Chapter 5

Oxidative Stress and Antioxidants in the Risk of Osteoporosis — Role of the Antioxidants Lycopene and Polyphenols

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Additional information is available at the end of the chapter

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1. Introduction

Osteoporosis is a metabolic bone disease known as "the silent thief" because the gradual loss of bone associated with this disease usually occurs over the years, and there are usually no noticeable symptoms until the bones are so fragile that a fracture occurs [1]. Although most statistics on the prevalence of osteoporosis quoted in the literature are from those published in 1991 to 2004 [2,3] the projection is nevertheless very consistent. Thus, osteoporosis is estimated to affect over 200 million people worldwide and 75 million people in Europe, the United States, and Japan [4]. Approximately 1 in 2 women and 1 in 5 men older than 50 years will eventually experience osteoporotic fractures [5] An increase in the worldwide incidence of hip fracture by 240% in women and 310% in men is projected by the year 2050 [6]. Osteoporosis is "a major public health threat" that is projected to results to 8.1 million fractures (78 % women, 22 % men) during the period between 2010 and 2050 [7]. The condition costs our healthcare system $18 billion per year [8]. Records show that Osteoporosis has been known to exist since the Egyptian mummies have been found with suspected dowager's hump [9]. Newer findings on all aspects of osteoporosis have increased exponentially. The more importantly ones are the introduction and improvement in more sensitive diagnostic instruments, discovering an ever increasing number of risk factors including oxidative stress, opening up new knowledge on the involvement of the bone forming cells osteoblasts and the bone resorbing cells osteoclasts in the development of osteoporosis and finding new drugs and the nutritional alternatives for the prevention and treatment of osteoporosis. Advances in knowledge on osteoporosis is not without pitfalls. Hormone Replacement Therapy (HRT), once a first line of treatment for osteoporosis has been discontinued due to side effects [10]. It is becoming more evident that the drugs known as bisphosphonates, although effective in

stopping the resoption of bone and preventing osteoporosis in women, are associated with a number of side effects [11,12]. The side effects have been alarming a number of women with osteoporosis in such a way that they are now resorting to other mode of treatment, including that from natural food components. Our laboratory has carried out studies on the use of antioxidants such as lycopene and polyphenols as possible alternatives and/or complementaries to drugs in the treatment and prevention of osteoporosis. This chapter will include an overview on osteoporosis, the role of oxidative stress in bone cells osteoclasts and osteoblasts, oxidative stress as a risk factor in the development of osteoporosis and a review of studies on the use of antioxidants in counteracting oxidative stress in the prevention of osteoporosis. These topics should put our research in perspective and offer a rationale to our study approaches. Finally we will highlight our pioneering studies on the effects of the lipid-soluble antioxidant lycopene and the water-soluble antioxidant polyphenols present in a nutritional supplement in an *in vitro* cultures of osteoblasts and osteoclasts and our clinical studies in the prevention of risk for osteoporosis in postmenopausal women.

2. Osteoporosis — Overview

2.1. Bone cells involved in the development of osteoporosis

Bone as a dynamic tissue continuously renews itself throughout life by the process of bone remodeling carried out by a functional and anatomic structure known as the basic multicellular unit (BMU) that requires the coordinated action of three major types of bone cells: osteoclasts, osteoblasts and osteocytes [13,14]. The remodeling process is the result of interactions between these cells and multiple molecular agents, including hormones, growth factors, and cytokines. Bone remodeling is a physiological process that follows a time sequence lasting approximately six months wherein osteoclasts eliminate old or damaged bone which is subsequently replaced with new bone formed by osteoblasts, while the osteocytes functions in the transduction of signals necessary to sustain mechanical loads. The coupled process of bone formation and bone resorption in mature, healthy bone is tightly regulated and maintained in order to prevent a significant alterations in bone mass or mechanical strength after each remodeling cycle [14,15]. At menopause when estrogen production is decreased, the increase in resorption cavities due to increased bone resorption, but insufficient increase in bone formation, results to incomplete filling of resorption cavities with new bone leading to a permanent loss of bone mass. Disturbances in the remodeling process of this nature can lead to metabolic bone diseases. One such disturbance caused by oxidative stress, shown to control the functions of both osteoclasts and osteoblasts, may contribute to the pathogenesis of skeletal system including osteoporosis, the most prevalent metabolic bone disease [16].

2.2. Prevalence of osteoporosis

Women over the age of 50 become susceptible to osteoporosis because of the loss of estrogen at menopause [17]. As well, men's susceptibility to osteoporosis is due to low levels of the sex hormone testosterone [18-20]. In the past, a majority of men view osteoporosis as solely a

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"woman's disease." This is because men in their fifties do not experience the rapid loss of bone mass that women do in the years following menopause, and therefore men osteoporosis does not set in until later in life [21,22]. However, by age 65 to 70, men and women are losing bone mass at the same rate [23]. The World Health Organization (WHO) aptly defined osteoporosis as a systemic disease that is characterized by low bone mass and deterioration of the micro-architecture of bone, resulting in an increased risk of fracture. Bone mass or bone mineral density (BMD) is measured using a dual-energy x-ray absorptiometry, or DXA, at various skeletal sites, including the spine, hip and wrist [24].

2.3. Bone mineral density as predictor of osteoporosis

BMD is expressed as T-score which is a value compared to the expected value for young adults of the same sex and race. WHO has established that for normal BMD, the T-score is between standard deviation of +2.5 and -1.0; for osteopenia/low BMD, T-score is between -1.0 and -2.5, inclusive; for osteoporosis, T-score is lower than -2.5 and for severe osteoporosis,T-score is lower than -2.5 with the presence of one or more fragility fractures [25]. Thus BMD values can identify osteoporosis, determine the risk for fractures (broken bones), and measure the response to osteoporosis treatment [26]. In the case of severe osteoporosis, minimal trauma such as a minor fall or just a hug from a loved one can result to fragility fracture. Fragility fracture is defined by WHO as "a fracture caused by injury that would be insufficient to cause fracture normally. The spine, hip and distal forearm are the most common sites of fragility fracture [27]. Some doctors recommend that people be tested on a regular basis for bone loss. For women, those tests should begin after menopause. For men, they should begin after the age of sixty-five. Such tests are important since there are seldom other signs of osteoporosis. Therefore, those who have a higher rate of bone loss and are at higher risk for a fracture need a better diagnostic tool. Recently, the WHO introduced a prognostic tool to evaluate fracture risk of patients called the FRAX® [28]. The FRAX tool takes into account country, bone mineral density of the hip (when available), age, sex, and 8 clinical risk factors to calculate the 10-year probability of a major osteoporotic fracture and the 10-year probability of a hip fracture [29]. It assesses the 10-year risk of osteoporosis based on individual patient models that combines clinical risk factors (CRF) as well as BMD at the femoral neck [30].

2.4. Bone turnover markers for detecting osteoporosis

During bone remodeling in healthy young adult, bone formation by osteoblasts equals bone resorption by osteoclasts. However In postmenopausal bone loss, the remodeling process becomes significantly more active with a primary increase in bone resorption and a corresponding, but an insufficient increase in bone formation [31]. Enzymes and/or other proteins are released into the blood that are considered to reflect either bone formation or bone resorption [32] and are termed as bone turnover markers. Molecular markers of bone turnover have been developed as a product of bone remodeling [31] in the diagnostic and therapeutic assessment of metabolic bone disease [33]. They are now used for the individual monitoring of osteoporotic patients treated with antiresorptive agents [34]. Specific and sensitive assessment of the rate of bone formation and bone resorption and prediction of fracture [35] can now

be possible using commercially available biochemical markers [36]. It remained to be seen whether bone turnover markers might contribute a useful independent risk factor for inclusion in FRAX [30]. The bone turnover markers we used for our clinical studies were crosslinked N-telopeptide of type I collagen (NTx) [37-39] and crosslinked C-telopeptide of type I collagen (CTx) [40,41] as bone resorption markers and bone alkaline phosphatase (BAP) [37-39] and Procollagen type I N-terminal propeptide (PINP) [40,41] for a measure of bone formation in the serum of participants.

Although BMD is considered the best parameter for determining the osteoporotic status of men and women, BMD is static and cannot predict changes that may occur post-measurement [42]. As well, changes in BMD occur slowly and can take up to one to two years to be detected during the course of therapy [43,44]. An alternative or additional parameters now measured clinically as either formation or resorption markers in the urine or serum of participants are bone turnover markers which can reveal changes much earlier in the course of therapy compared to changes in BMD [34]. When combined with BMD measurement, changes in bone turnover markers have been significantly linked to fracture risk due to a significant positive correlation between high bone turnover markers and loss of BMD [35]. Bone turnover markers are therefore very useful in assessing treatment protocol for a short duration period, e.g., 3 to 6 months. Measurement of bone turnover markers was therefore utilized in our clinical study during which postmenopausal women were given the antioxidant lycopene for 3 months [37-39] and in another study during which nutritional supplement greens+bone builder™ were administered for a period of eight weeks [40,41]. As will be reviewed in later sections, during the short period of treatment, positive changes were measured that correlated decreased bone resorption markers with decreases in oxidative stress parameters and thereby to decrease of risk for osteoporosis in postmenopausal women.

2.5. Risk factors of osteoporosis

Some of the risk factors for osteoporosis [45,46] are presented in Table 1 [47]. The risk factors that are of interest in our studies are oxidative stress-generating factors, including smoking, alcohol intake, low antioxidant status, nutrition deficiency, excessive sports activity and excessive caffeine intake. Oxidative stress will be reviewed in detail below.

2.6. Prevention and treatment of osteoporosis

Up until 10 years ago, the first line of treatment for women who have gone through menopause and was diagnosed with osteoporosis was hormone replacement therapy (HRT). However, results of the Women's Health Initiative (WHI) warned women that HRT leads to higher risks for breast cancer, cardiovascular events, blood clots, cognitive decline, and more [10]. This treatment for osteoporosis has since been discontinued and is prescribed only for a short period of time to alleviate hot flashes in menopausal women [48]. A wide range of pharmaceuticals are available for the treatment of osteoporosis. The current antiresorptive treatments approved by the Food and Drug Administration (FDA) include a number of bisphosphonates under specific trademarks which inhibit bone resorption [49]. Some are taken daily while others are formulated for weekly, monthly or intermittent oral use [50,51]. The newer bisphophonates are injecta-

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bles such as ibandronate and Zoledronate [51] Other drugs available include calcitonin, strontium renalate and the Selective Estrogen Receptor Modulator (SERM), Raloxifene (Evista) [52]. Parathyroid hormone, PTH1-34 or teriparatide (Forteo), is the only anabolic agent currently approved for use by the FDA [24,53]. The new class of osteoporosis medications now approved for use is a fully human monoclonal antibody (Denosumab) which bind to RANKL, imitating the effects of OPG and acting as an inhibitor of RANKL [54]. A number of other drugs are being tested clinically for osteoporotic treatment and prevention.[24].

Unmodifiable	Modifiable
Race	Chronic inactivity
Sex	Low body weight
Age	Low lifetime calcium intake
Genetics	Medication used
Body size	Oxidative stress-related factors
Family History	Smoking
Previous Fractures	Alcohol intake
	Low antioxidant status
	Nutrition deficiency
	Excessive sports activity
	Excessive caffeine intake

Table 1. Risk Factors for Osteoporosis

None of the drugs are without side effects. Side effects that emerged in clinical trials include esophageal irritation with oral administration and acute phase response with iv treatment or high-dose oral therapy. Uncommon side effects that have been noted with wide clinical use include osteonecrosis of the jaw, musculoskeletal complaints, and atypical fractures. The numbers of events are small, and a clear cause-and-effect relationship between these events and bisphosphonate treatment has not been established. Because Bisphosphonates accumulate in the bone, they create a reservoir leading to continued release from bone for months or years and provide some residual antifracture reduction when treatment is stopped. For this reason, there is a recommendation for a drug holiday after 5 –10 yr of bisphosphonate treatment [12,55]. The length of the holiday is based on fracture risk and previous duration of treatment and BMD status. Studies with risedronate and alendronate suggest that if treatment is stopped after 3–5 yr, there is persisting antifracture efficacy, at least for 1–2 yr. For those who are not on holiday, the consensus from expert panels [12] suggest not stopping the use of drug since the side effects are often rare, and that the benefits outweigh the side effects. In the balance, most individuals who have osteoporosis are much better taking an osteoporosis medication [11].

2.7. Alternative approach to prevention and treatment of osteoporosis

Considering the possible adverse side effects of HRT and the ever increasing reports on the side effects of bisphosphonates in the management of postmenopausal osteoporosis, there is

an increasing demand for complementary and alternative medicine (CAM) for the prevention and treatment of osteoporosis [56]. CAM is the term for medical practices, services and products that are not a part of standard care. Some of the approaches include exercise, acupuncture, diet, herbs rich in polyphenols and nutritional supplements including calcium, zinc, magnesium boron and other vitamins and minerals. Recent dietary guidelines for the prevention of chronic diseases have recommended an increase in the consumption of fruits and vegetables worldwide [57] that are good sources of dietary antioxidants [58]. The beneficial effects of antioxidants in bone health and osteoporosis are demonstrated epidemiologically and through clinical intervention. Given that many nutrients have been identified as being beneficial to bone health [59,60], there is strong scientific support for the potential benefits of incorporating therapeutic nutritional interventions with contemporary pharmaceutical treatments [61]. Diet is now recognized as an important life-style factor in the management of bone health [62]. As will be reviewed in this chapter, our clinical studies on lycopene treatment and nutritional supplements containing polyphenols and other nutritional components showed positive results on bone health.

3. Oxidative stress

Oxidative stress is caused by reactive oxygen species (ROS) which are the main by-products formed in the cells of aerobic organisms that can initiate autocatalytic reactions in such a way that the target molecules gets converted into free radicals causing a chain of damage [63]. There is ample evidence to show that oxidative stress induced by ROS increases the rate of bone loss and is therefore a risk factor for osteoporosis. Epidemiological evidence in humans and studies in animals indicate that aging and the associated increase in ROS are responsible for bone loss [64]. As will be reviewed in later sections, oxidative stress is associated with the activity and function of both the osteoblasts and osteoclasts cells, the two major bone cells involved in the pathogenesis of osteoporosis.

Oxidative stress results from the weakening of antioxidant defense or an over production of ROS in the body. ROS contains one or more unpaired electrons, a state that makes them highly reactive as they seek out another electron to fill their orbital and stabilize their electron balance [65]. Therefore, ROS are a family of highly reactive, oxygen-containing molecules and free radicals, including hydroxyl (OH –), superoxide radicals (O2 –), hydrogen peroxide (H_2O_2), singlet oxygen, and lipid peroxides [66]. ROS have an extremely short half-life and are difficult to measure in humans, but it is possible to measure the damage they cause to protein, lipids, and DNA and the damage is manifested as chronic diseases including osteoporosis [67]. This can occur by the induction of apoptosis, reduction of cellular proliferation, cell cycle arrest and modulation of cellular differentiation [68]. The major intracellular sites for the generation of ROS are via electron transport chains in the mitochondria, endoplasmic reticulum and nuclear membranes [69]. Oxidative stress may result from normal metabolic activity [70]; during acute or chronic immune responses [71]; lifestyle factors such as cigarette smoke [72,73], high alcohol intake [74,75], low antioxidant status [76], nutrition deficiency [74] excessive sports activity [77], excessive caffeine [78]; and environmental factors such as ultraviolet radiation, chemicals,

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pollution and toxins [79]. ROS production increases with age [80,81] and is associated with several chronic diseases including osteoporosis.

4. Antioxidants

Under normal physiological conditions, the cells can fight free radical attack or oxidative stress by promoting antioxidant defenses. A number of endogenous defense mechanisms are present in the body, including the metal chelating proteins and the endogenous antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). [82]. Exogenous antioxidants come from dietary sources present in fruits and vegetables containing several phytonutrient antioxidants such as the carotinoids potent antioxidant lipid-soluble lycopene; the water-soluble antioxidant polyphenols; and vitamins such as C and E [83]. In cases where the endogenous antioxidants or antioxidants from diet fail to prevent oxidative damage, the repair antioxidants come into play which include DNA repair enzymes, lipase, protease and transferase [69]. When antioxidants loses its fight with oxidative stress, diseases associated with oxidative stress develop, which include cardiovascular disease, cancer, diabetes, neurological diseases and osteoporosis [84].

The phytochemical antioxidants that are naturally present in plant- and animal-derived foods include the carotenoids, which are lipid-soluble, to which the potent antioxidant lycopene belongs and the water-soluble antioxidants such as polyphenols [85]. Figure 1 is a cartoon depicting the production of oxidative stress from ROS, the damaging effects they exert on DNA, lipid and protein which subsequently leads to chronic diseases and the protection afforded by antioxidants.

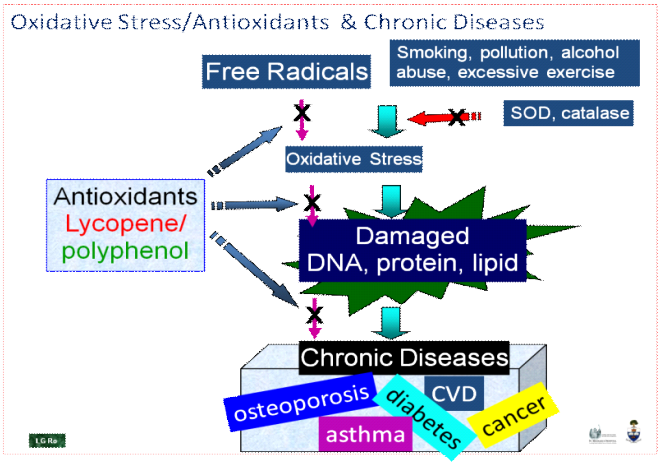

Figure 1. Oxidative Stress/Antioxidants & Chronic Diseases

4.1. Lycopene, a carotinoid lipid-soluble antioxidant

Lycopene is a potent antioxidant that is not synthesized in the body. It is a carotenoid acyclic isomer of ß-carotene, with no vitamin A activity [86]. It is a highly unsaturated, straight-chained hydrocarbon containing a total of 13 double bonds, of which 11 are conjugated, making it one of the most potent antioxidants [84,87]. The singlet oxygen-quenching ability of lycopene is twice that of ß-carotene and 10 times that of α-tocopherol [88]. The chemistry and antioxidant properties of lycopene have been comprehensively reviewed [87]. Other than from tomatoes and processed tomato, the dietary lycopene source of 85% of North Americans, lycopene can also be obtained from watermelon, pink guavas, and pink grapefruit [85]. Lycopene in an all-trans configuration such as that found in raw tomatoes, is not readily absorbed. Lycopene is absorbed more efficiently from processed tomato products than from raw tomatoes because it is converted from the all-trans to the cis-isomeric configuration with heat processing [89,90]. Since lycopene is a lipid-soluble compound that is absorbed via a chylomicron-mediated mechanism [91], the presence of small amounts of lipids further enhance its absorption [92]. The health benefits of lycopene may be due to its potent antioxidant property, although there is evidence for other mechanisms such as its effects on gap junction communication [93] and cell cycling [94]. The reported average daily intake levels of lycopene vary considerably from country to country, from 0.7 mg per day in Finland to 25 mg per day in Canada. However, a generally accepted universal level of daily intake is 2.5 mg. There is no official recommended daily intake of lycopene, but based on published research, a daily intake of 7 mg is suggested [95].

The role of lycopene in the prevention of human diseases is supported by a number of evidence [96]. Giovannicci was the first to publish the initial epidemiological observations suggesting an inverse relationship between the intake of tomatoes and lycopene and the incidence of prostate cancer [97]. Since then, there have been several epidemiological as well as clinical intervention studies showing the relationship between lycopene intake and the prevention of cancers at other sites, as well as coronary heart disease, hypertension, diabetes, macular degenerative disease, male infertility, and neurodegenerative disease [84]. The role of lycopene in bone health has so far been based on its potent antioxidant properties, the well known role of oxidative stress in bone health, and the limited studies on the effects of lycopene in bone cells in culture (see below) and more recently, the results of epidemiological studies [39,98]. To date our clinical intervention studies at St. Michael's Hospital on the role of lycopene and elucidation of its mechanism in lowering the risk for osteoporosis in postmenopausal women (aged 50 to 60 years) are so far the only studies reported in the literature.

4.2. Polyphenols, the water-soluble antioxidant

Polyphenols are a class of water-soluble molecules naturally found in plants. They are defined as compounds having molecular masses ranging from 500 to 3000–4000 Da and possessing 12 to 16 phenolic hydroxy groups on five to seven aromatic rings per 1000 Da of relative molecular mass [99]. It is estimated that there are 10,000 different phytonutrients (phyto, meaning from plants). To date, over 8000 polyphenols have been identified [100]. Polyphenols can be divided into 2 main groups: flavonoids and non-flavonoids [101-103]. The health benefits associated with fruits, vegetables, red wine, tea, and Mediterranean diets are probably linked to the

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polyphenol antioxidants they contain [59,104,105]. The polyphenols of interest in our study are a mixture of flavonoids such as quercetin, apigenin, kaempferol and luteolin present in the supplement greens+™ [106]. greens+™ in combination with another supplement, bone builder™, were used in our study on osteoblasts cells and in clinical intervention studies on the prevention of risk of osteoporosis in postmenopausal women. Studies on polyphenols and bone will be reviewed in later sections.

5. Studies on the damaging effects of oxidative stress and the beneficial effects of antioxidants

(Studies involving lycopene and polyphenols will be reviewed at a later section)

5.1. Studies on osteoblasts

The evidence being reported on the role of oxidative stress in osteoblasts has increased exponentially. Up until 2002, only a few studies were reported. Thus, it was reported that treatment of rat osteosarcoma ROS 17/2.8 cells with tumor necrosis factor-alpha (TNF-a) suppressed bone sialoprotein (BSP) gene transcription through a tyrosine kinase-dependent pathway that generates ROS [107]. Osteoblasts can be induced to produce intracellular ROS [108,109], which can cause a decrease in alkaline phosphatase (ALP) activity that is partially inhibited by vitamin E and cause cell death [108,109]. The intracellular calcium (Ca++) activity in osteoblasts is modulated by H_2O_2 by increasing Ca++ release from the intracellular Ca++ stores [110]. High concentrations of ROS can damage osteoblast cells to prevent normal growth and development [111] and have been shown to induce osteoblast death [112]. In osteoblasts, H_2O_2 has been shown to decrease cell growth, ALP activity, calcification, mineralization and gene expression of osteogenic markers such as ALP, bone sailoprotein (BSP) and runt-related transcription factor 2 (Runx2) [113,114]. More recently, Ueno et al induced oxidative stress by adding 100 microM H_2O_2 to osteoblasts cultured from rat bone marrow, and showed that this treatment substantially impaired the proliferation, differentiation, and mineralization and that addition of the antioxidant N-acetyl cysteine into the culture restored these damages to a near normal level [115]]. With their study on hydrogen sulphide, Xu et al concluded that hydrogen sulfide (H_2S) protected MC3T3-E1 osteoblastic cells via a MAPK (p38 and ERK1/2)-dependent mechanism against hydrogen peroxide (H_2O_2)-induced oxidative injury that cause the suppression of proliferation and differentiation of the cells [116].

More recently reported inducers of oxidative stress include Arsenic trioxide [117], Cobalt and Chromium ion [118] and Vanadium Compounds [119].

In vitro studies suggest an important role for antioxidants in abrogating the effects of oxidative stress on bone. Trolox, a water soluble vitamin E analogue, was shown to enhance ALP activity in MC3T3-E1 osteoblast-like cells, thus enhancing osteoblast differentiation by decreasing the generation of ROS [111]. The addition of metallothionein, a metal-chelating preventative anti-oxidant, to primary mouse bone marrow stromal cells impaired H_2O_2-stimulated NfκB signal-

ling, consequently preventing any inhibition of osteoblast differentiation [114]. Osteoblasts have been shown to produce antioxidants such as GPx which can protect against the damaging effects of ROS [120]. In MC3T3-E1 osteoblast-like cells, treatment with H_2O_2 to induce oxidative stress was associated with the prolonged up-regulation in gene expression of the transcription factor nuclear factor E2 p45-related factor 2 (Nrf2), which regulates antioxidant enzymes by assisting with recognition of the antioxidant-response element [113]. Using xanthine/xanthine oxidase to generate ROS, Fatokun *et al*.[120] showed that damage induced by ROS, as evidenced by decreased cell viability, was prevented by CAT in MC3T3-E1 osteoblast-like cells. This effect is attributed to the ability of CAT to neutralize H_2O_2 [120]. Newer mechanism of action of ROS is beginning to come to light. It has been shown that increased ROS production diverts the limited pool of β-catenin from TCF/LEF to FOXO-mediated transcription, converting the beneficial effects of Wnt/β-catenin on bone, eventually leading to decrease osteoblasts number and activity and eventually leading to osteoporosis [121-124].

In recent years, the number of antioxidants reported to prevent oxidative stress in osteoblasts are as follows: Tetrahydrostibene [125,126], Curculigoside [127], Green tea [128], Simvastatin [129], N-acetylcysteine [115], flavonoids from parsimmon [130], prevastatin [131], Linarin [132], Panaxnotaginseng saponin [133], crysoeriol from surya cilliata leaves [134], quercetin [135] Drynaria fortunei [136], cathamus tinctorium flower extract [137], estrogen [138], diazoxide, atractylodes japonica root extract [139]. and Myrcetin, a naturally occurring flavonoid [140]. The mechanism of osteblastic defense against oxidative stress was shown to involve β-Catenin which serves as a cofactor of the forkhead box O (FOXO) transcription factors [121].

5.2. Studies on osteoclasts

The mechanisms involved in the differentiation of osteoclasts and their ability to resorb bone is beginning to be unraveled, and evidence shows that ROS may be involved in this process [141]. Superoxide was detected both at the osteoclast-bone interface and intracellularly using nitroblue tetrazolium (NBT), which is reduced to purple-colored formazan by ROS, suggesting the participation of superoxide in bone resorption [142]. Both the H_2O_2 produced by endothelial cells [143] intimately associated with osteoclasts and the H_2O_2 that is produced by osteoclasts [144] increase osteoclastic activity and bone resorption. H_2O_2 may also be involved in osteoclast motility [144], differentiation of osteoclast precursors [145] and the regulation of osteoclast formation [146]. Osteoclastic superoxide is produced by NADPH oxidase [147]. The degradation of collagen and other proteins is caused by highly destructive ROS as a result of the reaction of H_2O_2 with tartrate-resistant acid phosphatase (TRAP), found on the surface of osteoclasts [148]. 1,25-Dihydroxyvitamin D3 had a direct nongenomic effect on the generation of superoxide anion (O2_), which was inhibited by estrogen [149]. Estrogen has been reported to have an antioxidant property [150]. Hormones known to stimulate bone resorption, such as parathyroid hormone (PTH) [151] and 1,25(OH)2D3, have stimulatory effects on ROS production in osteoclasts [149] and hormones known to have inhibitory effects on bone resorption, such as calcitonin, inhibit ROS production [151].

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Antioxidants also play a role in osteoclast activity. Osteoclasts produce the antioxidant enzyme SOD in the plasma membrane [152]. ROS production in osteoclasts was inhibited after treating the cells with antioxidant enzymes such as SOD [142] and catalase [146]. ROS production in osteoclasts was also inhibited by estrogen [149], the superoxide scavenger deferoxamine mesylatemanganese complex [153], pyrrolidine dithiocarbamate (PDTC), and N-acetyl cysteine (NAC) [154].

Other more recent antioxidant shown to affect osteoclasts include polyphenol extracts from dried plums [155], curcumerin [156], ascorbic acid [157], salvia miltorrhiza [158], coffee diterpene Kahweol [159], delthametrin [160], to name a few. The use of antioxidants from natural sources, such as fruits and vegetables, could be another way of inhibiting ROS. The use of lycopene and polyphenols in this regard is reviewed in a later section.

5.3. Studies of on animal

OVXed rats were treated with Strontium ranelate and at the end of the treatment, oxidative parameters including malondialdehyde (MDA) level, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined by biochemical analysis methods. Their results showed that Sr has preventive effect on oxidative damage in ovariectomized rats [161]. Yin et al showed that protection against osteoporosis by statins is linked to a reduction of oxidative stress and restoration of NO formation in aged and ovariectomized rats [162]. To investigate the anti-osteoporosis effect of Rhizoma Drynariae (RD), an effectively traditional Chinese medicine and its action mechanism, Liu et al administered with or without RD extract at a therapeutic dose to a group of rats for 12 weeks and showed that the anti-osteoporosis effect of RD has been reliably confirmed by the metabonomics method and that the osteoporosis might be prevented by RD via, among other things, through intervening antioxidant-oxidation balance in vivo in rats [163]. Treatment of OVXed rats with Salvia miltiorrhiza ethanol extract significantly ameliorated the decrease in BMD and trabecular bone mass according to DEXA and trabecular bone architecture analysis of trabecular bone structural parameters by μ-CT scanning. As well, SM decreased the released TRAP-5b, an osteoclast activation marker and oxidative stress parameters including MDA and NO induced by OVX [164]. Oxidative stress (OS) was assessed 100 days postovariectomy by measuring the activity of several enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase, as well as the concentrations of malondialdehyde (MDA), nitric oxide (NO), and total sulfhydryl groups in plasma and bone homogenates of OVXed rats treated with or without vitamin C. Their results suggest that ovariectomy may produce osteoporosis and oxidative stress in females, and vitamin C supplementation may provide alterations regarding improvement in OS and BMD values [165]. Curcumin was shown to inhibit OVX-induced bone loss, at least in part by reducing osteoclastogenesis as a result of increased antioxidant activity and impaired RANKL signaling [166]. In order to investigate the pathologic significance of oxidative stress in bones, Nojiri et al showed that mice deficient in cytoplasmic copper/zinc superoxide dismutase exhibited a distinct weakness in bone stiffness and decreased BMD, aging-like changes in collagen cross-linking, and transcriptional alterations in the genes associated with osteogenesis. They further demonstrated that intra-

cellular oxidative resulted in the decrease in osteoblast number and accompanied by suppression of RANKL/M-CSF osteoclastogenic signaling in bone; treatment with an antioxidant, vitamin C, effectively improved bone fragility and osteoblastic survival [167].

5.4. Epidemiological and clinical studies on osteoporosis

The detrimental effect of oxidative stress and the beneficial role of antioxidant in osteoporosis have been reviewed [58,83,168,169]. There is now ample evidence to suggest that ROS-induced oxidative stress is associated with the pathogenesis of osteoporosis. Thus, epidemiological studies demonstrated the adverse effect on bone of oxidative stress produced during strenuous exercise [170]; among heavy smokers [171] and that antioxidants including vitamin C, E and β-carotene may counteract these adverse effects and reduce the risk of osteoporosis [170-173]. A study of severe osteoporotic syndrome in relatively young males showed evidence linking osteoporosis to an increase in oxidative stress [174]. Maggio et al [175] demonstrated that women with osteoporosis had markedly decreased plasma antioxidants. A biochemical link between reduced bone density and increased oxidative stress biomarker 8-iso-prostaglandin F alpha (8-iso-PGF) has been reported [176,177]. Positive correlation was found between the severity of osteoporosis and the level of oxidative stress marker lactic acid in 2 men with mitochondrial deletion (mtDNA) [178].

Evidence points to the fact that Postmenopausal women are more prone to osteoporosis due to reduction in estrogen, but there is also ample evidence to support the theory that oxidative stress which accompanies the reduction in estrogen level may be the cause of osteoporosis [179]. Indeed, estrogen has been shown to have antioxidant properties [150]. Earlier reports on the association of oxidative stress with osteoporosis were confined mainly to epidemiological studies. Vitamins C, E, and A, uric acid, the antioxidant enzymes SOD in plasma and erythrocytes and GPx in plasma were consistently lower in osteoporotic than in control subjects. An epidemiological study by Hahn *et al* [180] found that GPx activity was significantly higher in postmenopausal women with osteopenia than that of postmenopausal women with a normal BMD, likely as a primary defense against the high levels of H_2O_2 in osteopenic women [180]. Osteoporotic women were found to have significantly depressed activities of CAT, GPx and SOD, compared to those found in healthy control women [177,181,182]. Furthermore, concentrations of the antioxidant enzymes SOD and CAT have been positively correlated with BMD which demonstrates a link between antioxidant status and BMD in postmenopausal women[182]. Surprisingly, a cross sectional analysis in healthy postmenopausal women aged 60-78 years revealed a negative association between 8-OH-dG levels and BMD of the lumbar spine, total hip, femoral neck, and trochanter and positive association with type I collagen C-telopeptide (ICTP) levels, showing that oxidative stress is associated with increased bone resorption and low bone mass even in otherwise healthy women. Medications used to treat postmenopausal osteoporosis such as HRT and Raloxifene [183] may act in part to decrease oxidative stress by acting in part as antioxidants. A 3-month course of Raloxifene therapy significantly decreased the lipid peroxidation and increased the CAT activity in women with postmenopausal osteoporosis [177,181,182]. Raloxifene treatment for 12 months significantly decreased protein oxidation in osteoporotic participants compared to matched, non-osteopor-

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otic controls [183]. Supplementation of ascorbic acid and alpha-tocopherol was found useful in preventing bone loss linked to oxidative stress in elderly [181].

Postmenopausal women with osteoporosis have been shown to have markedly reduced serum concentrations of retinol, β-cryptoxanthin, zeaxanthin and α- and β-carotene, compared to healthy postmenopausal women [175]. Overall carotenoid intake has been found to be inversely associated with risk of fracture [184]. Sadly, the effect of β-carotene on the risk of osteoporosis is still controversial. There are studies which suggest that β-carotene has beneficial effect on bone [98,185,186], while other studies suggest a null or even detrimental effect, most probably due to its association with vitamin A [187].

In summary, the studies presented above provide evidence of the detrimental effects of oxidative stress and beneficial effects of antioxidants on the risk of osteoporosis.

6. Studies on lycopene

The direct role of lycopene in osteoblasts and osteoclasts, the cells involved in the pathogenesis of osteoporosis is now being unraveled. This involvement is further supported by both epidemiological and clinical intervention with lycopene in postmenopausal women men who are at risk of osteoporosis.

6.1. *In vitro* studies of lycopene in osteoblasts

Only few studies on the effects of lycopene in osteoblasts have been reported. This is most likely because lycopene is not soluble in the culture medium and needed to be solubilized in organic solvent before it can be added to the cell culture. In our study, we used lyc-o-mato preparation that is partially dispersed in micelle form in water. When added to the human osteoblast-like SaOS-2 cells, lycopene had a stimulatory effect on cell proliferation as well as a stimulatory effect on alkaline phosphatase activity, a marker of osteoblastic differentiation in more mature cells but, depending on the time of addition, it had an inhibitory or no effect on younger SaOS-Dex cells. These findings comprised the first report on the effect of lycopene on human osteoblasts [188]. In another study, the effect of lycopene on MC3T3 cells (the osteoblastic cells of mice) was contrary to the findings of Kim et al. [188] in that lycopene had an inhibitory effect on cell proliferation [189]. The discrepancy in the effects of lycopene on cell proliferation could be a result of species differences, age of the cells when lycopene was added or experimental conditions. Both studies, however, reported an effect of lycopene on the differentiation of the cells by stimulating the alkaline phosphatase activity [188,189] and gene expression of BSP [189]. The lycopene used in our study is the trans-configuration (95% trans, 5% cis). Subsequently, we studied which configuration of lycopene will prevent the damaging effect of oxidative stress as well as repair this damage in human osteoblast cultures. Lycopene with varying content of cis- and trans- configuration (45:55, 28:72 or 5:95 *cis*:*trans* lycopene) were added to cell cultures before and after challenging with H_2O_2 and the effect on the generation of ROS and stimulation of mineralized bone nodule were assessed. Our results demonstrated that the addition of H_2O_2 resulted in significant increase in generation of ROS

($p<0.001$), which long-term resulted in a decreased number and area of mineralized bone nodules (both: $p<0.001$). Pre- and post-treatment with 45:55 or 28:72 *cis*:*trans* lycopene resulted in significantly lower ROS generation ($p<0.001$) and higher mineralized bone nodule area ($p<0.05$), compared to treatment with H_2O_2 alone, vehicle or 5:95 *cis*:*trans* lycopene. These findings support the hypothesis that the *cis* isomers of lycopene are capable of preventing and repairing the damaging effects of H_2O_2-induced oxidative stress on the formation of mineralized bone nodules (unpublished observation) [169,190].

6.2. *In vitro* studies of lycopene in osteoclasts

To date, there have been only 2 studies on the effects of lycopene in osteoclasts [191,192]. Rao et al. cultured cells from bone marrow prepared from rat femur in 16 well, calcium phosphate-coated Osteologic™ multi-test slides. Lycopene was added to the cells in the absence or presence of the resorbing agent parathyroid hormone (PTH) (1-34) and mineral resorption, TRAP+ multinucleated osteoclast formation, and NBT-staining were measured. Lycopene inhibited TRAP+ multinucleated cell formation in both vehicle- and PTH-treated cultures. The cells that were stained with the NBT reduction product formazan were decreased in number after treatment with lycopene, indicating that lycopene inhibited the formation of ROS-secreting osteoclasts [192]. The effect of lycopene on osteoclast formation and bone resorption was also reported by Ishimi et al in murine osteoclasts formed in co-culture with calvarial osteoblasts [191]. Their results differed from those of Rao et al [192] in that they found that lycopene inhibited PTH-induced, but not basal, TRAP+ multinucleated cell formation. Furthermore, they could not demonstrate any effect of lycopene on bone resorption. They also did not study the effect of lycopene on ROS production.

6.3. Lycopene intervention studies in animals

Other than our intervention studies to be discussed in the next section, most of the intervention studies with lycopene were carried out in animals. Liang et al investigated the beneficial effect of lycopene on bone biomarkers in ovariectomized (OVX) rats. Their results showed that administration of lycopene (20, 30 and 40 mg/kg b.w.) for 8 weeks to OVXed rats significantly enhanced BMD, concluding that the consumption of lycopene may have the most protective effect on bone in OVX [193]. Ke et al fed OVXed rats for 3 months with EM-X, an antioxidant beverage derived from ferment of unpolished rice, sea weeds and papaya with combinations of microorganisms and contains, among other things, lycopene. Results showed that rats receiving EM-X for 3 months after sham operation or ovariectomy had increased bone density of the middle of femur that was statistically significantly different from unreated rats [194].

6.4. Epidemiological studies on lycopene

A systematic review of the experimental studies on Mediterranean diet and disease prevention was made and analyzed [195]. Although the Mediterrenean diet comprised of many different food components, it is striking that one of the components is abundance of plant foods including fruits, vegetables [195]. The two possible active components in its properties to prevent diseases are lycopene [196] and polypenols [197]. Epidemiological evidence support

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the beneficial effects of tomatoes and tomato products in the prevention of osteoporosis in the Mediterranean population [104].

The role of lycopene in the prevention of risk for osteoporosis has recently been reviewed [58, 83,168,169]. Maggio et al [175] and Yang et al [198] both demonstrated that serum lycopene concentrations are lower in women with osteoporosis than in healthy women of the same age. The antioxidant mechanistic effect of lycopene is demonstrated by Misra *et al.* who have shown that HRT has the same antioxidant effects as lycopene in postmenopausal women by demonstrating that lipid peroxidation was significantly decreased while GSH significantly increased by both [199]. Epidemiological studies revealed the relationship between lycopene and BMD [185,186,200]. A cross-sectional and longitudinal analyses in men and women were carried out to evaluate the associations between total and individual carotenoid intakes (a-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin) with BMD at the hip, spine, and radial shaft and the 4-y change in BMD. Their analyses showed significant associations between lycopene intake and 4-y change in lumbar spine BMD for women [200] protective associations by total carotenoids against 4-y loss in trochanter BMD in men and in lumbar spine in women [98]. On the other hand, radial BMD was not correlated with serum lycopene in postmenopausal Japanese participants, while there was a weak correlation between radial BMD and β-cryptoxanthin and β-carotene [185]. These discrepancy maybe resolved by further in-depth study into the effect of lycopene on BMD. On the positive note, lycopene was shown to contribute to a decrease in the risk of fragility fracture related to osteoporosis.

We carried out a cross-sectional study in which 33 postmenopausal women aged 50–60 years provided seven-day dietary records and blood samples for analysis of oxidative stress parameters and bone turnover markers. Our results showed that postmenopausal women who consumed an average of 7.4 mg of lycopene per day had significantly higher serum lycopene. Our finding that the estimated dietary lycopene had a significant and direct correlation with serum lycopene suggests that lycopene from the diet is bioavailable. Our finding that a higher serum lycopene was associated with a low NTx ($p<0.005$) and lower protein oxidation ($p<0.05$). supports the antioxidative properties of lycopene involvement in its mechanisms of action in bone [39].

The overall conclusions from the epidemiological studies support the beneficial role of lycopene in the prevention of risk for osteoporosis. Further clinical studies described below support this conclusion.

6.5. Clinical intervention studies on lycopene

Since our laboratory is the only one to this date that reported clinical intervention studies with lycopene, this section will focus on reviewing our studies on the role of lycopene in the prevention of risk for osteoporosis in postmenopausal women.

We carried out 4 different clinical studies. In the first study, the objective was to determine the effects of a lycopene-restricted diet on oxidative stress parameters and bone turnover markers in postmenopausal women [38]. To avoid the effects of compounding factors with antioxidants, women who smoked or were on medications which may affect bone metabolism or have anti-

oxidant properties were excluded from participating. Twenty-three healthy postmenopausal women, 50-60 years old, provided blood samples at baseline and after a one-month lycopene-depletion period. Serum samples were analyzed for carotenoids; the oxidative stress parameters protein thiols and lipid peroxidation TBARS; the antioxidant enzymes SOD, CAT and GPx, and the bone turnover markers BAP and NTX. Results revealed that lycopene restriction resulted in significant decrease in serum lycopene, lutein/zeaxanthin and α-/β-carotene as shown in Table 2, However, the overall percent change in these serum carotenoids was not as high as that seen for lycopene. Figure 2 demonstrates that all configurations of lycopene (all trans, 5-cis- and other cis lycopene) were all decreased after lycopene restriction. The antioxidant enzymes CAT and SOD were significantly depressed (data not shown). These changes were accompanied by a significant increase in the bone resorption marker NTx [Figure 3].

Carotenoid	Concentration in serum (nM)		Results of paired t-test[1]	Average % change (mean ± SEM)
	Baseline (mean ± SEM)	lycopene restricted , (mean ± SEM)		
α-carotene	408.4 ± 131.4	334.4 ± 110.6	p<0.05	-13.03 ± 6.88
β-carotene	1443.0 ± 278.9	1035.0 ± 221.7	p<0.0005	-22.84 ± 5.09
β-cryptoxanthin	403.2 ± 58.4	367.6 ± 47.3	p = 0.229	-1.29 ± 8.21
Lycopene	1171.0 ± 111.1	494.9 ± 48.46	p<0.0001	-54.86 ± 3.59[a]
Lutein/zeaxanthin	516.4 ± 49.57	443.0 ± 47.04	p<0.01	-12.77 ± 5.03

[1] Wilcoxon matched pairs test used for these non-normally distributed data sets.

[a] Average percent change in lycopene was significantly higher than that seen for all the other carotenoids (p<0.0001), as determined by unpaired t-test or Mann-Whitney test.

Table 2. Change in serum carotenoid concentrations after postmenopausal women were assigned to Lycopene-restricted diet for a period of 1 month.

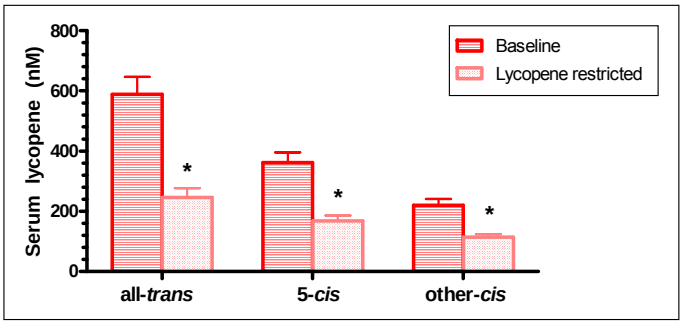

Figure 2. Decrease in all configurations of lycopene (all trans, 5-cis- and other cis lycopene) in the serum of postmenopausal women after lycopene restriction.

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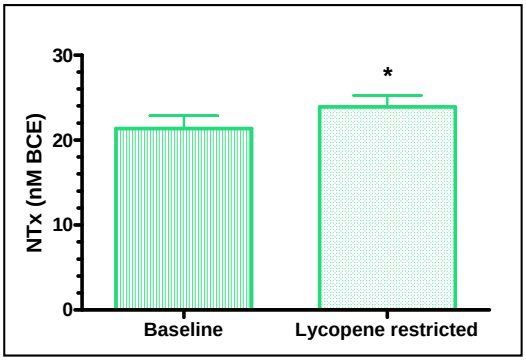

Figure 3. Increase in the concentrations of the bone resorption marker, NTx, in the serum of postmenopausal women after lycopene restriction.

To our knowledge, this is the first study on the effects of dietary lycopene restriction on increasing the risk for osteoporosis in postmenopausal women which proves that lycopene may be beneficial in reducing this risk. It can be speculated that this significant increase in the bone resorption marker NTx may lead to a long-term decrease in BMD and increased fracture risk as was observed by Brown et al [42], and that a longer restriction period may be detrimental to a group of postmenopausal women who were already at high risk for osteoporosis. It can also mean that shorter wash-out periods of no lycopene consumption is all that is needed in clinical trials examining the effects of lycopene on bone health. In addition, lycopene is present in a select number of foods; therefore not consuming these products as a part of the regular daily diet may result in negative health consequences to bone health.

In a second study [37], clinical intervention was carried out to investigate directly the effects of supplementation with lycopene on decreasing the risk for osteoporosis. Sixty postmenopausal women, 50-60 years old, were recruited for a fully randomized controlled intervention. Following a one-month washout without lycopene consumption, participants consumed either (N=15/group): (1) regular tomato juice, (2) lycopene-rich tomato juice, (3) tomato lycopene capsules or (4) placebo capsules, twice daily for total lycopene intakes of 30, 70, 30 and 0 mg/day, respectively for 4 months. Serum collected was assayed for oxidative stress parameters and bone turnover markers. Lycopene-supplementation for 4 months significantly increased serum lycopene compared to placebo ($p<0.001$). Since the increase in serum lycopene was similar for all three supplements, the participants were pooled into a "LYCOPENE-supplemented" and PLACEBO-supplement group for further statistical analyses. LYCOPENE-supplementation for 4 months resulted in significant increase in total antioxidant capacity as shown in Figure 4, decreased in oxidative stress parameters protein oxidation [Figure 5] and lipid peroxidation [Figure 6] which correlated to a decrease in NTx [Figure 7] in the LYCOPENE-supplemented group; all changes were significantly different from the PLACEBO group. These findings suggest that it did not matter whether lycopene was in the form of tomato juice or capsule to exert its potent antioxidant properties beneficial in reducing the risk of osteoporosis in postmenopausal women [37].

In a third study [169]. Serum lycopene, bone turnover markers and oxidative stress parameter data were compared between postmenopausal women who were supplemented with lycopene and those who obtained lycopene from both a low and high daily food intake of lycopene to determine whether the elevated dose obtained through supplementation was more beneficial in reducing bone turnover markers than intakes typically obtained from the usual daily diet. Table 3 showed that women supplemented with lycopene had significantly lower TBARS and marginally significant lower NTx values than participants who obtained a low intake (or high intake lycopene, data not show) through their usual daily diets. These differences in NTx and TBARS may be attributed to a significantly higher concentration of serum 5-*cis* in lycopene-supplemented participants compared to low or high usual daily intake participants. This suggests that it is the 5-*cis* isomer, with the most potent antioxidant capacity which, at higher concentrations, decreases bone turnover markers due to its ability to provide the greatest protection against oxidative stress. It also appears to show that supplementation with lycopene may be necessary in spite of the daily intake of lycopene.

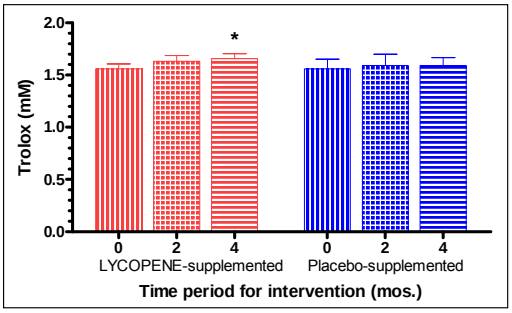

Figure 4. Increase in the serum total antioxidant capacity of postmenopausal women supplemented with LYCOPENE compared to placebo capsules for 4 months. Values are mean ± SEM. Values compared within supplement group was determined to be statistically significant using repeated-measures ANOVA (*p<0.05).

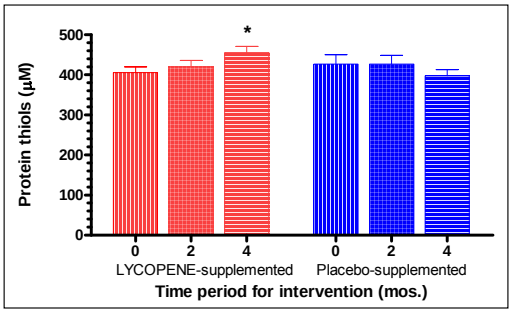

Figure 5. Increase the serum concentration of thiol (meaning decreased protein oxidation) in postmenopausal women supplemented with LYCOPENE compared to placebo capsules for a period of 4 months. Values are mean ± SEM. Values compared within supplement group was determined to be statistically significant using repeated-measures ANOVA (*p<0.001).

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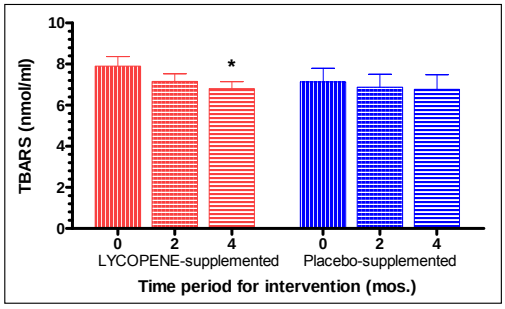

Figure 6. Decrease in the serum concentration of TBARS or lipid peroxidation in postmenopausal women supplemented with LYCOPENE compared to placebo capsules for a period of 4 months. Values are mean ± SEM. Values compared within supplement group was determined to be statistically significant using repeated-measures ANOVA (*p<0.001).

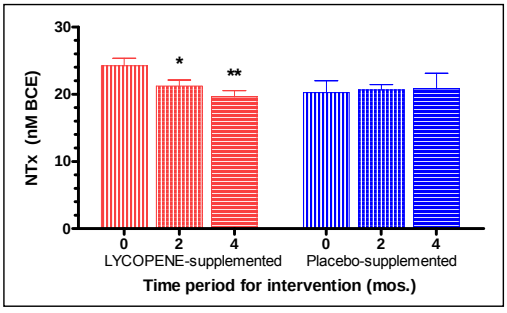

Figure 7. Decrease in the serum concentration of bone resorption marker NTx in postmenopausal women supplemented with LYCOPENE compared to placebo capsules for a period of 4 months. Values are mean ± SEM. Values compared within supplement group was determined to be statistically significant at 2 and 4 months using repeated-measures ANOVA (*p<0.01 and **p<0.001).

In a fourth study, we investigated whether the 172T→A or 584A→G polymorphisms of the paraoxonase 1 (PON 1) modulated the effects of serum lycopene on bone turnover markers, oxidative stress parameters and antioxidant capacity in women between the ages of 25-70 years. We showed that the PON1 polymorphism modified the association between lycopene and NTx and BAP ($p<0.02$ and $p<0.05$ for interaction). In the combined 172TT and 584G genotype, high serum lycopene was associated with decreased BAP ($p<0.01$) and NTx ($p<0.05$). Among those with the combined 172A and 584G genotype, however, increased serum lycopene was associated with increased BAP ($p<0.05$) and NTx ($p<0.05$). These findings show that PON1 polymorphisms modified the association between serum concentrations of lycopene and oxidative stress parameters and bone turnover markers and may, therefore, moderate the risk of osteoporosis [201].

		Mean ± SEM		p value[1]
		Low usual daily intake group (N=48)	Lycopene-supplemented group (N=45)	
Lycopene intake (mg/day)		2.59 ± 0.32[a]	43.33 ± 2.84	<0.0001
Serum lycopene (nM)	Total	1094 ± 80.24	2012 ± 88.56	<0.0001
	all-*trans*	539.0 ± 40.07	979.0 ± 48.48	<0.0001
	5-*cis*	342.4 ± 26.13	685.0 ± 30.22	<0.0001
	other-*cis*	212.6 ± 15.96	347.7 ± 17.36	<0.0001
Total antioxidant capacity (mM)		1.65 ± 0.03	1.66 ± 0.05	0.190
Bone turnover markers	NTx (nM BCE)	21.97 ± 1.11	19.19 ± 0.79	0.047
	BAP (U/L)	24.42 ± 1.40	23.56 ± 1.02	0.900
Oxidative stress parameters	Protein thiols (μM)	454.7 ± 15.37	455.2 ± 15.50	0.982
	TBARS (nmol/mL)	9.06 ± 0.35	6.80 ± 0.35	<0.0001
Antioxidant enzymes	CAT (K/g Hb)	81.50 ± 4.13	58.59 ± 2.06	<0.0001
	SOD (U/mg Hb)	47.60 ± 2.44	39.22 ± 4.72	0.001
	GPx (U/g Hb)	15.44 ± 1.40	32.82 ± 2.85	<0.0001

[1] Data that were not normally distributed were compared using the Mann-Whitney test

[a] The range of lycopene intake for the low usual daily intake group is 0.0-6.07 mg/day.

Table 3. Comparison of lycopene values, oxidative stress parameters and bone turnover markers between women who were supplemented with lycopene with those who obtained a low lycopene (not shown) intake from their usual daily diet (unpaired t-test).

A similar investigation was carried out in a fifth study to assesses whether the PON1 172T→A polymorphism affects the response to dietary intervention with lycopene. We showed that supplementation in the TT genotype and carriers of the A allele significantly increased serum lycopene (both: $p<0.0001$) while decreasing protein oxidation ($p<0.005$ and $p<0.05$, respectively) and lipid peroxidation ($p<0.005$ and $p<0.0005$). However, participants with the TT genotype responded more favourably to lycopene, with corresponding significant increase in total antioxidant capacity (TAC) ($p<0.01$) and significant decrease in NTx ($p<0.001$); this effect was not significant in carriers of the A allele. Further analyses showed that there was a significant interaction between PON1 genotype and change in TBARS ($p<0.05$) suggesting that supplementation with lycopene resulted in decreased lipid peroxidation, which interacted with the PON1 genotype to decrease bone resorption markers in postmenopausal women. These findings provide mechanistic evidence of how intervention with lycopene may act to decrease lipid peroxidation and thus the risk of osteoporosis in postmenopausal women [169,202].

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6.6. Concluding remark

There is now ample evidence to show that oxidative stress brought about by the accumulation of ROS in the body is one of the causes of the development of several chronic diseases including osteoporosis and that antioxidants such as lycopene can counteract this damaging effect. The evidence includes studies on their role in osteoclastic resorption and osteoblastic bone formation, animal intervention studies, epidemiological studies and, more recently, clinical intervention studies. Considering the possible adverse side effects of the conventional therapy (eg, HRT and bisphosphonates) in the management of postmenopausal osteoporosis, there is an increasing demand for the use of antioxidants naturally present in foods. The results of these studies indicate that lycopene maybe useful either as a dietary alternative to drug therapy or as a complement to the drugs presently used by women at risk for osteoporosis.

7. Studies on polyphenols

Polyphenols have long been known to have a role in the prevention of chronic diseases such as cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis. Only in the last 10 years has there been an increase in the interest on polyphenols and bone health [203-206]. Horcajada [204] has recently reviewed the anabolic role of phytonutrients and especially polyphenols in bone while Trzeciakiewicz [205] reviewed the mechanisms of action of polyphenol in osteoblast function and its interaction with osteoclasts. The beneficial effects of green tea polyphenols has been reviewed [207,208].

Currently, most of the research on polyphenols and their effects have emerged from *in vitro* and *in vivo* animal studies with only a few clinical studies available. In our recent review, we have included tables listing all the studies on polyphenols *in vitro* bone cell culture and the epidemiologic studies on the protective effects of polyphenol consumption against osteoporosis [209] and only a few studies will be reviewed here.

7.1. *In vitro* studies on polyphenols in bone cells

The most commonly studied polyphenol abundant in green tea is epigallocatechin-3-Gallate (EGCG). We have shown that epigallocathechin-3-gallate (EGCG) increased the formation of mineralized bone nodules by human osteoblast-like cells [210]. EGCG has been shown to inhibit the expression of matrix metalloproteinase 9 (MMP-9) and the formation of osteoclasts [211]. H_2O_2-induced alterations of osteoblast viability and reduction in alkaline phosphatase activity were prevented by pre-incubating the osteoblasts with green tea polyphenol [212]. Green tea was shown to protect human osteoblasts from cigarette smoke-induced injury [128]. EGCG was shown to inhibit thyroid hormone-stimulated osteocalcin synthesis in osteoblasts [213], suppressed the differentiation of murine osteoblastic MC3T3-E1 cells [214], inhibit rat osteoclast formation and differentiation [215] and induces apoptosis via caspase activation in osteoclasts differentiated from RAW 264.7 cells [216]. Horcajada suggested that most studies investigating the effects of polyphenols on osteoblast cells have reported involvement of complex networks of anabolic signaling pathways such as BMPs or estrogen receptor mediated

pathways [204]. Trzeciakiewicz describing a more detailed mechanisms, suggested that polyphenols modulate the expression of transcription factors in osteoblasts such as runt-related transcription factor-2 (Runx2) and Osterix, NFkappaB and activator protein-1 (AP-1) [205]. In agreement with Hocajada (2012), Trzeciakiewicz (2009) stated in his review that polyphenol may act on cellular signaling such as mitogen-activated protein kinase (MAPK), bone morphogenetic protein (BMP), oestrogen receptor and osteoprotegerin/receptor activator of NF-kappaB ligand (OPG/RANKL) and thus may affect osteoblast functions. The two reviews complement each other and paint a better understanding of the mechanisms of action of polyphenols in bone cells, with the warning that it is also important to take into account the possible interaction of these compounds on osteoblasts metabolism.

Other polyphenols/sources of polyphenols which were found to have beneficial effects on bone cells include the dried plum polyphenols found to attenuate the detrimental effects of TNFalpha on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I and increasing lysyl oxidase expression, and at the same time attenuate osteoclastogenesis signalling [217]; black tea polyphenol which affects the MMP activity and osteoclast formation and differentiation in vitro [215]; phenolic leaf extract of Heimia myrtifolia (Lythraceae) found to stimulate mineralization of SaOS-2 osteosarcoma cells) [218]; Oleuropein which enhances osteoblastogenesis and inhibits adipogenesis and the effects on differentiation in stem cells derived from bone marrow [219] and the polyphenol component of red wine resveratrol which promotes osteogenic differentiation and protects against dexamethasone damage in murine-induced pluripotent stem cells [220] and facilitates in vitro mineralization and in vivo bone regeneration [221]. A number of animal studies have been reported and this was reviewed by Rao et al [209].

Other good sources of polyphenols that are frequently studied are extracts containing combinations of polyphenols. One such source is the nutritional supplement greens+™, a blend of several herbal and botanical products containing a substantial amount of polyphenols including quercetin, apigenin and luteolin [106] which act as antioxidants and therefore should be able to counteract oxidative stress. Our laboratory has shown that the polyphenolic extracts from greens+™ have stimulatory effect on mineralized bone nodule formation in human osteoblast cells in a dose- and time- dependent manner and is more effective than epicatechin (EC) as shown in Figure 8 [222]. We have further shown that this stimulatory effect is accompanied by decreases in the reactive oxygen species H_2O_2 shown in Figure 9 [223], thus proving that greens+™ is able to counteract oxidative stress in human osteoblastic cells and may therefore be a good candidate as a nutritional supplement to prevent the risk of osteoporosis.

Two additional nutritional supplements have since been formulated which may prove to be good for bone health. These are the bone builder™ and the greens+bone builder™ ; the latter is the original greens+™ product that has been supplemented with the bone builder™ formula containing several compounds including vitamins, minerals, and antioxidants. These various components have been separately shown to have some beneficial effect on bone [224]. Using the human osteoblast SaOS-2 cells, we showed that similarly to the greens+™, the water-soluble bone-builder™ extract had a significant dose-dependent stimulatory effect on bone nodules formation (Figure 10) [225]. Figure 11 shows that when the two supplements, greens

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+^{TM} and bone builder^{TM}, were tested as combination, the effects were six times more effective than either one alone in stimulating bone formation in osteoblast culture [226].

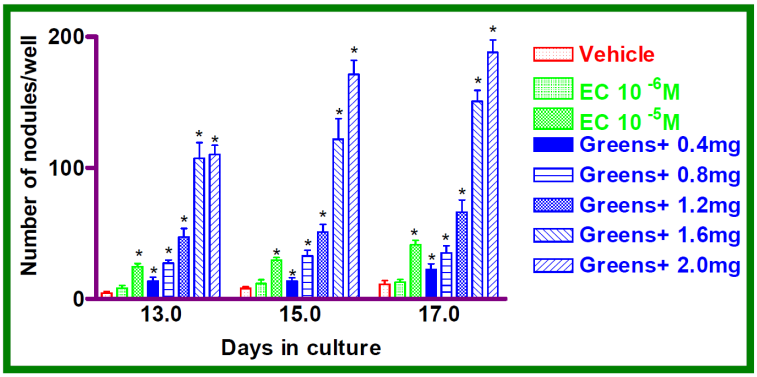

Figure 8. Effect of continuous addition of greens+^{TM} extract on the number of SaOS-2 cells cultured in the presence of EC, or varying dilutions of greens+^{TM} at an early time points and number of nodules analyzed at the indicated time points. An asterix, *, on a bar indicates statistical significance ($p < .05$) between a treatment and the control.

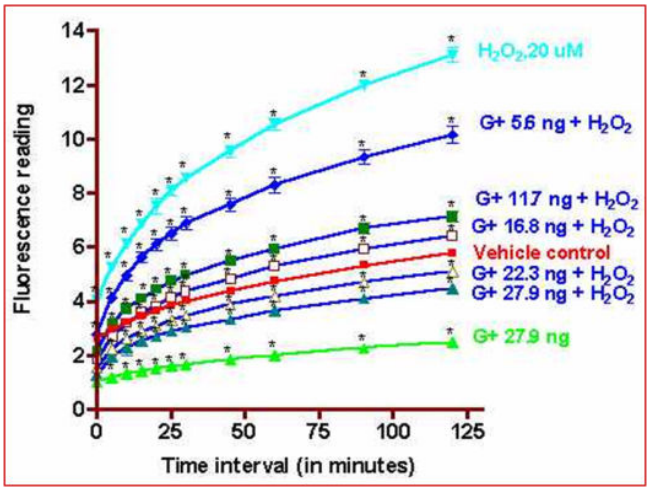

Figure 9. Dose-dependent inhibitory effects of phenolic extracts of greens+^{TM} on intracellular ROS levels stimulated by 20 uM H_2O_2 in SaOS-2 cells. Data are mean ± SEM of 6 replicates. *$p = < 0.05$.

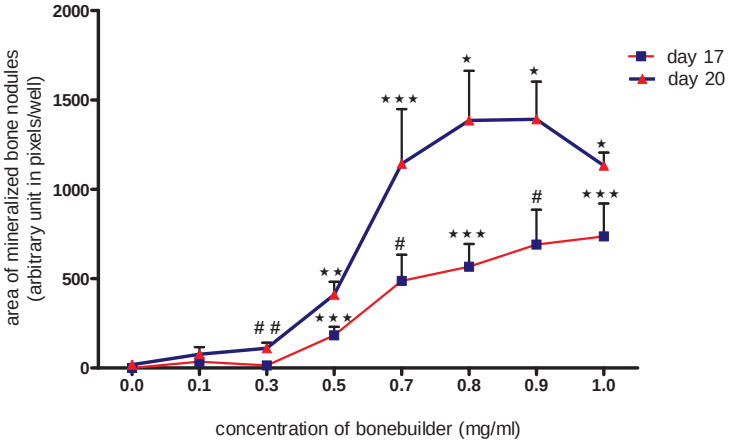

Figure 10. Time and Dose-dependent Effects of bone builder™ on mineralized bone nodule area in SaOS-2 cells. indicates significant difference from vehicle: p<0.0001; p<0.0005; p<0.005, #, p<0.01 and ##, p< 0.05. There is a significant dose-dependent effect both at day 17 and day 20, according to One-way ANOVA; p<0.0001.

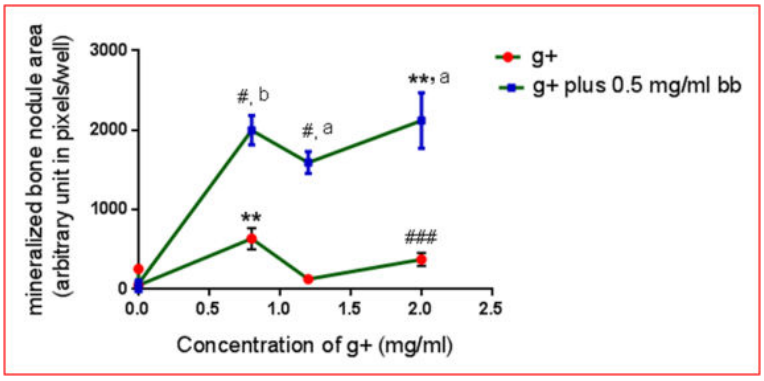

Figure 11. Dose dependent effect of greens + (g+) with and without 0.5 mg/ml of bone builder (bb) on the area of mineralized bone nodules in osteoblasts SaOS-2 Cells. Significant differences were found compared to respective controls. g+bb was more effective than either g+ or bb alone.

7.2. Clinical intervention studies of polyphenol

We have recently reviewed earlier clinical studies on polyphenols and osteoporosis [209]. Only the more recent reports, as well as our own clinical studies will be reviewed here. Shen et al [227] have extended their studies in osteopenic women and showed that dietary supplement in the form of green tea combined with tai-chi, a mind-body exercise, can alleviating bone loss in osteopenic women. The effect of catechin was studied in perimenopausal Scottish women

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and it was found that catechin was negatively associated with bone-resorption markers, association between energy-adjusted total flavonoid intakes and BMD at the femoreal neck and lumbar spine while annual percent change in BMD was associated with intakes of procyanidins and catechins [228].

Other than these clinical studies in the last two years, there has not been not been anymore reported clinical studies on polyphenols in human subjects except our studies.

Our results on the in vitro effects of greens+™, bone builder™ and greens+bone builder™ on bone formation in osteoblasts encouraged us formed the rationale for our clinical studies to test whether these products can prevent the risk of osteoporosis in postmenopausal women. We chose to study the greens+bone builder™ since of the three products, it gave the greatest stimulatory effect on bone formation, being six times more effective than the other two. The first randomized cross-sectional clinical intervention study was carried out to test whether a daily supplementation with greens+ bone builderTM may be important in reducing oxidative damage in postmenopausal women at risk for osteoporosis. [40]. Forty-seven postmenopausal women, 50-60 years old were randomized to either Treatment group consuming 1 scoop (equivalent to ¼ cup) daily of greens+ bone builder™ (N=23) or Placebo (N=24) group for a period of 8 weeks. Blood samples were collected at 0, 4 and 8 weeks of supplementation, processed and assayed for serum total antioxidant capacity (TAC), lipid peroxidation and protein oxidation as markers of oxidative stress. Results revealed that there was an increase in total antioxidant capacity (Figure 12, as well as a decrease in both protein oxidation (Figure 13) and lipid peroxidation (Figure 14) over a 4 and 8-weeks of intervention with greens+ bone builder™ compared to placebo. This suggests that the nutritional supplement may have a beneficial effect on bone health by counteracting the effects of oxidative stress [40].

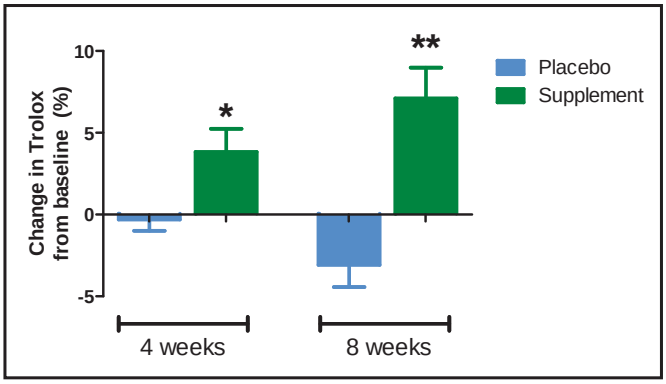

Figure 12. Change relative to baseline in serum concentrations of trolox, a measure of total antioxidant capacity, in greens+bone builder™-treated postmenopausal women was significantly increased after 4 and 8 weeks while that in the placebo-treated control was marginally decreased. Treated values were also higher than the placebo [unpaired t-test (*p<0.01, **p<0.0001)]. Values are mean ± SEM.

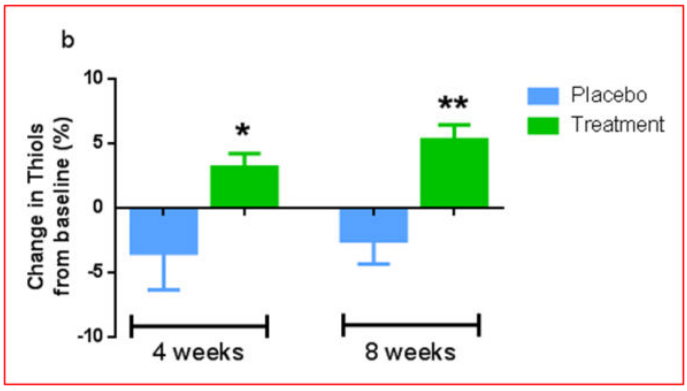

Figure 13. Change relative to baseline in serum concentrations of thiol in greens+bone builder-treated postmenopausal women was significantly increased after 4 and 8 weeks (meaning decreased protein oxidation) while that in the placebo-treated control was unchanged; treated values were also higher than the placebo. Mann-Whitney test (*p<0.05, **p<0.001). Values are mean ± SEM.

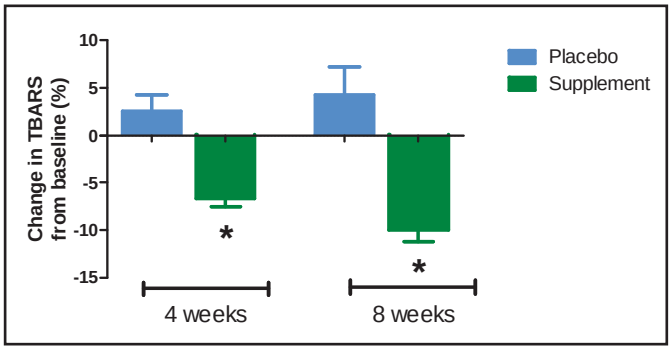

Figure 14. Change relative to baseline in serum concentrations of TBARS in greens+bone builder™-treated postmenopausal women was significantly decreased after 4 and 8 weeks (meaning decreased lipid peroxidation) while that in the placebo-treated control was unchanged; treated values were also lower than the placebo. Mann-Whitney test (*p<0.05, **p<0.001). Values are mean ± SEM.

In order to test whether the antioxidant properties of greens+bone builder™ can prevent the risk of osteoporosis in postmenopausal women, we also measured the serum bone turnover markers, C-terminal telopeptide of type I collagen (CTX) as indicator of bone resorption, and procollagen type I N-terminal propeptide (PINP) as indicator of bone formation, in addition to the serum antioxidant capacity, and the oxidative stress parameters lipid peroxidation, protein oxidation. As shown in Figure 15, statistical analysis showed that at 8 weeks, the greens +bone builderTM supplement group significantly decreased the bone resorption marker CTX, while the Placebo group showed no significant changes. The supplement group was also significantly different from that of the Placebo group in all parameters measured. This decrease

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in CTX correlated to the increase in their serum total antioxidant capacity [Figure 12] and decreases in oxidative parameters protein oxidation [Figure 13] lipid peroxidation [Figure 14]. These results suggest that a daily supplementation with polyphenols and micronutrients may be important in reducing oxidative damage by reducing bone resorption, thereby reducing the risk of osteoporosis in postmenopausal women [41].

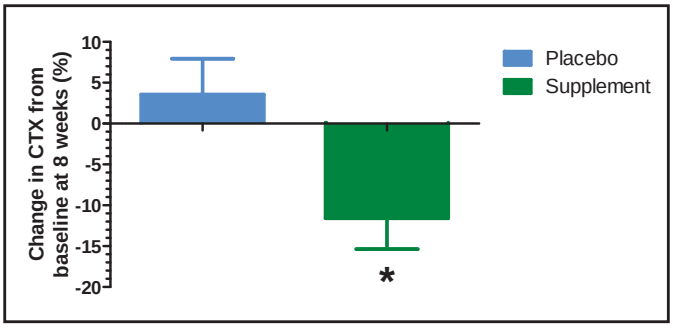

Figure 15. Change relative to baseline in serum concentrations of CTX in greens+bone builder™-treated postmenopausal women was significantly decreased after 8 weeks (meaning decreased bone resorption marker) compared to that of the placebo-treated control (a paired t-test (*p<0.05).. Values are mean ± SEM.

7.3. Concluding remarks

Studies reported in the literature on the role of polyphenols in bone health have exploded in the last 10 years, but most of the reports involved in vitro studies in osteoclasts and osteoblasts, animal studies and epidemiologicai studies. There is little doubt from the excellent studies reported that oxidative stress is one of the primary culprits responsible for the pathogenesis of osteoporosis via its role in osteoclastic resoption and the detrimental effects on the bone-forming osteoblasts. To date, only four clinical intervention studies have been reported, including ours. It is easy to see why it is very difficult to evaluate the role of polyphenols since, as we learned from this review, there are at least 8,000 different polyphenols identified to date, and each one probably having different effects on humans. Additionally, polyphenols are present in food with other constituents that may also be beneficial to bone health. In our clinical study, we combined the effects of a combination of polyphenols present in the nutritional supplement from greens+™ with the nutritional components present in bone builder™ such as minerals, vitamins and other nutrients. It is possible that the effects of greens+bone builder™ in increasing total antioxidant capacity, decreasing the oxidative stress markers protein oxidation and lipid peroxidation which correlated to the decrease in bone turnover marker for bone resorption is a result of the combined effects of the different polyphenols it contained with those of the other nutritional components present in the bone builder™. It remained for future studies to zero in on specific component that is responsible for its beneficial effect.

8. General summary and conclusion

In conclusion, we showed that oxidative stress due to ROS that are shown to cause the development of osteoporosis may be prevented by supplementation with the antioxidants lycopene and polyphenols. Results of in vitro studies in osteoblasts and osteoclasts, animal intervention studies, epidemiological studies and clinical intervention studies on lycopene and polyphenols are evidence for their potential use as alternative or complementary agent with other established drugs approved for the prevention or treatment of osteoporosis in postmenopausal women.

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