters are significantly different from each other (P < 0.05). Abbreviations used: SHAM: SHAM operated; OVX-CTRLovariectomized control; OVX-BB 5% : Ovariectomized+blackberry 5%; OVX-BB 10% (w/w): Ovariectomized + blackberry 10 % (w/w) Cort Th: cortical thickness; Cort: cortical; Med: medullary

Analysis of data also showed that Ovx considerably compromised all parameters of

trabecular microarchitecture of the proximal tibia and 4th lumbar vertebra (**Figure 4.1**) except

tibial Tb.Th. Ovx-induced changes were characterized by decreased BV/TV (Fig A), Tb.N. (Fig

B), and Tb.Th. (Fig C) and increased Tb.Sp. (Fig D). Ovx decreased tibial and vertebral BV/TV

by 23% and 13%, respectively. C3G-rich blackberry treatment at the level of 5% (w/w)

significantly increased tibial BV/TV by 37% and had no effect on lumbar BV/TV. The higher

dose of BB 10% (w/w) had a negative impact to vertebral BV/TV significantly reducing bone

volume by 28% compared to Ovx+control.

Similarly, tibial and vertebral Tb.N. was decreased in response to Ovx by 11% and 1.7%,

respectively. C3G-rich BB at the level of 5% (w/w) resulted in significant positive effects on

tibial but not vertebral Tb.N. Tibial Tb.N. values significantly increased by 21% in the

Ovx+BB5% group but the higher BB dose had no such effect. Ovx reduced Tb.Th. by 1.7% and

6.3% in the tibia and vertebra bones, respectively. In regard to treatments, the low BB diet dose

increased vertebra Tb.Th by 6% to the level of the sham group but the higher dose had no effect;

and neither treatments had an effect on tibial TbTh. Ovx also increased tibial and vertebra Tb.Sp.

by 17% and the slight 0.6% respectively. C3G-rich BB treatment at the level of 5% (w/w)

prevented tibial Tb.Sp. reducing it by 22%, bringing it up to the sham level; but the same dose

had no positive effects on the vertebral bone. Tibial and vertebral Tb.Sp in animals fed

blackberry at the level of 10% (w/w) was similar to the Ovx control group.

Non-metric parameters of the tibial and lumbar trabecular bone, i.e., SMI and

connectivity density (**Figures 4.1E and 4.1F**) were modified by Ovx albeit not to a statistically

significant degree. Higher SMI indicates a more biomechanically compromised trabecular bone

pattern which is more rod-like as opposed to the favorable plate-like pattern. Tibial SMI

increased by 9.4%, and that of the vertebrae by 31%, while connectivity density decreased in both bones by 32% and 2.6% respectively, due to Ovx. Though the impact of C3G-rich BB5% (w/w) on tibial SMI did not reach significance level, this dose modestly prevented the change of trabecular to the unfavorable rod-like pattern by reducing the index by 18% compared to Ovx+control. The vertebra SMI was not changed by the lower dose of BB treatment but the high dose significantly increased SMI. Tibial but not lumbar connectivity density in animals fed a low dose of C3G-rich BB significantly increased to a value higher than the Ovx and sham groups. The higher dose of C3G-rich BB treatment had no positive effects on connectivity density of both bones.
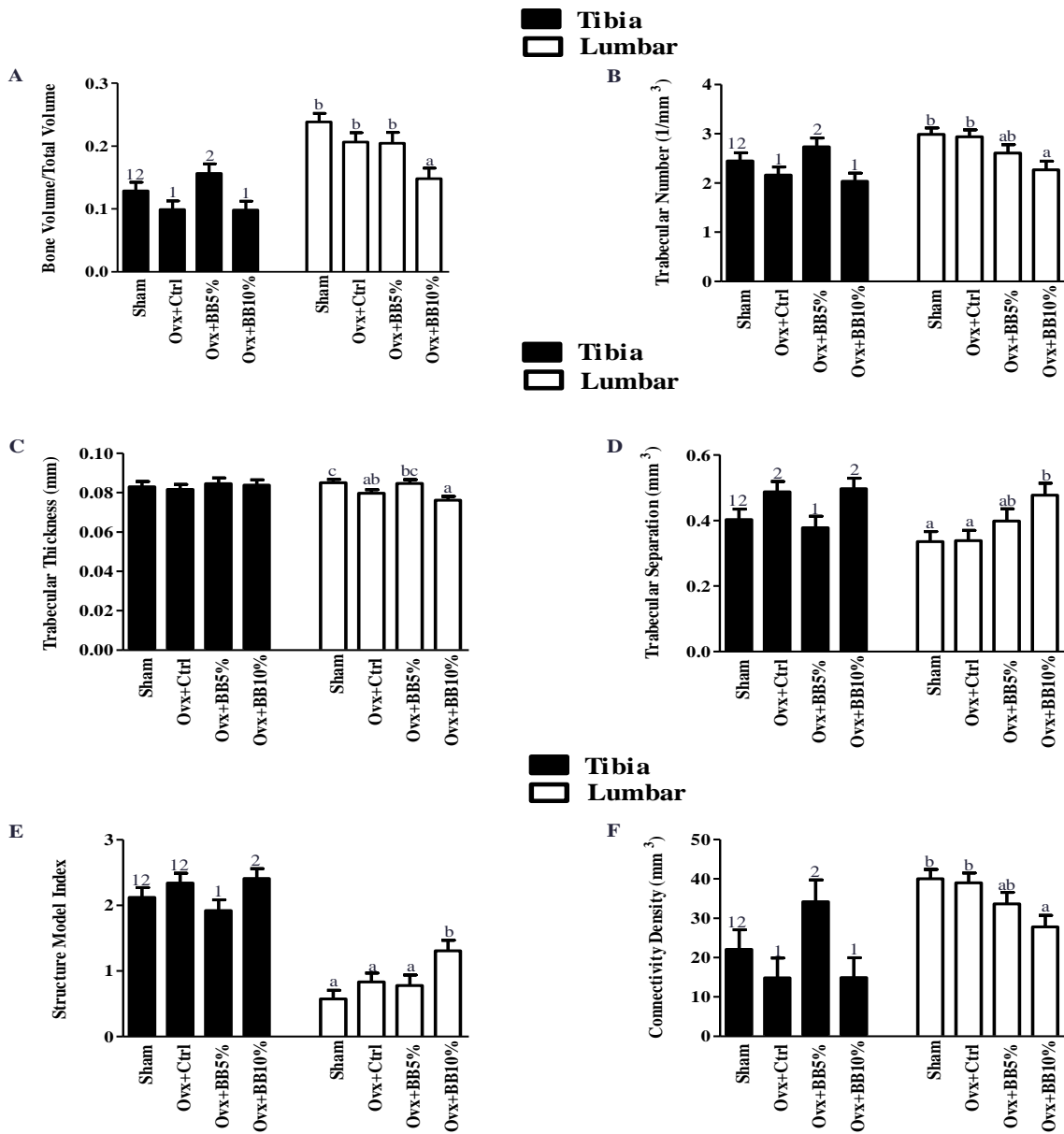
Figure 4.1. Effects of ovariectomy and dose-dependent C3G-rich blackberry treatments on tibia, vertebrae and trabecular bone. Values are means ± SEM, bars that do not share the same letters or numbers are significantly different ($P < 0.05$). Sham (n=9), OVX-Control (n=8) OVX-BB 5% and OVX-BB 10% (n=6)

Biomarkers of oxidative stress and bone metabolism

Effects of Ovx and C3G-rich BB treatments were assessed on three antioxidant enzymes, SOD, GPX, and catalase and a biomarker of oxidative stress, TBARS. Neither Ovx nor C3G-rich BB treatments had any significant effects on these measures.

Urinary DPD and serum osteocalcin, ALP, IGF-I were measured to assess their response to Ovx and C3G-rich BB treatments on bone resorption and bone formation, respectively (**Table 4.4**). DPD expressed per unit of creatinine was increased by Ovx, but C3G-rich BB treatments did not suppress the urinary excretion of these collagen by-products. Serum osteocalcin was significantly increased in the Ovx+control group (37%) in response to Ovx, but not altered by C3G-rich BB treatments. ALP was significantly higher in the Ovx rats compared to the sham + control group and C3G-rich BB treatment at the level of 10% but not 5% (w/w), decreased ALP level the level of the sham group. In response to Ovx, IGF-1 increased by 9%, and C3G-rich BB treatments further enhanced these levels in a dose dependent manner; however these changes represent only a trend and did not reach the level of statistical significance.

Table 4.4. Effects of ovariectomy and dose-dependent C3G-rich blackberry treatments on antioxidant enzymes and systemic biomarkers of bone metabolism

| Parameter	Sham + Control (n=9)	OVX + Control (n=8)	OVX + BB5% (n=6)	OVX + BB10% (n=6)
DPD (nmol /mmol creatinine)	16.29 ± 3.41^a	37.29 ± 3.42^b	37.6 ± 3.87^b	31.2 ± 3.87^b
Osteocalcin (ng/mL)	85.74 ± 19.46^a	136.89 ± 19.47^b	143.93 ± 25.16^b	144.2 ± 25.16^b
ALP (U/L)	34.82 ± 3.97	43.50 ± 3.98	47.83 ± 5.51	43.57 ± 5.51
IGF-1 (pg/mL)	12.14 ± 0.77	13.33 ± 0.78	13.47 ± 1.40	14.41 ± 1.40

Values are means ±SEM. Within a row, values that do not share the same superscript letters are significantly different from each other ($P < 0.05$). Abbreviations used: SHAM: SHAM operated; OVX-CTRLovariectomized control; OVX-BB 5% : Ovariectomized+blackberry 5%; OVX-BB 10% (w/w): Ovariectomized + blackberry 10 % (w/w); SOD: superoxide dismutase; GPX: glutathione peroxide; TBARS: thiobarbituric acid reactive substances; DPD: deoxypyridinoline; ALP: alkaline phosphatase; IGF: insulin-like growth factor

DISCUSSION

Bone loss in response to Ovx is partially due to increased oxidative stress and impaired antioxidant systems of the bone tissue [11, 42]. In this study, bone loss occurred at all sites measured in varying magnitudes. However, modest protection of tibial and femoral BMD by C3G-rich BB 5% (w/w) reduced fracture risk at these sites; the primary goal in osteoporosis therapy. Such findings are encouraging considering that the risk of any fracture in women is about twice as high as that of men [12]. Similar bone protective effects have been demonstrated in blueberries which have an equally high antioxidant capacity [17]

Micro-CT analyses indicated Ovx -induced changes of the cortical and trabecular microarchitecture as reported by others [15, 37, 43]. Moreover, evaluation of the metaphyseal region of the tibia demonstrated C3G-rich BB 5% (w/w)-protection against loss of BV/TV and Tb.N. As a consequence of preserved Tb.N, less Tb.Sp. and more connectivity were observed. Tibial Tb.Th. remained unaltered by both Ovx and treatment. We speculate that no effect on Tb.Th resulted from a compensatory mechanism to correct for loss in connectivity as proposed previously [37], and/or lack of statistical power due to limited sample size.

Rod-like trabeculae are more susceptible to fracture than plate-like patterns [44]. Therefore by preventing rod-like pattern formation as evidenced by lower SMI values, C3G-rich BB 5% (w/w) reduced fracture risk. Such findings are important in providing evidence that supports epidemiological studies [16, 45]that associate consumption of fruits and vegetables to improved bone health. Interestingly, response to treatment at the lumbar vertebrae was weak and these findings agree with previous studies [37,46]. However, compared to the proximal tibia this site was also less vulnerable to the trabeculae deterioration effects of Ovx.

In terms of biomarkers, concentrations of urinary DPD increased characteristic of bone turnover in favor of resorption due to Ovx. However, C3G-rich BB diets did not suppress bone resorption rate as would be expected. Also serum markers of bone formation ALP and osteocalcin both of which indicated increased bone turnover following Ovx were unaltered by BB treatment. Ovx and BB treatments had no effect on IGF-1. Suppression of Ovx-induced ALP and osteocalcin has been reported in blueberry treatment [17] but not in dried plum [15]. Similar to dried plums treatment [15], C3G-rich BB diets were unable to alter bone mineralization raising the question as to the mechanism of action. Systemic markers appear to differ with treatments and mechanism of action; therefore further investigations are necessary to elucidate the mechanisms by which C3G-rich BB prevent Ovx-induced bone loss.

Following Ovx, rats suffer depleted intracellular antioxidants, such as glutathione, and experience increased osteoclast activity [11]. Oxidative stress results in marked regulation and expression of antioxidant enzymes including SOD, GPX and catalase [47]. Overall, this study found no significant changes in these enzymes as result of either Ovx or C3G-rich treatments. However, a slight dose-dependent trend in reduction of SOD, GPX and catalase may be indicative of reduced need for their expression due reduced oxidative stress by C3G-rich BB. TBARS, an indicator of oxidative stress was reduced by C3G-rich BB 5% (w/w) but not 10% (w/w) to levels similar to the sham group demonstrating the antioxidant potential of BB phenolics.

Anthocyanin concentration in the blackberries in this study as determined by HPLC and HPLC-ESI-MS was 631.3 mg/100 g of dry weight (DW) and cyanidin 3-O-β-D-glucoside was 87% of total anthocyanins. Therefore we can hypothesize that the bone protective effects seen in this study are largely due to the high C3G content; which has one of the highest oxygen radical

absorbance capacity activities (ORAC) recorded [48]. It is plausible that the dose at 10% (w/w) was excessive and therefore ineffective in combating oxidative stress. Also high fiber content in blackberries may have inhibited the absorption of essential bone building minerals such as calcium, phosphorous. However the mechanisms by which C3G-rich blackberries prevent bone loss need to be explored in future studies. To our knowledge no other study has investigated the bone-protective effects of blackberry phenolic compounds.

CONCLUSION

In conclusion, blackberries supplemented in the diet at the level of 5% (w/w) may modestly reduce ovarian hormone-induced bone loss evident by improved tibial vertebral and femoral BMD values, and tibial bone microstructural parameters. Bone protective effects may be as a result of the synergistic effects of phenolic compounds; however, further work is required to determine blackberries' specific mechanisms of action.

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ANTIOXIDANT-RICH BERRIES EXERT MODEST BONE PROTECTIVE EFFECTS IN POSTMENOPAUSAL SMOKERS WITHOUT IMPROVING BIOMARKERS OF BONE METABOLISM

ABSTRACT

Smoking aggravates bone loss in postmenopausal women due to increased oxidative stress and inflammation. Berries have shown bone protective effects in animal studies partially due to antioxidant and anti-inflammatory properties of their phenolic compounds. We hypothesized that consumption of 45g of blackberries or blueberries for 9 months will prevent smoking-induced bone loss in postmenopausal women. Postmenopausal smokers (n=45) and nonsmokers (NS) (n=20) were recruited. Smokers were randomly assigned to one of three treatment groups: smoker control (S-C) n=21; smoker + 45 g freeze-dried blackberries (S-BB) n=8; or smoker + 45 g freeze-dried blueberries (S-BL) (n=16). We measured: bone mineral density (BMD) of total body, and other regions at 0 and 9 mo., and urinary deoxypyridinoline, bone alkaline phosphate, osteocalcin, thiobarbituric acid reactive substances and highly sensitive C-reactive protein at 0, 3, 6, and 9 month. Bone loss of total body BMD was statistically significant in S-C and S-BL groups, but not in S-BB and NS-C groups ($P = 0.0284$). No significant changes were noted on other parameters. These findings indicate that antioxidant rich blackberries but not blueberries modestly protected against smoking-induced bone loss in postmenopausal smokers. The mechanisms by which berries prevent bone loss need further exploration.

INTRODUCTION

Postmenopausal osteoporosis is one of the most prevalent and debilitating bone diseases, affecting over 200 million women worldwide [1]. With regard to etiology and pathogenesis of postmenopausal osteoporosis, oxidative stress and inflammation are established mechanisms demonstrated in ovariectomized (Ovx) rats [2]. Oxidative stress (OS) characterized by increased osteoclast production of oxygen derived free radicals occurs as a consequence to lowered thiol antioxidant defenses due estrogen loss [2,3]. In response to elevated levels of OS, there occurs increased expression of pro-inflammatory cytokines including, tumor necrosis factor-α (TNF-α) [3]. Jagger and others [3] have demonstrated that through TNF-α signaling, osteoclastogenesis is stimulated and promoted in a rat model of postmenopausal osteoporosis.

In light of this, postmenopausal smokers have increased risk for osteoporosis [4] due to an additional 10^{15} free radicals/g inhaled from cigarette smoke [5]. Oxidants from cigarette smoke also down-regulate the expression of important mediators of bone formation, osteocalcin, type 1 collagen and alkaline phosphatase that are essential for bone structure [6]. In fact, smokers have 5-10% less bone than nonsmokers by the age of 45-50 years [7]. Furthermore, smokers compared to nonsmokers have elevated levels of plasma inflammatory factors, and lipid peroxidation biomarkers [8].

Pharmacological therapies for postmenopausal osteoporosis are aimed at reducing future fracture risk, they include: bisphosphonates, raloxifene, strontium ranelate, denosumab and parathyroid hormone peptides [9]. Though effective, both poor access and adherence to these treatments have been reported [10, 11] Treatments such as insulin-growth factor-1, calcium, and vitamin D have also demonstrated modest anabolic responses [12]. Nonetheless, safe life-long

preventive interventions need to be embraced in overcoming the burden of osteoporosis in individuals and in the society.

Nutritional therapies using fruits and vegetables may also be effective in protecting against bone loss as suggested by some epidemiological, clinical and animal studies [13-17]. A growing body of evidence proposes that besides the nutrients, phenolic compounds in fruits and vegetables possess strong antioxidant and anti-inflammatory properties that can improve oxidant states *in vivo* [18] and exert health benefits.

With regard to fruits, dried plums, freeze-dried blueberries and freeze-dried blackberries have demonstrated bone protective effects in Ovx rats [16, 17, 19]. Furthermore, effects of dried plums [20] but not blackberries and blueberries on bone health have been explored in humans. The phenolic composition of blackberries (BB) and blueberries (BL) consists mainly of anthocyanins, flavonols, and chlorogenic acid (BL) and ellagitannins and ellagic acid (BB) [21]. Anthocyanins are known to be powerful antioxidants and make up a large proportion of these berries. However, with regard to health benefits, we must consider possible synergetic effects from all berry bioactive compounds and their metabolites.

Healthy postmenopausal smokers were used in the present study to test the hypothesis that antioxidant-rich BB and BL can prevent smoking-induced bone loss. To this end, we assessed the effects of BB and BL on: bone mineral density (BMD), and bone mineral content (BMC), three biomarkers of bone metabolism, a biomarker of oxidative stress and a biomarker of inflammation. To our knowledge, the present study is the first to explore bone health benefits in postmenopausal smokers after BB and BL consumption.

PARTICIPANTS AND METHODS

Participants

Guidelines of the Declaration of Helsinki were followed when conducting this clinical study and

the clinical protocol was approved by the University of Arkansas Institutional Review

Committee (Fayetteville AR, USA). Participants had to be healthy, 1-10 years postmenopausal,

and not on hormone replacement therapy for at least 6 months before initiation of the study.

Participants were not on medication affecting calcium or bone metabolism, and have a BMD t-

score above 2.5 SD of the mean at all sites. In addition, participants with chronic diseases

including skeletal disorders, diabetes mellitus, cardiovascular disease, gastrointestinal/digestive

problems, and respiratory problems, thyroid disorder, and impaired liver function were excluded.

Also excluded from the study were: participants on anabolic steroids, corticosteroids or

glucocorticoids, medications for bone, or hormone replacement therapy, and women consuming

a cup or more of blueberries or blackberries. A total of 212 women responded to advertisement

on local radio and newspaper, fliers, and brochures. Sixty five eligible women were enrolled in

the study. Participants signed an informed consent and completed a health and medical history

questionnaire related to the exclusion criteria. Recruited participants were smokers and

nonsmokers. Smokers (N=44) were defined as smoking daily and nonsmokers (N=21) were not

current smokers and none of those recruited had smoked in the previous 10 years.

Study design

The study design is summarized in **Figure 5.1**. Smokers were randomly assigned to one

of three groups: smoker control (n=21), smoker + 45 g/day freeze-dried blackberries (n=8), or

smoker + 45 g/day freeze-dried blueberries (n=16) and nonsmokers (n=20) was a control group.

Participants in berry groups were provided with 45 g BL and BB (WaterShed Foods Gridley, IL

USA) pre-weighed and vacuum-packed in moisture-proof bags to ensure food safety, retention of nutrition value, and for ease of storage and transportation. Participants were asked to consume 45 g of their respective berries daily. Participants on berry regimens were advised to adjust their daily food intake to account for the energy provided by the freeze-dried berries. Calendars were provided to record days on which berries were consumed and participants requested to return any unused berries in preceding visits. At baseline and after 9 months, BMD was measured, and at baseline, 3, 6 and 9 months, venous blood samples and 2nd void urine samples were collected.
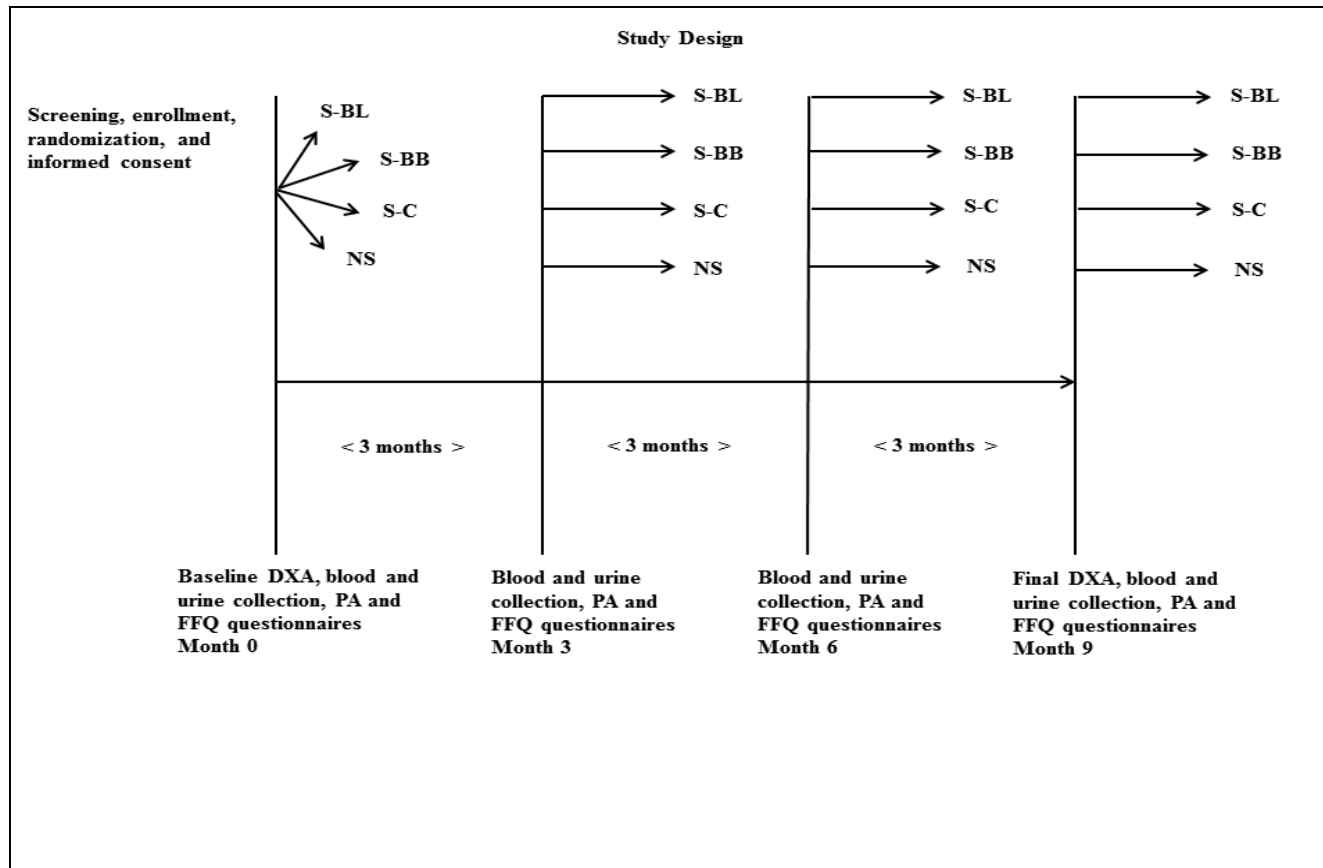
Figure 5.1. Study design showing the effects of daily consumption 45 g of freeze-dried blackberries and freeze-dried blueberries studied in postmenopausal smokers during a 9 month intervention. Abbreviations used: S-BL: smokers + 45 g blueberries/day; S-BB: smokers + 45 g blackberries/day; S-C: Smokers control group; NS-C: nonsmokers control group.

Nutrient and phenolic composition of freeze-dried berries

Macro and micro-nutrient composition of 45 g of FBB and FBL were analyzed by Nutrition Pro® (Axxya Systems; Stafford, TX) software based on USDA references. Proximate analyses of blackberries and blueberries were performed in duplicates by the Central Analytical Laboratory at the University of Arkansas (Poultry Science Center, Fayetteville AR). In addition flavonoid analysis of FBB and FBL was performed in triplicates using high performance liquid chromatography (HPLC) confirmed by mass spectroscopy methods as described by Cho and others [22].

Anthropometric measurements, dietary and physical activity assessment

At baseline, nutrition and medical histories were obtained from each participant by a trained staff member. Similarly, anthropometric data were collected at 0, 3, 6 and 9 month visits by the same trained staff member and a 114-item, 7-day food frequency questionnaire (FFQ) was administered to assess dietary intake. Nutrition data was analyzed by Nutrition Pro® (Axxya Systems; Stafford, TX) software based on USDA references. Nutrients of interest are presented in **Table 5.1.** The Five-City Physical Activity Recall was used to assess participants' seven-day hours of physical activity, sleep and other domestic and occupational activities [23] at 0, 3, 6, and 9 month time intervals. Data collected was used to compute hours spent on physical activity (PA) per week.

Bone biochemical measurements

Second void urine samples were collected, measured, aliquoted into 1 ml and stored at -20° C till analysis. Venous blood was obtained by a phlebotomist; 4 ml sample for plasma and 6 ml sample for serum after an overnight fast at baseline, 3, 6, and 9 months. Blood samples were

centrifuged at 3500 g for 15 minutes at 4° C, and the resultant plasma and serum aliquoted into 200 μl samples and stored at -80° C till analyses. Urine samples were used in evaluation of urinary deoxypyridinoline (DPD) which is biomarker of bone resorption. Urinary creatinine, which is required to normalize urinary DPD, was measured using a calorimetric assay (Quidel Corporation, San Diego, CA, USA). Two markers of bone turnover, serum bone-specific alkaline phosphatase (BALP) and serum osteocalcin (OC) were assessed using Elisa kits (Quidel Corporation, San Diego, CA, USA).

Assessment of oxidative stress and inflammation

Effects of berry treatments on oxidative stress levels were assessed using thiobarbituric acid reactive substances (TBARS), a biomarker of lipid peroxidation. TBARS were determined in duplicate using TBARS assay kit (Cayman Chemical; Ann Arbor, Michigan). To measure the potential anti-inflammatory effects of FBL and FBB, high-sensitivity C-reactive protein (hsCRP) was evaluated using a latex particle enhanced immuneturbidimetric assay measured on an ACE Alera clinical chemistry system (Alfa Wasserman, Inc.; West Caldwell, NJ).

Bone density measurements

At the beginning and end of the treatment period, BMD and bone mineral content (BMC) were obtained using dual X-ray absorptiometry (DXA; GE Lunar DXA, Waukesha, WI). The densitometry was equipped with enCore software for whole body, lumbar spine (L1-L4), hip and forearm BMD and BMC measurements. To ensure stability of the densitometer and accuracy of the data, the apparatus was tested using phantom scans before every data acquisition.

Statistical analysis

Since measurements were taken at four time points (months 0, 3, 6 and 9), a split plot model was used with treatments (i.e. NS, S-C, and S-BL and S-BB groups) as whole plot and time as a split plot. Least square means (LSM) were compared for significance and where significant effects were determined, means were compared. Data are reported as LSM ± SEM and statistical significance set at $P < 0.05$. Data analysis was done using ANOVA using PROC MIXED in SAS (Version 9.2; SAS Institute, Cary, NC, USA). A 2 sample t-test was also done to compare baseline whole body, lumbar spine, and femurs, and forearm BMD and BMC measurements of smokers versus nonsmokers.

RESULTS

Macronutrient and phenolic composition of freeze-dried berries

Results of proximate analysis were as follows: carbohydrates 88.4% and 92.1%; crude fiber 27.7% and 12.5%; protein 6.4% and 3.6%; and fat 6.3% and 2.3% for blackberry and blueberry respectively. Percent carbohydrates were calculated using the formula: 100 – percent protein –percent fat. The energy and macronutrients provided by 45 g of FBB and FBL computed from these percentages in addition to those calculated by nutrition software are shown on **Table 5.1**.

Table 5.1. Nutrient and phenolic compounds composition of 45 g of freeze-dried blackberries and blueberries obtained by Nutrition Pro® software, proximate analysis and HPLC analysis.

Nutrients	Freeze-dried blackberries		Freeze-dried blueberries	
	Calculated	Proximate analysis	Calculated	Proximate analysis
Energy (kcal)	118.94	195.00	97.95	185
Total fat (g)	1.07	3.00	0.88	1.00
Total carbohydrates (g)	28.96	39.00	23.85	42.00
Protein(g)	1.84	3.00	1.52	2.00
Fiber (g)	10.44	12.00	8.6	6.00
Crude fiber (g)	5.59	-	4.61	-
Vitamin E (IU)	2.19	-	1.81	-
Vitamin A (RE)	26.41	-	21.75	-
Vitamin C (mg)	74.86	-	61.65	-
Folate (µg)	53.19	-	43.8	-
Ca (mg)	46.81	-	38.55	-
P (mg)	39.89	-	32.85	-

K (mg)	384.32	-	316.5	-
Fe (mg)	1.06	-	0.88	-
Phenolic composition*		**HPLC analysis**	-	**HPLC analysis**
Total anthocyanins (mg)	-	284.085 ± 3.915	-	652.14 ± 53.865
Total flavonols (mg)	-	33.48 ± 0.225	-	35.919 ± 3.06
Total ellagitannins (mg)	-	111.96 ± 0.72	-	-
Chlorogenic acid (mg)	-	-	-	72.9315 ± 1.89

Calculated values from Nutrition Pro® (Axxya Systems, Stafford, TX); analysis based on USDA references. Proximate analyses performed in duplicates by the Central Analytical Laboratory at the University of Arkansas. ***** HPLC phenolic composition performed in triplicates; values are mean ± SEM

Estimates of the major phenolics consumed in 45 g of FBB and FBL per day are summarized in **Table 5.1.** In terms of the phenolic profile of FBB, 5 different anthocyanins were identified but approximately 87% of the anthocyanins were cyanidin-3-glucoside. In addition, 9 quercetin glycosides, and 4 ellagitannin compounds, were determined. Twenty nine different phenolic compounds were identified in FBL. Of these, 17 were anthocyanins, mainly, cyanidin, delphinidin, malvidin, peonidin, and petunidin glycosides. Six flavonols compounds determined in FBL were quercetin and myricetin glycosides.

Participants

Fifty women completed the 9 month study but data used was for 49 of the participants (31smokers and 18 nonsmokers). The overall attrition rate was 24.6%. The most common reasons for dropping out of the study were: inconvenience (n=6), medical reasons (n=2), relocation (n=1), and inability to consume berries as prescribed (n=5), and quit smoking (n=2). Attrition was noted to be highest at the 3 month visit (n=11) compared to 6 month (n=3) and 9 month (n=2) visits. Attrition affected the experimental groups as follows: S-C, S-BB, S-BL and NS reduced by 9, 2, 3 and 2, women respectively.

Baseline characteristics, anthropometric measurements, physical activity measurements and dietary intake

Characteristics, anthropometrics measurements are presented by treatment group in **Table 5.2.** The participants were predominantly white (98%) aged 46.5-68.0 years and the median age was 54.5 years. Baseline characteristics were similar for all smokers in the study. The average age of the NS-C was 4 years greater than each of the other experimental groups ($p = 0.0166$). All nonsmokers still fit the 1-10 years postmenopausal range specified for eligibility

into the study. Since PA improves bone function and strength, data was analyzed to detect

differences among experimental groups for number of hours spent on PA. No significant

differences existed in the activity levels of the women throughout the study period.

Table 5.2. Baseline characteristics of study participants

Measures	NS-C (n=20)	S-C (n=21)	S-BB (n=8)	S-BL (n=16)	*p* value
Age (yrs)	58.0 ± 0.9^b	54.4 ± 0.9^a	54.3 ± 1.6^a	54.2 ± 1.1^a	0.0166
Years postmenopausal	7.4 ± 0.7	5.6 ± 0.7	6.9 ± 1.2	6.7 ± 0.8	0.2049
Weight (kg)	66.0 ± 3.8	72.9 ± 3.6	68.2 ± 6.5	76.5 ± 4.4	0.2980
BMI (Kg/m^2)	23.7 ± 1.5	27.8 ± 1.4	25.2 ± 2.5	29.3 ± 1.7	0.1476
Physical activity (hrs/wk)	1.8 ± 0.3	1.6 ± 0.3	1.7 ± 0.6	1.59 ± 0.4	0.9533
Cigarettes/day	-	17.5 ± 1.7	17.9 ± 3.0	16.1 ± 2.1	0.7378
Body fat (%)	37.2 ± 1.7	41.5 ± 1.7	38.9 ± 3.8	40.2 ± 1.9	0.3668

Values represent means ± SEM. Values within a row that do not share the same letters are significantly different from each other ($P < 0.05$). Abbreviations used: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB: smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day. With the exception of age, there were no significant differences observed between the experimental groups for these measures at 3, 6 and 9 months

All *P*-values of time and treatments and their interaction derived from 7d FFQ data is presented in **Table 5.3**. Treatment effect was significant for vitamin A intake. S-C, S-BL, and S-BB groups consumed significantly lower amounts of vitamin A when compared to NS-C group. Consumption of dietary fiber, vitamin E and vitamin D were significantly different based on month and this may be due to seasonal variations. Means of total energy, vitamins and minerals as analyzed from the FFQ are presented in **Appendixes 3, 4 and 5**.

Based on a 2000 kcal diet, 45 g of FBB and FBL were estimated to contribute 5.9% and 4.9%, respectively to participants' total energy intake. Actual percent contribution of total energy based on the mean total energy intakes was 5.6% and 4.8%, for FBB and FBL, respectively. Blueberries had better acceptance than blackberries due to the large amounts of seeds in BB. Nonetheless, both berries were well tolerated. The mean self-reported compliance for S-BL and S-BL groups were 85% and 87% respectively.

Table 5.3. Entries are p-values for the F-tests of treatments and time on daily total energy and nutrient intake in the ANOVA.as calculated from a 7 d FFQ.

	Total energy	Protein	Total fat	Carbohydrates	Dietary Fiber	
Treatments	0.7674	0.9698	0.2577	0.6808	0.1413	
Time	0.7937	0.1896	0.9187	0.2770	**0.0121**	
Treatment*Time	0.7403	0.8888	0.8099	0.7808	0.2183	
	Vitamin C	**Vitamin A**	**Vitamin E**	**Vitamin K**	**Vitamin D**	
Treatments	0.3726	**0.0011**	0.0618	0.4782	0.0621	
Time	0.0654	0.6624	**0.0017**	0.5234	**0.0222**	
Treatment*Time	0.3151	0.8415	0.5920	0.6802	0.8145	
	Calcium	**Magnesium**	**Potassium**	**Phosphorus**	**Sodium**	**Biotin**
Treatments	0.1097	0.1045	0.4291	0.3482	0.8963	0.0929
Time	0.5182	0.5526	0.3170	0.4091	0.8335	0.4697
Treatment*Time	0.9053	0.8037	0.8676	0.9695	0.6778	0.2058

Treatments were: nonsmokers control group, smokers control group, smokers + 45 g blackberries/day and smokers + 45 g blueberries/day. Time was 0, 3, 6, and 9 months, and treatment*time is the interaction of treatment and time. Significance was established at $p < 0.05$.

Bone mineral density and content

A 2 sample *t*-test comparing baseline whole body, lumbar spine (L1-L4), hip and forearm BMD and BMC measurements showed no significant differences between smokers and nonsmokers (data not shown). After the 9 mo. treatment period, bone loss of total body BMD was statistically significant in S-C and S-BL groups, but not in S-BB and NS-C groups ($P = 0.0284$). Blackberries but not blueberries demonstrated modest bone protective effects in preventing smoking-induced bone loss. Both freeze-dried fruit regimens had no significant changes on BMC, and BMD of the lumbar spine, right and left femur and ulna.

Biomarkers of bone metabolism, TBARS and hsCRP

Berry consumption had no significant effect on neither urinary nor serum biomarkers of bone metabolism BAP and OC (**Appendix 6**). DPD is a stable product of lysine breakdown during bone resorption. Though not to a significant degree, DPD levels remained somewhat stable for the S-BB group, but slightly fluctuated over the nine month periods for the other experimental groups. Similarly, this trend was observed with the two serum biomarkers of bone turnover, BAP and OC for all groups.

Berry regimens had no significant effects on oxidative stress or inflammation levels in postmenopausal smokers as shown on **Appendix 7**. Levels of plasma hsCRP, a reliable marker of inflammation, fluctuated in the four experimental groups over 0, 3, 6, and 9 month periods.

TBARS a biomarker of lipid peroxidation decreased in the S-BB and S-BL groups and remained low thereafter compared to the levels in S-C and NS groups which increased in a time dependent manner.

DISCUSSION

There is paucity of studies regarding potential bone health benefits of blackberries and blueberries. Nonetheless, two studies [16, 17] suggest that BB and BL may suppress bone turnover and prevent bone loss in animal models.

Characteristic of ideal randomization, all smokers started with similar baseline attributes and they remained unaffected by berry intervention throughout the study. Considering data analyzed from the FFQ, there were two main observations: first, freeze-dried BB and BL supplied 12.5g and 6 g of fiber respectively which may have enhanced weight control [24] and prevented weight gain. Secondly, vitamin A, known to suppress bone resorption and stimulate bone formation [25, 26, 27] may have contributed to reduced bone loss in N-SC group.

In terms of BMC and BMD, we propose that age of nonsmokers' and lack of statistical power due to small sample sizes were possible causes for lack of differences in baseline BMD measures of smokers versus nonsmokers. However, as expected and evidenced by reduced total BMC and BMDs at all sites, all experimental groups lost bone within the 9 month period. However, S-C group lost significantly more bone mass than the S- BB and NS-C group but not S-BL group. Notably, the ulna experienced more bone loss than all other sites in all groups indicating increased fracture risk at this site. Overall nonsmokers had the least percent bone loss which is consistent with epidemiological findings that smoking aggravates bone loss [7]. Findings of this study suggest that blackberries showed modest bone protection of only total body BMD and blueberries had no effect on bone health. Blackberries supplied 284 mg of ACY, 33 mg of flavonols and, 112 mg of ellagitannins daily while blueberries supplied 652 mg ACY, 36 mg flavonols and 73 mg of chlorogenic acid daily. Although it is plausible that the ACY profile of the

berries may be a major factor, BB just like BL did not have a profound effect on BMD in other sites measured. These findings are contrary to others [20, 28] that demonstrate bone health benefits of consuming fruits and vegetables. Nonetheless, since only smokers received berries in this study the question remains as to whether these findings would be similar in nonsmokers.

Mechanisms by which berries may exert health benefits were investigated by measuring both markers of bone formation and bone resorption. Levels of BAP and OC, now considered bone turnover markers [29-31] are generally correlated with bone mass and describe rates of bone formation or resorption. Contrary to our expectations, berry regimens had no effect on formation and resorption rates in postmenopausal smokers matching the results of bone mass. In the S-BB group, levels of urinary DPD indicated a reduced rate of bone breakdown albeit not to a significant degree. Nonetheless, we can reasonably propose that the mechanism by which BBs prevented bone loss may involve suppression of bone resorption. This is a mechanism that has been postulated in other studies that have demonstrated protective effects of dried plum [19, 32].

Regarding oxidative stress, we expected that berry antioxidants would improve the basal level of oxidation that accompanies normal cellular metabolism in smokers. After three months of berry consumption, concentrations of TBARS in the berry groups (irrespective of the berry type) remained stable compared to control groups. Although this did not achieve statistical significance, it is indicative that berry regimens may prevent oxidative stress in postmenopausal smokers. To strengthen the argument, we observed that, TBARS concentrations in the control groups increased in time-dependent fashion as expected due to smoking[33] (S-C group) and estrogen loss[34](S-C and NS-C groups). With respect to inflammation, smokers had higher levels of hsCRP, similar to

findings by Gan et al., [35]. Berry regimens did not suppress inflammation resulting from smoking and postmenopausal status even though berry phenolic compounds and anthocyanins in particular have demonstrated anti-inflammatory properties in animal models [36, 37]. Conflicting findings concerning effects of phenolic compounds are reported in other studies [38,-40]. Karlsen et al., [41] found no effect on CRP concentrations after daily consumption of 300 mg/d of blueberry anthocyanins in a 3 week study using healthy nonsmokers.

Overall, outcomes of this study are atypical due to growing evidence that dietary antioxidants exert modest antioxidant effects *in vivo* and improve bone indices[14, 16, 17, 42]. Berry intervention may have been affected by one or a combination of several factors: (i) composition of gut microbiota in smokers receiving berries [43], (ii) our relatively small treatment groups which affected statistical power, (iii) smoking status of participants, and (iv) absorption and bioavailability of berry bioactive compounds and their metabolites upon which potency is dependent. BB and BL absorption and metabolism though unclear, is known to be different [40, 44, 45] and may influence health benefits.

CONCLUSION

In conclusion, the findings of this study and the existing literature on berry bioactive compounds are conflicting. Blackberries demonstrated only modest protection on total BMD and had no effect on other parameters; while and blueberries had no positive effects on all parameters determined in postmenopausal smokers. Future studies are necessary to explore mechanisms by which berries prevent bone loss and effective doses in both postmenopausal smokers and nonsmokers.

ACKNOWLEDGEMENTS

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V. SUMMARY AND CONCLUSIONS

This conclusions chapter gives a summary of each of the chapters of this dissertation.

The introduction and literature review establishes that postmenopausal osteoporosis is a public health burden that manifests in fragility fractures and affects over 200 million women worldwide [1]. In the United States alone nearly half of all women over the age of 50 suffer osteoporosis-related fractures, i.e. more than 1.5 million fractures a year [2].

Additionally, smoking in postmenopausal women increases the risk factor of osteoporosis [3] by reducing active vitamin D in plasma[4], causing malabsorption of calcium[5], and increased oxidative stress[6] and inflammation[6, 7]. Moreover, oxidants from cigarette smoke down regulate the expression of important genes that mediate bone formation and essential for bone structure [8].

Approximately 34,000 hip fractures that occur in the United States annually are attributable to smoking [9]. In addition, in females smoking can reduce bone mass at hip, lumbar spine, forearm and calcaneus, and increase the lifetime risk of hip fracture and lumbar spine by 31% and 13% respectively [7]. Moerover, heavy smoking in women, throughout adult life can result in 5-10% less bone by the age of 45-50 years, compared to non-smokers [5].

Also, osteoporosis drug therapies fall into two categories: the antiresorptive medications that slow the breakdown of bone and the anabolic treatments that help build bone. These drugs are effective and work with the body systems to protect bone mineral density and reduce the risk of fractures [10]. However, due to several factors including cost, fear, and side effects associated with these drugs, approximately 50% of patients

discontinue medication or fail to adhere to treatments regimens in the first year of use [10, 11].

The literature review emphasizes that phenolic compounds, particularly anthocyanins and flavonols found in fruits and vegetables have antioxidative and anti-inflammatory capacities [12]. Hence, consumption of diets rich antioxidants is associated with health benefits. Blueberry and blackberry phenolics function as antioxidants by easily donating hydrogen atoms from their aromatic hydroxyl groups and by supporting unpaired electrons [13].

The chapter on the review of blackberries reveals that blackberries are rich in phenolic compounds and their composition and concentration is influenced by several factors including variety, location of cultivation, and maturation. Blackberries are a rich source of phenolics including, anthocyanins [18], ETs [19], flavonols [20], flavan-3-ols, and procyanidins [21]. The berries also contain appreciable levels of phenolic acids [22] and low levels of lignans [23]. Total phenolics in blackberries have been shown to range from 114 to 1056 mg/100 g fresh weight[22].

Finally, the review demonstrates that blackberry phenolic compounds have protective effects against age-related neurodegenerative diseases, and bone loss in vivo, and inhibits LDL and liposomal oxidation *in vitro*. However, more research is needed to explore their metabolism, bioavailability and the mechanisms by which they confer health benefits. Furthermore, studies are necessary to elucidate the *in vivo* effective concentrations of blackberry phenolic compounds.

The animal study was based on the hypothesis that lowering oxidative stress by consumption of antioxidant rich foods may prevent bone loss by attenuating osteoclastogenesis and bone resorption [14-17]. Findings of the animal study demonstrate

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that blackberries supplemented in the diet at the level of 5% (w/w) may modestly reduce ovarian hormone-induced bone loss. Although the mechanism is still unclear, it is reasonable to speculate that the compounds work by lowering oxidative stress.

In chapter five, findings of the clinical study in which postmenopausal smokers consumed 45g of blueberries or blackberries were inconclusive, showing only modest protection on total BMD by blackberries and not blueberries. Effects of blackberry and blueberry consumption on bone health need further elucidation in pre- and postmenopausal smokers and nonsmokers.

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Appendix 1. Health and Medical History Questionnaire

Subject ID: _____

Interviewer: _____ Date_____

Age_____ Height_____ Weight_____ BMI_____

I. Medical History

A. Skeletal Health

Personal history of skeletal disorders:
      1. Not known _____
      2. Yes: uncontrolled _____
      3. Yes: Medications _____
      4. Yes: Exercise program _____
      5. Yes: Modified diet _____
      6. Yes: Surgery _____
      7. Yes: Combined program _____
      Give details _____
      Type of Medication(s)_____
      Current dosage _____ Years taken _____
      How does this condition affect your activity?

Family history of skeletal disorders:

      1. None _____
      2. One parent _____
      3. Both parents _____
      4. One close relative _____
      5. More than one close relative _____

      Relative(s) _____

      Comments: _____

B. Cardiovascular Function
Personal history of cardiovascular disease:
      1. Not known _____
      2. Yes: uncontrolled _____
      3. Yes: Medications _____
      4. Yes: Exercise program _____
      5. Yes: Modified diet _____
      6. Yes: Surgery _____
      7. Yes: Combined program _____
      Give details _____

Type of Medication(s)_____

Current dosage _____ Years taken _____

How does this condition affect your activity?

Family history of cardiovascular disease:
1. None _____
2. One parent _____
3. Both parents _____
4. One close relative _____
5. More than one close relative _____

Relative(s) _____

Comments: _____

C. Hypertension
1. None known _____
2. Yes: uncontrolled _____
3. Yes: Medications _____
4. Yes: Exercise program _____
5. Yes: Modified diet _____
6. Yes: Yes: combined program_____
7. Most recent blood pressure _____

Explain_____

Type of Medication(s)_____

Current dosage _____ Years taken _____

Ever taken thiazide diuretics? _____

D. Diabetes
1. No record or indication _____
2. In past, but not now _____
3. Yes, well controlled _____
4. Yes, not controlled _____

Explain _____

Type of Medication(s)_____

Current dosage _____Years taken_____

How does this condition affect your activity? _____

E. Gastrointestinal/Digestive Problems
1. No record or indication _____
2. In past, but not now _____
3. Yes, well controlled _____
4. Yes, not controlled _____

Explain _____

Type of Medication(s)_____

Current dosage _____Years taken_____

Ever taken steroids (i.e., prednisone)? _____

Currently taking antacids? _____

How does this condition affect your activity? _____

F. Liver Disease/ Problems
           1. No record or indication _____
    2. In past, but not now _____
    3. Yes, well controlled _____
    4. Yes, not controlled _____
    Explain _____
    Type of Medication(s)_____
Current dosage _____Years taken_____
How does this condition affect your activity? _____

G. Respiratory Problems
           1. No record or indication _____
    2. In past, but not now _____
    3. Yes, well controlled _____
           4. Yes, not controlled _____
    Explain _____
    Type of Medication(s)_____
    Current dosage _____Years taken_____
    How does this condition affect your activity? _____

H. Thyroid Disorder
    1. No record or indication _____
    2. In past, but not now _____
    Hyper? _____   Hypo? ___
    Explain _____
    Type of Medication(s)_____
    Ever taken thyroid hormones (i.e., Synthroid)? _____

I.   Reproductive history: LMP_____, No. of children_____

II. Medication or Drug Use
A. Previous or Present Use of Any of the Following (Specify):
           1. Anabolic steroids _____
    2. Corticosteroids or glucocorticoids _____prednisone _____
    3. Thiazide diuretics _____   Other diuretics _____
    4. Vitamin D
    5. Medications for bone   Fosamax:
               Evista (Roloxaphin): _____
               Miacalcin (Calcitonin): _____
               Teriparatide (Forteo):_____
    6. Others:

               _____
               _____
               _____

B. Previous or Present Use of Alcoholic Beverages (Beer, wine, hard liquor).
    Please indicate:

Frequency of intake (Times/week or times/month): _____
Number of servings at a sitting: _____
Number of years of use: _____

C.   *Estrogen or Hormone Replacement (ERT or HRT)*
     1. Never ___ move along to section D.
     2. Yes, in past ___ at what age? ___ & How long _____

D. *Currently /previously a smoker?_____If yes, number of cigarettes per*
     day_____

III. Physical Activity

A. Occupational Intensity (respond to 1, 2, 3, or 4):
     1. Majority of time: Sitting ___   Standing ___   Walking ___
     2. Equal amount of time: Sitting and Standing ___
                               Walking and Sitting ___
                               Standing and Walking ___
     3. Combination: Sitting, Standing, and Walking ___
     4. Much of time: Lifting & Carrying ___

Appendix 2. Telephone Screening Exclusion Criteria

Effect of Dried Fruits on Indices of Bone Status in Postmenopausal Women

Interviewer: study coordinator or trained personnel
Hello,
Good morning/good afternoon/good evening
How are you?
Thank you for your interest in our study
Is it a good time for me to go over a few questions with you now?
Are you a postmenopausal woman? If yes, when was your last period?
Do you know if you have current or previous history of:
Bone disorders (osteomalacia, severe osteoporosis, unexplained or stress fractures)
cancer
Diabetes mellitus (only Insulin Dependent)
Eating disorders (anorexia nervosa or bulemia)
Gastrointestinal or chronic digestive disorders (chrohn's disease, ulcerative collitis)
Hypertension (exclude if taking thiazide diuretics)
Active liver disease
Parathyroid disorders
Renal disease/problems
Thyroid disease/problem
Other current illnesses?

Are you taking or have taken any of the following medications since the time of cessation of your menses?

	Glucocorticoids
Anabolic steroids (testosterone, androgens)	
Anticonvulsants	Current use of oral contraceptives
Calcitonin	Progestagens, progesterone (HRT)
Bisphosphonates	
Estrogens (estrase, premarin, prempro)	
Teriparatide (Forteo)	Recent (<6 months) initiation on of thyroid hormones

Are you a smoker? If so, how many cigarettes per day?

Appendix 3. Seven Day Food Frequency Questionnaire

Subject Number_____   **Date**_____

<u>Vitamin and Mineral Supplement</u>

1. Do you take any vitamin or mineral supplement(s)?   Yes ___No _____

2. If **Yes**, please, list all **names** of vitamin or mineral supplements, and **how often** do you take

the supplement(s)?

Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week

This questionnaire asks you about your consumption of foods and beverages over the past week, which includes the time from exactly one week ago until the last meal you had before you fill out this questionnaire. The "How Often" columns are for day, week, or rarely/never. We want you to think back over the past week and tell us how many times (per day, if you consume the item every day, or per week) you consumed each item. A medium serving is in parentheses.

EXAMPLES:
Ate 1/2 grapefruit about twice last week.
Ate 1 large hamburger four times last week.
Drank 2 cups of whole milk each day.

Type of Food	How Often			Size		
(Medium Serving)	Day	Week	Rarely/ Never	S	M	L
Grapefruit (1/2)		2			X	
Hamburger, regular (1 patty, 3 oz)		4				X
Whole milk (1 cup, 8 oz)	2				X	

Type of Food	How Often			Size		
(Medium Serving)	Day	Week	Rarely/ Never	S	M	L
DAIRY FOODS						
Whole milk (1 cup, 8 oz)						
2% milk (1cup, 8 oz)						
Skim milk (1 cup, 8 oz)						
Cream, whipped (1 Tbsp)						
Sour cream (1 Tbsp)						
Coffee cream (1 Tbsp)						

Type of Food	Day	Week	Rarely/Never	S	M	L
Ice cream (½ cup)						
Low fat ice cream (½ cup)						
Frozen yogurt (½ cup)						
Yogurt (1 cup)						
Low fat yogurt (1 cup)						
Cottage cheese (½ cup)						
Cream cheese (1 oz)						
Low fat cream cheese (1 oz)						
Other cheese (1 slice or 1 oz)						
Low fat cheese (1 slice or 1 oz)						
Margarine (1 tsp)						
Butter (1 tsp)						
Reduced fat margarine (1 tsp)						
FRUITS, FRUIT JUICES						
Raisins (1 oz or 1 sm box)						
Grapes (20)						
Prunes (½ cup)						
Bananas						
Cantaloupe (¼ melon)						
Watermelon (1 slice)						
Apples, applesauce or pears (1 fresh, ½ cup)						
Dried Apples (1 oz)						
Apple juice (½ cup)						
Oranges						
Orange juice (½ cup)						
Grapefruit (½ cup)						
Grapefruit juice (½ cup)						
Other fruit juices (½ cup)						
Strawberries—fresh, frozen, or canned (½ cup)						
Blueberries—fresh, frozen, or canned (½ cup)						
Type of Food	**How Often**			**Size**		
(Medium Serving)	**Day**	**Week**	**Rarely/ Never**	**S**	**M**	**L**
Peaches (1 fresh, ½ cup canned)						
Apricots (1 fresh, ½ cup canned)						
Plums (1 fresh, ½ cup canned)						
Honeydew melon (¼ melon)						

VEGETABLES, **VEGETABLE JUICE**						
Tomatoes (1)						
Tomato juice (½ cup)						
Tomato sauce (½ cup)						
Spaghetti sauce (½ cup)						
Red chili sauce, taco sauce, or salsa (1 Tbsp)						
Tofu or soybeans (3-4 oz)						
String beans, green beans (½ cup)						
Broccoli (½ cup)						
Cabbage (½ cup)						
Cole slaw (½ cup)						
Cauliflower (½ cup)						
Brussels sprouts (½ cup)						
Carrots, raw (½ carrot or 2-4 sticks)						
Carrots, cooked (½ cup)						
Corn (1 ear or ½ cup frozen or canned)						
Peas (½ cup fresh, frozen or canned)						
Lima beans (½ cup frozen, or canned)						
Mixed vegetables (½ cup)						
Beans or lentils, baked or dried (½ cup)						
Summer or yellow squash (½cup)						
Winter squash (½ cup)						
Zucchini (½ cup)						
Yam or sweet potato (½ cup)						
Spinach, (cooked ½ cup, raw 1 cup)						
Iceberg lettuce, romaine or leaf (1 cup)						
Celery (4" stick)						
Beets (½ cup)						
Alfalfa sprouts (½ cup)						
Kale, mustard, or chard greens (½ cup)						
Vegetable, vegetable beef, minestrone or tomato soup (1 cup)						

Type of Food	How Often			Size		
(Medium Serving)	**Day**	**Week**	**Rarely/ Never**	**S**	**M**	**L**
EGGS, MEAT, ETC.						
Eggs (2)						
Chicken or turkey, roasted or broiled						

with skin (3-4 oz)						
Chicken or turkey, roasted or broiled skinless (3-4 oz)						
Chicken, fried with skin (3-4 oz)						
Bacon (2 slices)						
Hot dogs (2)						
Low fat hot dogs (2)						
Sausage (2 patties or 2 links)						
Bologna (1 slice)						
Other processed luncheon meat (1 slice)						
Liver, chicken or beef (3-4 oz)						
Hamburger, regular (1 patty, 3-4 oz)						
Hamburger, lean (1 patty, 3-4 oz)						
Meat loaf (3-4 oz)						
Pork, chops, roasts (3-4 oz)						
Lamb (3-4 oz)						
Beef, roast, steak (3-4 oz)						
Beef stew with vegetables (1 cup)						
Ham (3-4 oz)						
Tuna fish (3-4 oz)						
Tuna salad (½ cup)						
Fish, baked or broiled (3-4 oz)						
Fish, fried or fish sandwich (3-4 oz)						
Shrimp, Lobster, Scallops						
Pizza (2 slices)						
Mixed dishes with cheese (1 cup)						
Lasagna or meat pasta dishes (1 cup)						

Type of Food	How Often			Size		
(Medium Serving)	Day	Week	Rarely/ Never	S	M	L
BREADS, CEREALS, STARCHES						
Cold breakfast cereal (1 cup)						
Cold breakfast cereal—fortified (1 cup)						
Cooked oatmeal (1 cup)						
Other cooked breakfast cereal (1 cup)						
White bread (1 slice)						
Pita bread (1 piece)						
Dark bread (1 slice)						
English muffin (1)						
Bagel (1)						
Dinner roll (1)						
Hamburger or hotdog bun (1)						
Muffin (1)						
Biscuit (1)						
Corn bread, corn muffin (1)						
Brown rice (1cup)						
White rice (1cup)						
Spaghetti noodles (1 cup)						
Macaroni noodles (1 cup)						
Other pasta noodles (1 cup)						
Bulgar, kasha, couscous (1 cup)						
Pancakes or waffles (2)						
Potatoes, french fries or fried (½ cup)						
Potatoes, baked or boiled (1)						
Mashed potatoes (1 cup)						
Potato chips or corn chips (small bag or 1 oz)						
Saltine crackers (5)						
Saltine crackers, low sodium (5)						
Saltine crackers, fat free (5)						
Other crackers (5)						
Other crackers, low fat (5)						

Type of Food	How Often			Size		
(Medium Serving)	Day	Week	Rarely/ Never	S	M	L
BEVERAGES						
Regular soft drink (1)						
Diet soft drink (1)						
Caffeine free soft drink (1)						
Caffeine free, Diet soft drink (1)						
Lemonade or other non-carbonated drink (1 glass, bottle, or can)						
Water (1 cup)						
Coffee (1 cup)						
Decaffeinated coffee (1 cup)						
Tea (1 cup)						
Herbal tea (1 cup)						
Beer (1 glass, bottle, or can)						
Red wine (4 oz glass)						
White wine (4 oz glass)						
Whiskey, gin, or other liquor (1 drink or shot)						
SWEETS, BAKED GOODS, MISC.						
Chocolate (1 small bar or 1 oz)						
Candy bar (1 small bar)						
Candy without chocolate (1 oz)						
Cookies, home baked (2)						
Cookies, ready-made (2)						
Brownies (2)						
Doughnuts (2)						
Cake, home baked (1 slice)						
Cake, ready-made (1 slice)						
Sweet roll, coffee cake, or other pastry ready-made (1 serving)						
Sweet roll, coffee cake, or other pastry home baked (1 serving)						
Pie, homemade (1 slice)						
Pie, readymade (1 slice)						
Jam, jelly, preserves, syrup, or Honey (1 Tbsp)						
Peanut butter (1 Tbsp)						
Popcorn (1 cup)						
Popcorn, air popped (1 cup)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
Nuts (small packet or 1 oz)						
Bran, added to food (1 Tbsp)						
Wheat germ (1 Tbsp)						
Chowder or cream soup (1 cup)						
Oil and vinegar dressing (1 Tbsp)						
Mayonnaise or other creamy salad dressing, Regular (1 Tbsp)						
Mayonnaise or other creamy salad dressing, Low Fat or Reduced Calorie, Lite (1 Tbsp)						
Mayonnaise or other creamy salad dressing, Fat Free (1 Tbsp)						
Mustard, dry or prepared (1 tsp)						
Salt (1 shake)						
Pepper (1 shake)						

Can you think of any other food or drink that you had in the past week that was not on this form? If so, what was it? What was the amount? How many times did you have it this past week?

Food_____

Amount_____, **How often per day**_____, **per week**_____

Food_____

Amount_____, **How often per day**_____, **per week**_____

Food_____

Amount_____, **How often per day**_____, **per week**_____

Food_____

Amount_____, **How often per day**_____, **per week**_____

Nutrition History Questionnaire

Subject ID:_____

Interviewer:_____

Effect of Dried Fruits on Indices of Bone Status in Postmenopausal Women

Date:_____

Nutrition History

Modified/Specialized Diet(s)

Followed:_____

Recommended By_____ Length of time Followed_____

Recent Changes in Appetite?_____ Due to_____

Foods Avoided?_____ Due to_____

History of Problems Digesting Milk?_____ When?_____ How Long?_____

Diagnosis of Lactose Intolerance?_____ When?_____ By whom? _____

Intake of Milk/Dairy Products & Calcium-Containing Foods (No Times/Day, Wk or Month,

Portion Size):

Milk_____ Yogurt_____ Frozen Yogurt_____

Ice Cream/Milk_____ Pudding/Custard_____ Hard Cheese_____

Mixed Dishes with Cheese_____ Soft Cheese_____ Grated Cheese_____

Donuts/Cakes/Cookies_____ Eggs_____ Dark Breads_____

How has this intake changed during that past three years?

Intake of High Fiber Foods (Specify Type, No Times/Day, Week or Month, Portion Size):

Whole Grain Products: Breads_____ Bran (wheat, oat)_____

Cereals_____ Crackers_____ Grains (i.e., popcorn)_____

Nuts_____ Seeds_____ Fruits_____

Dried Fruits (prunes/dried apples)_____Vegetables_____

Beans/Legumes (i.e., chili)_____

How has this intake changed during the past three years?

Nutritional Supplements (Type, Dose, Times/Week, No Years):

Vitamin Supplements_____

Mineral Supplements_____

Vitamin/Mineral Supplements_____

Other Supplements (e.g., cod liver oil, protein powder)_____

Daily Intake *(Typical weekday)*

Time   Food/Description   Serving Size   Food Code

Sodium Intake (amount of salt used (tsp)/day): in cooking_____ at the table_____

Estimate of Na^+ Intake/Day: Salt Shaker_____ High Na^+ foods:_____

Beverages Consumed (servings per day):

Coffee: reg_____ decaf_____ Cocoa:_____ Tea: reg_____ decaf_____

Soda: reg (+caf)_____ diet(+caf)_____ reg (caf free)_____ diet (caf

free)_____

Milk (oz/day): reg (3.5%)_____ low fat (2%)_____ skim(1% or <1%)_____

During what period(s) of life have you been a milk drinker? As a(n):

____1. Child ____2. Teenager ____3. Young adult (20-29) ____4. Adult (30-39)

____5. mid-life adult (40-59) ____6. Older adult (60+)   ____7. Never

If you excluded milk during any part of your life, was (is) this due to digestive problems?

(Explain):_____

Milk consumed/day: child_____ teenager_____ young adult (20-29)_____ adult (30-39)_____

mid-life adult (40-59)_____ older adult (60+)_____*Juice* (type): _____ *Calcium*

fortified Juice: _____ *Other:*_____

*Alcoholic Beverages:*_____ If yes, please specify:

Type	Times/week or Mo	Serving Size	No Servings	Total/Week or Mo

Beer: Reg
　　　　Lite
　　　　Dark

Wine: White
　　　　Red
　　　　Rose
　　　　(Blush)

*Mixed Drink (*specify):
Other (specify):

How did your food or beverage intake differ from the above during your younger years (teenager and young adulthood)?

Appendix 4. **Physical Activity Recall Items**

Subject ID: _____   Interviewer: _____
Date: _____

First we would like to know about your physical activity during the past 7 days. But first, let me ask you about your sleep habits.

1. On the average, how many hours did you sleep each night during the last five weekday nights (Sunday-Thursday)? _____hours

2. On the average, how many hours did you sleep each night last Friday and Saturday nights? _____hours

  Now I am going to ask you about your physical activity during the past 7 days, that is, the last 5 weekdays and last weekend, Saturday and Sunday.

  We are not going to talk about light activities such as slow walking, light housework, or non-strenuous sports such as bowling, archery, or softball.

  Please look at this list which shows some examples of what we consider moderate, hard, and very hard activities. (interviewer: hand subject the following card and allow time for the subject to read it over.)

  People engage in many other types of activities, and if you are not sure where one of your activities fits, please ask me about it.

3.   First, let's consider moderate activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these moderate activities or others like them?

     Please tell me to the nearest half-hour. _____hours

4. Last Saturday and Sunday, how many hours did you spend on moderate activities and what did you do?

    (Probe: Can you think of any other sports, job, or household activities that would fit into this category?)
     _____hours

5. Now, let's look at hard activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these hard activities or others like them?

    Please tell me to the nearest half-hour. _____hours

6. Last Saturday and Sunday, how many hours did you spend on hard activities and what did you do?
(Probe: Can you think of any other sports, job, or household activities that would fit into this category?)
_____ hours

7. Now, let's look at very hard activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these very hard activities or others like them? Please tell me to the nearest half-hour.

(Probe: Can you think of any other sports, job, or household activities that would fit into this category?)
_____ hours

8. Last Saturday and Sunday, how many hours did you spend on very hard activities and what did you do?

(Probe: Can you think of any other sports, job, or household activities that would fit into this category?)
_____ hours

9. Compared with your physical activity over the past 3 months, was last week's physical activity more, or less, or about the same?
_____ 1. More
_____ 2. Less
_____ 3. About the same

Interviewer: Please list below any activities reported by the subject, which you don't know how to classify. Flag this record for review and completion.

Activity(brief description)   Hours: workday   Hours: weekend day

_____   _____   _____
_____   _____   _____
_____   _____   _____
_____   _____   _____

Examples of Activities in Each Category
Moderate activity (3-5 mets)

Occupational Tasks:
1) Delivering mail or patrolling on foot
2) House painting
3) Truck driving (making deliveries, lifting/carrying light objects

Household Activities:
1) Sweeping, mopping, cleaning windows
2) Mowing the lawn with a power mower
3) Raking the lawn and yardwork
4) Light carpentry

Sports:
(actual playing time)
1) Table tennis or Ping-Pong
2) Softball, baseball
3) Volleyball
4) Dancing: folk, square, aerobics (low impact & intensity)
5) Brisk walking (3 to 4 mile/hr; 15-20 min/mile)
6) Bicycling on level ground (10-15 mile/hr)
7) Golfing (walking and pulling/carrying own clubs)
Calisthenics exercise and weight lifting

Hard Activity (5.1 – 6.9 METS)

Occupational Tasks:
1) Heavy carpentry
2) Construction work

Household Tasks:
1) Scrubbing floors
2) Shoveling snow
3) Moving (lifting furniture and boxes)

Sports:
(actual playing time)
1) Racket Sports: badminton, paddleball, tennis (double)
2) Basketball
3) Rowing or canoeing leisurely
4) Dancing: disco, jazz, aerobics (medium impact & intensity
5) Power walking (>mile/hr; <15 min/mile) or hiking
6) Vigorous bicycling (16 – 20 mile/hr
7) Jogging (≥5 mile/hr)
8) Swimming
9) Roller or ice skating
10) Stationary bicycling

Very Hard Activity (≥7.0 METS)

Occupational Tasks:
1) Digging or chopping with heavy tools
2) Carrying heavy loads, such as bricks or lumber

Sports
(actual playing time)
1) Racket Sports: handball, racketball, squash, tennis (singles)
2) Soccer
3) Snow skiing (downhill and cross country)
4) Dancing: aerobics (high impact & intensity)

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5) Jumping rope
6) Vigorous bicycling on hillsJogging or running (≥8 mile/hr)

Physical Activity Recall

1. _____ HOURS week-day sleep

2. _____ HOURS week-end sleep   ——— Total (sum of #1 and #2) _____

3. _____ HOURS week-day moderate/5 = _____

4. _____ HOURS week-end moderate/2 = _____

5. _____ HOURS week-day hard/5 = _____

6. _____ HOURS week-end hard/2 = _____

7. _____ HOURS week-day very hard/5 = _____

8. _____ HOURS week-end very hard/2 = _____

9. 1 _____ 2 _____ 3 _____

     1. Total sleep hours _____ x 1 = _____

     2. Average = hours moderate act._____ x 4 = _____

     3. Average = hours hard act. _____x 6 =   _____
     4. Average = hours very hard act. _____ x 10 = _____

     5. Sum of hours _____ - 24 _____x 1.5   = _____

10. Sum of 1,2,3,4, & 5

Appendix 5. Daily Total Energy and Macro-nutrient Intake Calculated from a 7 D FFQ of Control Smokers and Nonsmokers, and Smokers Consuming 45 g of Blackberries and Blueberries at Baseline, 3, 6 and 9 Months.

	Month	NS-C	S-C	S-BB	S-BL
Total Energy (kcal)	0	2051.5 ± 142.5	1818.7 ± 206.9	2095.8 ± 212.1	2051.6 ± 154.7
	3	1997.8 ± 176.4	1789.9 ± 158.4	2367.5 ± 95.8	2141.8 ± 286.5
	6	2253.8 ± 257.0	1957.4 ± 277.9	2217.3 ± 254.2	1939.5 ± 147.0
	9	2139.9 ± 196.2	1953.6 ± 235.1	1855.6 ± 299.8	2026.4 ± 231.5
Protein (g)	0	89.0 ± 6.0	93.1 ± 13.1	88.7 ± 4.4	88.6 ± 11.3
	3	82.2 ± 6.4	79.9 ± 7.8	91.0 ± 3.6	88.7 ± 16.7
	6	86.2 ± 6.6	80.0 ± 11.7	87.9 ± 13.0	71.5 ± 5.5
	9	84.0 ± 5.9	82.0 ± 11.2	71.0 ± 14.7	76.1 ± 9.3
Total fat (g)	0	89.2 ± 8.7	72.8 ± 10.8	88.7 ± 14.7	84.0 ± 10.0
	3	87.2 ± 9.3	68.3 ± 11.1	95.1 ± 14.7	87.0 ± 10.0
	6	103.9 ± 9.7	73.3 ± 11.1	93.5 ± 15.6	77.4 ± 10.3
	9	96.0 ± 9.3	76.3 ± 11.5	79.2 ± 15.6	79.6 ± 10.6
Carbohydrates (g)	0	240.5 ± 20.1	196.7 ± 24.9	223.6 ± 34.0	219.2 ± 23.2
	3	242.0 ± 21.2	200.8 ± 25.6	266.0 ± 34.0	261.8 ± 23.2
	6	242.6 ± 22.0	229.9 ± 25.6	230.4 ± 35.6	250.9 ± 23.7
	9	236.8 ± 21.2	224.4 ± 26.3	236.5 ± 35.6	247.8 ± 24.2
Dietary Fiber (g)	0	27.2 ± 2.3	19.8 ± 2.9	27.0 ± 3.9	20.2 ± 2.5
	3	27.5 ± 3.2	19.7 ± 1.9	36.5 ± 2.0	29.8 ± 4.1
	6	29.7 ± 2.9	23.5 ± 2.9	34.4 ± 2.8	26.9 ± 2.4
	9	30.5 ± 3.9	21.6 ± 2.8	29.1 ± 3.6	29.9 ± 4.3

Values are means ± SEM. Abbreviations used: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB: smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day. NS-C: baseline: n=20, 3 mo. n=19, 6 mo. n= 19, and final n=18; S-C: baseline n=21, 3 mo. n=15, 6 mo. n= 12 and final n=12; S-BB: baseline n=8, 3 mo. n=6, 6 mo. n= 6 and final n=6; and S-BL baseline n=16, 3 mo. n=14, 6 mo. n= 14 and final n=13 due to attrition.

Appendix 6. Daily Intake of Selected Vitamins Calculated From a 7 D FFQ of Control Smokers and Nonsmokers, and Smokers Consuming 45 g of Blackberries and Blueberries at Baseline, 3, 6 and 9 Months.

	Month	NS-C	S-C	S-BB	S-BL
Vitamin C (mg)	0	200.6 ± 21.1	145.0 ± 29.2	143.1 ± 18.7	140.1 ± 15.8
	3	185.5 ± 27.3	139.6 ± 19.6	235.8 ± 32.2	207.0 ± 30.4
	6	202.2 ± 23.7	152.7 ± 28.1	185.9 ± 16.9	197.2 ± 26.0
	9	174.9 ± 29.6	139.3 ± 9.5	174.3 ± 15.4	182.5 ± 23.1
Vitamin A (RE)	0	1844.8 ± 212.1	1095.8 ± 156.2	1114.0 ± 402.7	1162.6 ± 147.4
	3	1497.2 ± 158.6	906.184 ± 139.4	1122.8 ± 159.4	1108.0 ± 193.4
	6	1712.0 ± 179.9	1071.8 ± 215.2	744.3 ± 142.2	891.4 ± 189.8
	9	2010.0 ± 445.8	1220.3 ± 271.2	712.8 ± 230.6	963.9 ± 238.7
Vitamin E (mg)	0	6.4 ± 0.9	4.4 ± 0.6	6.7 ± 1.2	4.3 ± 0.7
	3	6.6 ± 0.9	5.2 ± 0.7	9.2 ± 2.1	6.0 ± 1.0
	6	8.2 ± 1.5	4.6 ± 0.9	12.6 ± 2.8	7.4 ± 1.2
	9	9.7 ± 1.6	6.2 ± 1.3	9.6 ± 2.6	8.1 ± 1.7
Vitamin K (μg)	0	95.6 ± 15.8	75.8 ± 13.1	56.5 ± 14.8	90.8 ± 16.1

	3	85.8 ± 16.5	52.8 ± 5.9	59.7 ± 15.7	92.9± 36.1
	6	88.6 ± 15.3	62.6 ± 17.7	49.3 ± 16.2	72.5 ± 20.6
	9	70.6 ± 9.9	76.9 ± 17.2	56.37 ± 13.1	46.5± 12.0
Vitamin D (ug)	0	3.8156 ± 0.7	3.8 ± 0.8	2.7 ± 0.7	1.7 ± 0.5
	3	3.3128 ± 0.7	3.5 ± 0.9	2.7 ± 0.8	1.7± 0.4
	6	3.9821 ± 0.8	3.6 ± 0.9	3.2 ± 1.0	1.2 ± 0.4
	9	2.2758 ± 0.6	3.0 ± 0.7	1.7 ± 0.6	1.1 ± 0.3

Values are means ± SEM. Abbreviations used RE; retinol equivalents: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB: smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day. NS-C: baseline: n=20, 3 mo. n=19, 6 mo. n= 19, and final n=18; S-C: baseline n=21, 3 mo. n=15, 6 mo. n= 12 and final n=12; S-BB: baseline n=8, 3 mo. n=6, 6 mo. n= 6 and final n=6; and S-BL baseline n=16, 3 mo. n=14, 6 mo. n= 14 and final n=13 due to attrition.

Appendix 7. Daily Intake of Selected Minerals Calculated From a 7 D FFQ of Control Smokers And Nonsmokers, and Smokers Consuming 45 g of Blackberries and Blueberries at Baseline, 3, 6 And 9 Months.

	Month	NS-C	S-C	S-BB	S-BL
Ca (mg)	0	1066.9 ± 94.4	897.6 ± 111.2	821.8 ± 77.4	790.8 ± 73.1
	3	1126.3 ± 172.3	801.2 ± 126.5	925.1 ± 77.2	787.2 ± 106.6
	6	1017.6 ± 79.3	946.2 ± 159.6	810.8 ± 112.9	784.5 ± 125.6
	9	913.5 ± 77.9	805.4 ± 126.8	760.7 ± 127.2	760.7 ± 97.5
Mg (mg)	0	411.6 ± 30.2	316.2 ± 34.03	428.2 ± 42.6	332.1 ± 29.7
	3	414.1 ± 33.6	328.9 ± 34.0	437.2 ± 31.0	367.9 ± 46.1
	6	442.8 ± 41.3	351.2 ± 43.3	434.1 ± 49.4	334.1 ± 23.1
	9	420.8 ± 41.3	320.1 ± 42.5	348.3 ± 64.4	369.0 ± 31.9
K(mg)	0	4041.6 ± 284.5	3468.3 ± 318.1	3978.0 ± 260.7	3583.3 ± 283.5
	3	4121.8 ± 363.7	3397.7 ± 387.8	4428.8 ± 301.0	3859.2 ± 449.9
	6	4207.6 ± 303.0	3650.7 ± 498.7	4127.5 ± 388.4	3490.8 ± 288.1
	9	4009.7 ± 342.4	3287.8 ± 454.7	3388.2 ± 599.5	3750.9 ± 347.3
P (mg)	0	1606.4 ± 102.3	1500.2 ± 185.4	1547.8 ± 125.1	1369.5 ± 134.0
	3	1610.3 ± 147.7	1349.7 ± 152.2	1624.67 ± 81.2	1428.6 ± 220.1
	6	1616.2 ± 124.6	1428.6 ± 199.1	1558.3 ± 187.6	1283.5 ± 108.6
	9	1562.3 ± 121.0	1353.1 ± 191.2	1286.1± 259.4	1325.9 ± 123.4
Na(mg)	0	2132.4 ± 178.8	1981.3 ± 289.2	2044.6 ± 243.2	2134.9 ± 237.9

		NS-C	S-C	S-BB	S-BL
	3	1835.6 ± 186.6	2084.3 ± 322.1	2429.4 ± 137.5	1991.3 ± 232.5
	6	2128.2 ± 265.3	2205.4 ± 476.1	2360.3 ± 399.7	1914.7 ± 140.5
	9	2018.1 ± 198.5	2285.1 ± 298.0	1978.1 ± 334.6	1751.0 ± 255.6
Biotin (µg)	0	21.7 ± 2.4	23.3 ± 7.0	12.6 ± 2.1	15.0 ± 1.5
	3	23.4 ± 3.5	12.4 ± 2.9	16.0 ± 3.1	15.4 ± 2.2
	6	25.2 ± 5.4	15.2 ± 2.7	13.3 ± 2.1	11.2 ± 1.9
	9	19.0 ± 2.4	13.8 ± 2.5	14.3 ± 1.8	13.1 ± 1.9

Values represent least square means ± SEM. Abbreviations used: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB: smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day. NS-C: baseline: n=20, 3 mo. n=19, 6 mo. n= 19, and final n=18; S-C: baseline n=21, 3 mo. n=15, 6 mo. n= 12 and final n=12; S-BB: baseline n=8, 3 mo. n=6, 6 mo. n= 6 and final n=6; and S-BL baseline n=16, 3 mo. n=14, 6 mo. n= 14 and final n=13 due to attrition.

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Appendix 8. Effects of freeze-dried blackberries and blueberries on bone biochemical measurements in postmenopausal smokers

	Month	NS-C	S-C	S-BB	S-BL	*p* values
nmol DPD/mmol creatinine	0	5.77 ± 0.40	7.16 ± 0.41	6.10 ± 0.75	8.14 ± 1.10	0.3415α
	3	6.74 ± 0.64	6.30 ± 0.50	6.32 ± 0.33	8.02 ± 0.91	0.8273β
	6	6.74 ± 0.39	6.87 ± 0.67	6.14 ± 0.54	6.36 ± 0.83	0.1229γ
	9	6.57 ± 0.39	7.39 ± 0.62	6.12 ± 0.94	7.35 ± 0.46	
BAP (U/L)	0	29.45 ± 4.89	28.88 ± 1.90	25.56 ± 3.21	39.96 ± 3.02	0.3777α
	3	31.28 ± 5.63	28.82 ± 2.21	24.67 ± 2.83	34.02 ± 2.42	0.5284β
	6	30.92 ± 3.41	29.52 ± 2.34	30.37 ± 3.76	29.95 ± 2.42	0.0811γ
	9	29.00 ± 3.11	29.32 ± 2.05	24.19 ± 2.44	32.72 ± 3.45	
OC (ng/mL)	0	8.35 ± 1.13	9.14 ± 0.86	10.13 ± 1.64	9.37 ± 1.20	0.6848α
	3	9.18 ± 1.27	9.30 ± 1.63	6.28 ± 2.25	11.18 ± 1.09	0.6146β
	6	11.30 ± 1.06	10.62 ± 0.83	9.22 ± 1.77	8.34 ± 1.09	0.0521γ
	9	10.12 ± 0.93	9.95 ± 1.37	6.99 ± 1.52	9.43 ± 1.02	

Values represent means ± SEM. α, β, and γ represent, treatment, time, and treatment x time p values respectively. NS-C: baseline: n=20, 3 mo. n=19, 6 mo. n= 19, and final n=18; S-C: baseline n=21, 3 mo. n=15, 6 mo. n= 12 and final n=12; S-BB: baseline n=8, 3 mo. n=6, 6 mo. n= 6 and final n=6; and S-BL baseline n=16, 3 mo. n=14, 6 mo. n= 14 and final n=13 due to attrition. Abbreviations: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB:

smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day: BAP: bone alkaline

phosphatase; DPD: deoxypyridinoline; OC: osteocalcin

Appendix 9. Effects of freeze-dried blackberries and blueberries on biomarkers of oxidative stress and inflammation in postmenopausal smokers

	Month	NS-C	S-C	S-BB	S-BL	*p* values
TBARS	0	10.46 ± 0.89	9.95 ± 0.61	11.77 ± 0.74	11.19 ± 1.10	$0.5359\ \alpha$
	3	9.32 ± 0.82	12.44 ± 1.17	11.16 ± 1.25	10.58 ± 0.95	0.3396β
	6	11.41 ± 1.17	13.51 ± 1.78	10.70 ± 0.92	13.21 ± 1.87	0.7531γ
	9	13.26 ± 3.19	15.35 ± 2.60	10.94 ± 1.79	11.53 ± 1.06	
hsCRP	0	1.54 ± 0.59	3.02 ± 0.53	2.97 ± 0.97	1.92 ± 0.67	0.1256α
	3	1.00 ± 0.61	2.67 ± 0.62	2.56 ± 0.97	1.78 ± 0.66	$0.1638\ \beta$
	6	1.31 ± 0.59	3.03 ± 0.62	3.73 ± 1.01	2.30 ± 0.67	0.9905γ
	9	1.12 ± 0.59	2.57 ± 0.64	2.41 ± 1.01	1.81 ± 0.67	

Values represent means $\pm$ SEM. α, β, and γ represent treatment, time, and treatment x time p values respectively. NS-C: baseline: n=20, 3 mo. n=19, 6 mo. n= 19, and final n=18; S-C: baseline n=21, 3 mo. n=15, 6 mo. n= 12 and final n=12; S-BB: baseline n=8, 3 mo. n=6, 6 mo. n= 6 and final n=6; and S-BL baseline n=16, 3 mo. n=14, 6 mo. n= 14 and final n=13 due to attrition. Abbreviations: NS-C: Nonsmokers control group; S-C: Smokers control group; S-BB: smokers + 45 g blackberries/day; S-BL: smokers + 45 g blueberries/day: TBARs: thiobarbituric acid reactive substances; hsCRP: high-sensitivity C-reactive protein.