Oxidative Stress and the Risk of Osteoporosis: The Role of Dietary Polyphenols and Nutritional Supplements in Postmenopausal Women

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

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2012

Abstract

Previous findings have indicated that oxidative stress plays a role in the development of osteoporosis and that individual polyphenols, by virtue of their antioxidant properties, may mitigate these damaging effects. Nutritional supplements, greens+ bone builder$^{TM}$, containing polyphenols and other micronutrients beneficial for bone health are of recent interest as complementary strategies in the management of osteoporosis. A randomized controlled study was conducted to explore the combined effects of the nutrients found within the supplement on bone health for 8 weeks. Total polyphenol content and antioxidant capacity increased whereas oxidative stress parameters and the bone resorption marker, crosslinked C-telopeptide of type I collagen decreased after supplementation. There was no significant change in the bone formation marker, procollagen type I N-terminal propeptide. This thesis shows an association of polyphenols with other micronutrients acts through their antioxidant capacity to decrease oxidative stress parameters and bone resorption, thus potentially reducing the risk for osteoporosis.
Acknowledgments

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>( ^1 \text{O}_2 )</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>8-iso-PGF(_2\alpha )</td>
<td>8-iso-Prostaglandin F(_2\alpha )</td>
</tr>
<tr>
<td>8-OH-dG</td>
<td>8-Hydroxy-2′-deoxyguanosine</td>
</tr>
<tr>
<td>A, B, C</td>
<td></td>
</tr>
<tr>
<td>ABTS</td>
<td>2, 2, azinobis (3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>ABTS(^-)</td>
<td>2, 2, azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOPP</td>
<td>advanced oxidation protein product</td>
</tr>
<tr>
<td>BAP</td>
<td>bone alkaline phosphatase</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BMU(s)</td>
<td>basic multicellular unit(s)</td>
</tr>
<tr>
<td>BTM(s)</td>
<td>bone turnover marker(s)</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CO(_2 )</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>CRF(s)</td>
<td>clinical risk factor(s)</td>
</tr>
<tr>
<td>CTX</td>
<td>collagen type I cross linked C-telopeptide</td>
</tr>
<tr>
<td>D, E, F, G</td>
<td></td>
</tr>
<tr>
<td>DPD</td>
<td>deoxypyridinoline</td>
</tr>
<tr>
<td>DXA</td>
<td>dual X ray absorptiometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>Grx-5</td>
<td>glutaredoxin 5</td>
</tr>
<tr>
<td>H, I, J, K</td>
<td></td>
</tr>
<tr>
<td>H(_2 )O</td>
<td>water</td>
</tr>
<tr>
<td>H(_2 )O(_2 )</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HClO</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>ICTP</td>
<td>type I collagen C-telopeptide</td>
</tr>
<tr>
<td>IL-1/6</td>
<td>interleukin-1/6</td>
</tr>
<tr>
<td>INRA</td>
<td>Institut National de la Recherche Agronomique</td>
</tr>
<tr>
<td>L, M, N</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>monocyte/macrophage colony–stimulating factor</td>
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<tr>
<td>Mn-SOD</td>
<td>manganese superoxide dismutase</td>
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</tbody>
</table>
MDA  malondialdehyde  
MSC  mesenchymal stem cells  
NFκB  nuclear factor-κB  
NO  nitric oxide radical  
NTX  crosslinked N-telopeptides of type I collagen  

**O, P**

O$_2^-$  superoxide radical  
O$_3$  ozone  
OCN  osteocalcin  
OH$^-$  hydroxyl radical  
OPG  osteoprotegerin  
OSI  oxidative stress index  
PICP  procollagen type I C-terminal propeptide  
PINP  procollagen type I N-terminal propeptide  
PTH  parathyroid hormone  
PYD  pyridoline  

**Q, R, S**

RANκ  receptor activator of nuclear factor κB  
RANκL  receptor activator of nuclear factor κB ligand  
REB  research ethics board  
RO$^·$  alkoxyl radical  
ROO$^·$  peroxyl  
ROS  reactive oxygen species  
Runx2  runt-related transcription factor 2  
SOD  superoxide dismutase  

**T**

TAC  total antioxidant capacity  
TAS  total antioxidant status  
TBARS  thiobarbituric acid reactive substances  
TEAC  Trolox equivalent antioxidant capacity  
TNF  tumor necrosis factor  
TOS  total oxidative status  
TRAP5b  type 5 tartrate resistant acid phosphatase  

**U, V, W, X, Y, Z**

USDA  United States Department of Agriculture  
WHO  World Health Organization  

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CHAPTER 1
INTRODUCTION

The research work undertaken in this thesis focuses on the role of the additive effects of polyphenol antioxidants with other micronutrients (Appendix I), which have been shown to be important for bone health, in lowering the risk of osteoporosis due to oxidative stress. For better understanding of these relationships, the following sections include a discussion of postmenopausal osteoporosis, oxidative stress and polyphenol antioxidants.
CHAPTER 2

LITERATURE REVIEW
I. Postmenopausal Osteoporosis

IA. Overview

Osteoporosis is a skeletal disease characterized by low bone mass and structural deterioration of bone tissue and structure, leading to an increase in bone fragility and susceptibility to fractures, most frequently in the hip, wrist and spine [1]. Osteoporosis is commonly referred to as the “silent thief” as there are no noticeable symptoms associated with its progression until it manifests in the form of a fragility fracture [2]. It has been estimated that approximately 1 in 3 women over the age of 50 will suffer a fragility fracture at some point in their life-time [3].

There are two types of osteoporosis, primary and secondary types. Primary osteoporosis is classified into two subtypes: (a) type I osteoporosis (also known as postmenopausal osteoporosis), which is a common bone disorder in postmenopausal women and is mainly due to estrogen deficiency resulting from menopause, and (b) type II osteoporosis (also referred to as age-related osteoporosis or senile osteoporosis), which is related mainly with aging in both women and men [4]. Alternatively, secondary osteoporosis refers to bone disorders that are the consequence of various other medical conditions, or of changes in physical activity, or are adverse outcomes of therapeutic interventions, generally pharmological, for certain disorders [4, 5]. The gold standard for diagnosis of osteoporosis is dual energy X ray absorption (DXA) scan, which measures the bone mineral density (BMD). Using DXA measurements, BMD is expressed as the Z-score, which measures the variance between the patient and the expected value for patient’s age and sex, and T-score, which measures the variance between the patient the young adult of the same sex and race [6]. The World Health Organization (WHO) developed guidelines for the use of clinically diagnosing osteoporosis based on BMD T-scores: +1 to -1 (normal BMD), -1 to -2.5 (osteopenia/low BMD), -2.5 or lower (osteoporosis), and -2.5 or lower with fragility fractures (severe osteoporosis) [7].
Postmenopausal osteoporosis in particular, is becoming more prevalent as the population ages and therefore the impact of this condition on public health is becoming increasingly critical [8]. Approximately 1.6 million hip fractures occur worldwide each year and by 2050, this prevalence could reach between 4.5 million and 6.3 million [3]. Postmenopausal women experience an increased rate of bone turnover, which in turn has a detrimental effect on bone microarchitecture and is associated with an increased risk of osteoporotic fractures [9]. Specifically, early postmenopausal women experience an accelerated bone loss; an average bone mineral loss at a rate of 0.5-2% per year, which then slows down later with age [10]. In 2005, Johnell and Kanis showed that an average 50 year old woman has approximately 40–50% lifetime risk of an osteoporotic fracture leading to significant personal and societal burden [11].

**IB. Bone biology: Remodelling and pathophysiology of osteoporosis**

Bone is a dynamic tissue that is constantly being remodelled to preserve a healthy, functional skeleton. Remodelling is essential to maintain bone mass, to repair microdamage of the skeleton, to avoid accumulation of too much old bone, and for mineral homeostasis [5]. There are two major types of bone: (a) cortical, which is protective and provides a mechanical function, and (b) trabecular, which provides strength and is more metabolically active (25% per year) than cortical bone (3% per year) [4, 12]. The higher turnover may be due to the large bone surface area, which is in close contact with the bone marrow in which regulatory factors are present (discussed below) [12-15]. Given its higher metabolic activity, trabecular bone is the primary site of bone remodelling; hence, it is the main site for various bone diseases to occur, particularly metabolic bone diseases including osteoporosis [4]. However, it is also noteworthy that metabolic bone diseases affect both trabecular and cortical bone.

Bone remodelling is carried out by a functional and anatomic structure known as the basic multicellular unit (BMU) and requires the coordinated action of three major types of bone cells: osteoclasts, osteoblasts and osteocytes [4].
Cells of Bone

Osteoclasts are large, multinucleated cells that differentiate from mononuclear cells of the monocyte/macrophage lineage upon stimulation by two integral factors: the monocyte/macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor κB ligand (RANKL) [4]. M-CSF enables proliferation, survival and differentiation of osteoclast precursors, whereas RANKL is the most important cytokine that prepares the precursor cells for osteoclast differentiation. RANKL binds to the receptor RANK on the surface of osteoclast precursors and osteoclasts, and is the primary activator of osteoclast formation and action [16]. Osteoclasts have a catabolic role and break down components of bone through the action of lysosomal enzymes at specific sites [17]. In contrast, osteoblasts are anabolic, and thus, promote the formation of new bone by producing the organic constituents of bone (unmineralized bone matrix), and subsequently take part in its two stage mineralization process [16]. Mesenchymal stem cells (MSCs) yield osteoprogenitors, which grow and differentiate into pre-osteoblasts and then mature into osteoblasts via the Wnt signaling pathway [4, 17]. Even though the synthesis of unmineralized bone matrix is relatively fast and can take place within 6–12 hours from initiation, the secondary mineralization takes much longer (1–2 months) [17]. When bone formation is complete, the osteoblasts become surrounded by the new bone matrix and differentiate into osteocytes [17]. Osteoblasts also secrete osteoprotogerin (OPG), a circulating inhibitor of RANKL [17]. OPG binds to and sequesters RANKL and so, prevents its binding to RANK; thus, OPG acts as a potent anti-resorptive cytokine by inhibiting osteoclast formation and thus, activity [17, 18]. Osteocytes are the most abundant cells in bone and may play an important part in transmitting local strain information to allow BMUs to regulate the bone content in response to the local need. They might function as ‘mechanostats’ and under conditions of strain would prompt the remodelling of bone [4, 17].

Bone remodelling or turnover in an individual BMU is a physiological process that follows a time sequence lasting approximately four months wherein old or damaged bone is eliminated by osteoclasts then replaced by new bone formed by osteoblasts [4, 16]. There are four overlapping phases [4, 16]: (1) Osteoclast precursors are require factors produced by marrow stromal cells, osteoblasts or T-lymphocytes to differentiate into multinuclear osteoclasts.
Two essential factors are the M-CSF and RANKL [19]. (2) Osteoclasts resorb bone, producing a resorption cavity – a process that takes approximately 3 weeks: once the multinucleated osteoclasts have matured, they attach to a bone surface to seal off a resorption zone. Osteoclasts then secrete numerous enzymes (e.g. the protease Cathepsin K) and acid into this zone causing bone resorption. (3) Osteoblasts produce collagenous bone matrices and complete its mineralization, resulting in the synthesis of such bone matrix proteins such as collagen type 1, osteopontin, osteocalcin, bone specific alkaline phosphatase and bone sialoprotein. Additionally, the osteoblasts produce different factors that are stored within the newly synthesized bone for future use and released during subsequent remodelling cycles. Following behind the osteoclasts during bone resorption are the newly recruited osteoblasts to fill the site of resorption. Thus, osteoclasts consist of cells that are of different ages and the successive set of osteoblasts are of the same age [20]. (4) As bone formation persists, osteoblasts (i) become entrenched more deeply into the bone, eventually becoming surrounded by bone and are henceforth defined as osteocytes; (ii) enter apoptosis; or (iii) stay on the bone surface to become bone-lining cells.

A model illustrating the 4 phases of bone remodelling is show in Figure 2.1.
Figure 2.1: Model for bone remodelling (Adapted from [4])

It is important to note that the organization of the BMU found in trabecular bone is different from cortical bone. In cortical bone, the front (cutting cone) of the BMU forms a cylindrical canal through the bone, which is filled by osteoblasts (closing cone) [21]. Unlike cortical bone, the BMU travels across the trabecular surface by creating a trench rather than a canal, and osteoblasts fill the resorption cavity. The lifespan of a BMU is approximately 2-8 months [13, 22]. The whole remodelling process renews about 10% of bone per year, thus renewing the entire skeleton in approximately 7-10 years [20, 21].

Postmenopausal Homeostasis of Bone and its Dysregulation
In mature, healthy bone, the balance between bone resorption and bone formation is tightly regulated and maintained to ensure that there are no significant alterations in bone mass or
mechanical strength after each remodelling cycle [4, 16]. However, an imbalance between bone resorption and formation may arise under certain pathological conditions. This dysregulation of the homeostatic relationship between bone forming and bone resorbing cells leads to abnormal bone remodelling and the development of bone disorders including osteoporosis [4]. The greatest change in bone remodelling occurs at menopause, where there are more resorption cavities due to an increase in bone resorption. Moreover, bone formation does not increase proportionately with the increases seen in resorption and so resorption cavities are not completely refilled with new bone. This deficit in bone replacement during menopause causes increasing loss of bone mass, which if left untreated, is permanent [5, 16].

This might be related to the fact that at menopause, estrogen, which normally exerts an inhibitory effect on osteoclast function, becomes deficient [17]. A more detailed discussion regarding the pathophysiology of osteoporosis is below.

Pathophysiology of Osteoporosis: a brief overview
The exact pathogenesis of osteoporosis is not known. It is recognized that both bone resorption and bone formation are amplified in postmenopausal osteoporosis; nonetheless, there is an imbalance between bone resorption and bone formation in favour of bone resorption [4]. Lerner and colleagues suggest that it is the increased frequency of resorption cavities and the reduced ability of individual osteoblasts to synthesize new bone that cause the decrease in bone mass as well as in bone strength [5]. Alternatively, Raisz et al. presumed that impairment of osteoblast renewal, rather than a defect in osteoblast function, was involved [23]. Consequently, the number of new osteoblasts that differentiate and lay down successive lamellae of new bone is reduced.

Indeed, an increase in bone resorption seems to be the key stimulus for bone loss in the setting of acute estrogen deficiency. It is evident that osteoblasts, osteocytes, and osteoclasts express functional estrogen receptors [18]. These receptors are also expressed in MSCs, the precursors of osteoblasts, which physically support future osteoclasts, T-cells, β-cells, and most other cells in the human bone marrow [18]. It is well established that activation of estrogen receptors in osteoblasts by estrogen induces their anabolic activities and reduces the
pathway by which osteoblasts can activate osteoclasts activity [5]. In contrast, stimulation of estrogen receptors in osteoclast progenitor cells diminishes osteoclast formation, and activation of estrogen receptors in mature osteoclasts prevents their bone-resorbing activity. Furthermore, via a complex interaction between bone cells and the immune system, lack of estrogen stimulates T-cells to release various inflammatory cytokines, which estrogen normally inhibits [18]. Some (e.g., interleukin [IL]-1, IL-6, and tumor necrosis factor [TNF]-α) promote osteoclast recruitment, differentiation, and prolonged survival, while others (e.g., IL-7) inhibit osteoblast differentiation and activity, and cause apoptosis of osteoblasts [18]. The ultimate outcomes of estrogen deficiency are therefore increased bone resorption and impaired bone formation and hence, bone loss.

Thus, the pathogenesis of osteoporosis in women likely involves augmented bone resorption by osteoclasts, related to changes in estrogen levels at menopause. Another factor that is involved in the development of osteoporosis is age. Age is centered on bone formation by osteoblasts, and involves various distinct factors linked to the aging process in both men and women such as the formation and accumulation of reactive oxygen species (ROS) [4] inducing oxidative stress. ROS, the radical forms of oxygen, are by-products of respiration and oxidase enzyme activity in the mitochondria, and of cellular responses to numerous external stimuli ranging from inflammatory cytokines to ionizing radiation [4]. There is an age-related rise in ROS levels that stems from age-related increases in ROS production and/or a reduction in antioxidants, which counteract these adverse effects [4, 24]. Age-dependent increases in ROS have been shown to reduce osteoblastogenesis and hence, bone formation [4, 24]. A proposed signaling pathway of ROS affecting osteoblasts and osteoclasts is shown in figure 2.2. The role of oxidative stress and antioxidants in osteoporosis will be discussed further in the following sections.
**Figure 2.2:** ROS-activated signalling pathways affecting mesenchymal stem cells (MSC), osteoblasts (OB) and osteoclasts (OC).

**IC. Risk Factors for Osteoporosis**

Osteoporosis is a multi-factorial disease. Risk factors can be categorized into two main groups: non-modifiable risk factors and modifiable risk factors. The non-modifiable risk factors are those that cannot be changed, including age, sex, race, family history, early menopause, as well as medical comorbidities. The modifiable risk factors include geographical region and lifestyle (alcohol, smoking, exercise, and diet).

**Non-modifiable Risk Factors:**

**Age**
Approximately 90% of hip fractures occur in people who are over the age of 50 [25]. For any BMD, the fracture risk is much higher in the elderly than in the young [26-28]. This is partially because the bone remodelling balance tips toward bone mineral loss, leading to an increased risk of fracture.

**Sex Differences**
Women generally have a lower peak bone mass compared to men [29]. Furthermore, women are more susceptible to bone loss than men, especially postmenopausal women due to their
deficiency of estrogen. Estrogen plays a vital role in bone remodelling in that it promotes an increased osteoblast activity, favouring bone formation [24, 30]. These factors, among others contribute to the fact that postmenopausal women are at greater risk for the development of fracture than men of the same age. For instance, it has been shown that all else being the same, females have a 40-50% chance of developing fractures due to osteoporosis whilst males only have a 13-22% chance [11].

**Race**

Studies have shown that variations in the incidence of osteoporotic fractures incidence can also be explained by exist depending on race. Osteoporosis is considered to be more common amongst the Caucasian and Asian populations [31, 32] and one study also showed that vertebral fracture was more prevalent in Japanese women than in American Caucasian women [33]. As well, the incidence of osteoporosis and fractures of the hip and spine is lower in Black than in Caucasian subjects [34-36]. Furthermore, the lifetime risk of a hip fracture amongst the Black population is approximately 50% of the rate observed in the Caucasian population [37]. Interestingly, despite lower BMD measurements in Asians than Black women, the National Osteoporosis Risk Factor Assessment study noted that they shared a relatively low risk for osteoporotic fractures of the forearm when compared with American Caucasian, Hispanic and Native American women [38].

**Family and Medical History**

A family history of fractures also appears to be a risk factor for future fractures [39]. A family history of hip fracture has been shown to correlate to increased risk for fracture independent of BMD [40]. Furthermore, it has been shown that the risk of hip or wrist fracture was increased in women with a family history of wrist or hip fracture [41, 42]. As well, women with a family history of osteoporosis were more likely to develop osteoporosis in comparison to those who did not have such a family history [43].

Risk factors for fracture also include a previous fracture related to (presumably osteoporosis related) fragility [44], body mass index [41, 45, 46], resting pulse rate over 80 beats per minute [47], certain therapeutics in addition to diseases that directly or indirectly affect bone
remodelling (asthma, rheumatoid arthritis, Crohn’s disease, etc) and conditions that affect mobility and balance (e.g. poor vision) that contribute to increased risk of falling [37, 39, 48, 49].

**Modifiable Risk Factors:**

**Geographical Region**

Variation in the prevalence of osteoporosis has also been shown to differ on the basis of geographic location [50, 51]. For example, it has been reported that there is an increased incidence of osteoporosis in subjects living in northern countries as compared to those living in southern countries [50, 52, 53].

**Lifestyle: Diet and Exercise**

It is accepted that a healthful diet of minerals including, but not limited to manganese, copper, selenium, and zinc is important insofar as the prevention of many diseases and disorders are concerned. A diet low in calcium might lead to increases in the synthesis and secretion of parathyroid hormone, which activates osteoclast activity. This could conceivably lead to increased bone resorption so as to normalize serum calcium that might otherwise be reduced due to the dietary deficit [54]. As well, vitamin D is also essential because it mediates calcium absorption from the intestines into the blood. It is recommended in Canada for women over the age of 50 years to consume to that 800 to 2000 IU of vitamin D and 1,200 mg of calcium is consumed daily to prevent the development of osteoporosis [55, 56]. Therefore, a diet low in calcium and vitamin D may increase the risk of osteoporosis.

Exercising has also been shown to affect skeletal growth [57-59]. It has been documented that people with a more sedentary lifestyle are more likely to have a hip fracture than individuals who are active. For instance, women who sit for more than nine hours a day are twice more likely to have a hip fracture than those who sit for less than six hours a day [60]. In contrast, studies have reported that exercise had no effect on preventing or treating osteoporosis [61].
Cigarette smoking and excessive alcohol consumption are two other contributing lifestyle choices that are modifiable risk factors for osteoporosis. It has been documented that people who used to smoke or still currently smoke have a greater risk for any type of fracture, including one related to osteoporosis when compared to those who are never smoked [62]. When compared to individuals who consumed a moderate or no intake of alcohol, those who consumed an excessive amount of alcohol (> 2 units/day) were at an increased risk of sustaining any osteoporotic fracture [27, 63]. This may be caused by the effects of alcohol on osteoblasts, and could also affect the hormonal cascades involving calcium metabolism and vitamin D deficiency [64].

**ID. Bone biology: Biomarkers of bone turnover as predictors of bone loss and individual risk of fracture in participants with postmenopausal osteoporosis**

Bone turnover markers (BTM) reflect whole body rates of bone resorption and bone formation, and provide a dynamic analysis of ongoing changes within the skeleton. This is advantageous over BMD measurements using DXA for various reasons that include: (a) DXA offers a static measurement of bone mineral content and density [65, 66], even though bones are physiologically active and undergo a continuous process of remodelling in each BMU; (b) BMD changes occur more slowly (e.g. could take a couple of years) than changes in the levels of the biomarkers which can be seen in weeks in response to changes in activity of disease (with or without treatment) [67]; (c) from a clinical use perspective, half of the patients with incident fractures have baseline BMD measurements above -2.5 SD or below the average value of young healthy women, thus limiting the sensitivity of this particular outcome measure. In fact, as a consequence of this relatively poor sensitivity, other approaches have been developed to determine the risk for osteoporotic bone fractures to develop. In this regard, recently, the WHO introduced a prognostic tool to evaluate fracture risk of patients called the FRAX® [68]. It assesses the 10-year risk of osteoporotic-related fracture based on individual patient models that combines clinical risk factors (CRF) as well as BMD at the femoral neck.

An in depth review showed several studies that examined the association between baseline levels of biochemical markers and the rate of subsequent bone loss estimated by changes in
BMD by DXA over long periods of up to 10 years among elderly and younger postmenopausal women [69]. Many of these studies showed a significant association between high bone turnover with faster subsequent bone loss, although the association was inconsistent between the skeletal sites. Thus, a single measurement of bone markers cannot accurately identify individuals with rapid bone loss. However, this was acknowledged in one study where BMD and BTM were assessed, showing that by averaging serial measurements over time, it may be possible to improve the predictive value relative to bone loss in elderly women compared to a single baseline assessment [70].

Bone remodelling can be assessed accurately by the measurement of BTM in blood or urine. These have been useful in reflecting the effects of therapeutic agents on the cell’s activities related to either or both bone formation or resorption. Assessment of BTM alone cannot be used to diagnose osteoporosis because the values for osteoporotic and normal patients overlap considerably; hence DXA is a much stronger tool in this case, but as noted above, even in the case of DXA, there is considerable overlap [71]. However, measurement of BTM can give a good indication about the future risk for bone loss and fractures and is highly effective in monitoring the efficacy of antiresorptive therapy in patients with osteoporosis[67, 72, 73]. They can be categorized as bone formation markers and bone resorption markers (Table 2.1).
<table>
<thead>
<tr>
<th>Bone formation Markers</th>
<th>Bone Resorption Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Serum</td>
</tr>
<tr>
<td>Serum osteocalcin (OC)</td>
<td>collagen type I cross linked C-telopeptide (s-CTX)</td>
</tr>
<tr>
<td>Serum Total Alkaline Phosphatase (ALP)</td>
<td>Carboxyterminal telopeptide of type I collagen (ICTP)</td>
</tr>
<tr>
<td>Serum Bone-Specific Alkaline phosphatase (BALP)</td>
<td>Tartrate resistant acid phosphatase (TRACP)</td>
</tr>
<tr>
<td>Serum procollagen type I C-terminal propeptide (PICP)</td>
<td>Tartrate resistant acid phosphatase 5b (s-TRACP 5b)</td>
</tr>
<tr>
<td>Serum procollagen type N-terminal propeptide (PINP)</td>
<td></td>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

Bone formation markers reflect osteoblast activity. These include bone specific alkaline phosphatase (BAP), osteocalcin (OC) and procollagen type I C- and N-terminal propeptide (PICP and PINP, respectively). Bone resorption markers are indicative of either enzymes of the osteoclastic cells such as the 5b isoenzyme of tartrate resistant acid phosphatase (TRAP5b) or the proteolytic fragments of bone collagen matrix, including the type I collagen
crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD), and their telopeptides, carboxy terminal telopeptide (CTX) and amino terminal telopeptide (NTX) [65]. For the purpose of this thesis, PINP and CTX will be discussed in further detail as they were the markers used for the studies conducted.

Type I collagen is an important and most abundant component in the bone matrix where osteoblasts secrete its precursor proteins, procollagen and tropocollagen, during bone formation. Type I collagen is a triple helical structure consisting of two identical α1 chains and one α2 chain with a non-helical region where the amino (N)-telopeptide and carboxy (C)-telopeptide attach to the crosslinks [65, 67, 74]. As collagen is being synthesized, the N- and C-terminal extensions of the procollagen type 1 are cleaved by enzymes and are referred to as PINP and PICP respectively [75-77]. The PINP concentration has been shown to be proportional to the amount of new collagen laid down during bone formation. To note, type I collagen propeptides sources may vary, however, most of the non-skeletal tissues have illustrated a slower rate of turnover than bone, thus the propeptides seen in circulation are mainly derived from bone [74]. PINP is a highly specific and sensitive bone turnover marker for monitoring anabolic treatment [78]. One of the advantages of using this assay is that the only source of variation is dependent on the small circadian rhythm.

During bone resorption, an osteoclast enzyme such as cathepsin K hydrolyzes the crosslinks and releases the N (NTX)- or C (CTX)-telopeptide into circulation as markers of bone resorption [79, 80]. The CTX-telopeptide consists of two isomeric forms, the native (α CTX) and the age-related (β CTX) [74, 81]. The CTX-telopeptide is highly vulnerable to isomerization or racemization, resulting in αL, βL, αD, and βD [65, 74]. The degree to which collagen is isomerized may possibly provide information on the age-dependent changes of collagen in health and disease [82, 83]. As there are various assays to determine CTX, a sandwich enzyme-linked immunosorbent assay (ELISA) for the measurement of β-CTX in serum is one of the most commonly used measures. CTX is detected by using two monoclonal antibodies that recognize the β-isomerized octapeptide on the non-helical (C)-telopeptide [65]. To date, CTX is considered to be the most sensitive marker of bone resorption. However, CTX has some disadvantages in that the samples must be collected at a
specific period of the day and in a fasting state. For instance, CTX levels are much lower in the afternoon than in the morning and are related to the ingestion of food [75, 84].
II. Oxidative Stress

IIA. Overview

Oxidative stress is characterized by an increased level of ROS that disrupts the intracellular reduction–oxidation (redox) balance [85]. This balance heavily depends on the equilibrium between the concentrations of ROS and antioxidants, reacting with ROS, to protect other intracellular molecules from oxidative damage. ROS are oxygen-derived pro-oxidants that can damage cell structures that are comprised of lipids, proteins or DNA, as well as some enzymes that defend the cell [86]. Damaging any of these structures can lead to the damage of bone cells. The two common groups of ROS are radicals and nonradicals. The radicals have one or more unpaired electrons, making them highly reactive as they donate or obtain another electron from another free radical to become stable [87]. These include nitric oxide (NO$^\cdot$), superoxide ion (O$_2$$^\cdot$), hydroxyl (OH$^\cdot$), peroxyl (ROO$^\cdot$), and alkoxy radicals (RO$^\cdot$) and one form of singlet oxygen ($^1$O$_2$) [88]. Nonradicals, also highly reactive, include hypochlorous acid (HClO), hydrogen peroxide (H$_2$O$_2$), organic peroxides, aldehydes, ozone (O$_3$) and O$_2$ [88]. All of these oxygen radicals and nonradicals are created as by-products of aerobic metabolism [89]. Other factors which can generate ROS include cigarette smoke, physical stress, ultraviolet radiation, chemicals, fried foods, environmental pollutants and the aging process [77, 90-93].

IIB. Oxidative damage to cell components

**Oxidative Damage to Lipids**

All cellular membranes contain high concentrations of unsaturated fatty acids, therefore they are highly vulnerable to oxidation [94]. This damage is commonly referred to as lipid peroxidation, which occurs in three majors steps: initiation, propagation and termination. In general, initiation involves the attack of a ROS and removing a hydrogen atom from the lipid, thus creating a fatty acid radical [87]. When oxygen is present in the surroundings, the fatty acid radical will react with it creating a peroxy fatty acid radical, leading to the
production of other fatty acid radicals undergoing the same reaction. This initiates the propagation step and allows the process to continue [87, 95]. Finally, termination follows when two radicals interact or when a radical interacts with an antioxidant, producing a non-radical. This can occur when there is ample concentration of the radical species [87, 95]. The lipid peroxidation marker, malondialdehyde (MDA) has been used as a measure of osteoclast function [96] because osteoclasts generate $O_2^-$, which can result in the oxidation of lipids. This can be manifested as increased serum concentrations of MDA (or other lipid peroxidation markers) [1].

**Oxidative Damage to Protein**

Proteins can undergo oxidation by interacting with ROS; involving peroxidation, damage to specific side-chains, change in their tertiary structure and degradation [87, 97-100]. Several lines of research have indicated that OH’ and RO’ and nitrogen-reactive radicals are the main ROS that cause protein damage [97]. Oxidizing proteins can lead to the loss of enzymatic and structural activity, alteration in cellular functions (energy production), improper modulation of cell signaling, and induce apoptosis [97, 98, 100, 101]. Other common protein oxidation products are aldehydes, keto compounds and carbonyls [102].

**Oxidative Damage to DNA**

ROS can interact with DNA and cause alterations by modifying DNA bases, increasing the susceptibility to lose purines, and damaging the deoxyribose sugar as well as the DNA repair system. Notably, not all ROS can damage DNA. For instance, $O_2^-$ and $H_2O_2$ have been shown not to react with DNA [87]. In contrast, OH’ can react and affect all DNA bases, whereas $^1O_2$ selectively attacks only guanine [87, 103]. Damage to DNA can cause mutations and lead to various chronic diseases including osteoporosis [104-106].

**IIC. Defense mechanisms**

There are three main lines of defense against the development of ROS and their actions (i.e. oxidative stress). These include mechanisms of prevention, repair and defense against antioxidant effects. The preventative mechanism involves the use of metal chelating proteins
and the endogenous antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). Repair mechanisms include DNA repair enzymes, lipase, protease and transferase. These are responsible for repairing damage and reconstituting tissues [107]. The antioxidant defenses include the dietary antioxidants. These can function as singlet oxygen quenchers, radical scavengers, reducing agents, or radical chain-suppressors, breakers or terminators [107, 108]. Free radical scavengers in the human diet are derived mainly from plants and include carotenoids, flavonoids, vitamins C and E and certain plant polyphenols [109-111]. These antioxidants break the chain of oxidation during the propagation step by interacting with the free radical at the site of attack in that they become the targets for oxidation rather than important biomolecules or tissues. Therefore, these antioxidants prevent oxidation of nearby cellular components such as proteins, lipids and DNA [107].

IID. Oxidative stress and the risk for the development of osteoporosis

Oxidative stress occurs when the production of free radicals exceeds the ability of antioxidant defense system to eliminate these oxidants [104, 112]. When they are not counteracted or prevented from forming in the first place, free radicals can change the integrity of and thus, damage several biomolecules, such as DNA, proteins and lipids [104]. There is increasing evidence implying that oxidative stress is responsible, at least in part for the pathophysiological processes of the aging and may be involved in the pathogenesis of atherosclerosis, neurodegenerative diseases, cancer, and diabetes. Recently, it was proposed that ROS might be responsible for the development of osteoporosis. Several in vitro and animal studies have shown that oxidative stress diminishes bone formation by reducing the differentiation and survival of osteoblasts [104], while ROS also activate osteoclasts and thus, enhance bone resorption [104]. Clinical studies have also suggested that the involvement of ROS and/or antioxidant systems may play a role in the pathogenesis of bone loss [113-115]. Mackinnon et al. showed that supplementation with lycopene significantly reduced oxidative stress parameters and bone resorption among early postmenopausal women, suggesting that oxidative stress, induced by ROS, may be associated with the pathogenesis of bone loss [114].
Baek et al. found a significant negative association between the levels of 8-Hydroxy-2’-deoxyguanosine (8-OH-dG), an oxidative DNA adduct, and BMD at four bone sites (lumbar spine, total hip, femoral neck, and trochanter) [104]. The level of 8-OH-dG in urine, tissue, and serum is commonly used as a marker of oxidative stress in vivo. Furthermore, a positive correlation has been demonstrated between serum 8-OH-dG levels and levels of the bone resorption marker, type I collagen C-telopeptide (ICTP). This result and the aforementioned negative relationship between 8-OH-dG levels and bone mass imply that subjects with higher oxidant levels lose more bone through increased osteoclast-mediated bone resorption. In vitro studies conducted by Baek and colleagues demonstrated that ROS promoted production of RANKL and M-CSF, thus stimulating osteoclastogenesis and osteoclast activity in a primary human bone marrow cell culture. These results indicate that osteoporosis, at least in part, could be caused by oxidative damage.

Zhang et al. reported a negative correlation between BMD in the femur and the levels of the advanced oxidation protein product (AOPP) in the plasma as well as between femur BMD and plasma malondialdehyde (MDA) levels in rats [112]. It was also shown that AOPP prevents osteoblast cell differentiation. AOPP and MDA are markers of oxidative damage to proteins and lipids, respectively. Sontakke et al. showed that MDA has an effect on osteoclasts [96]. The contribution of oxidative stress in age-related bone loss was further established by the positive correlation between SOD activity and BMD in the femur [112]. In the broader sense, several studies have reported a negative correlation between lipid oxidation and BMD [1, 116]. Thus, these data suggest that the buildup of proteins and lipids that have been modified by oxidation are related to age-related bone loss, whereas the presence of antioxidant enzymes can prevent bone loss.

Experimental data have demonstrated that a rise in superoxide radical generation is related to induced osteoclast-mediated bone resorption, and vice versa [117, 118]. Garrett et al. reported that superoxide radicals are generated by osteoclasts and is associated with their ability to resorb bone, and in turn promotes the production of osteoclasts [119]. Superoxide radicals generated by osteoclasts in an extracellular compartment, even in the absence of
other enzymes can degrade bone matrix proteins [120]. In contrast, the superoxide scavenger desferal-manganese complex reportedly reduces the amount of osteoclastic superoxide radicals and decreases bone resorption in a dose-dependent manner [117].

Yang *et al.* and Dreher *et al.* provided further evidence that ROS participate in bone resorption, with direct involvement of osteoclast-generated superoxide and bone degradation[121, 122]. It has been documented that osteoblasts generate antioxidants such as GPx to defend against H$_2$O$_2$, as well as transforming growth factor-β (TGF-β), which plays a role in depressing bone resorption [122, 123]. GPx has also been reported to prevent RANκL-induced osteoclastogenesis [85]. Interestingly, recent studies suggested that ROS may be needed as a signaling intermediates for osteoclast differentiation, and that antioxidants could limit bone resorption *in vivo* [124, 125].

Attindag and others was able to determine the levels of oxidative stress found in osteoporotic patients. They reported lower levels of total antioxidative status (TAS), and higher levels of total oxidative status (TOS) as well as oxidative stress index (OSI) in osteoporotic patients as compared to healthy controls [126]. As well, there was a significant negative correlation between BMD and OSI in the lumbar and femoral neck region. Studies have also demonstrated that there is an association between oxygen-derived free radicals with osteoclastic bone resorption stimulated by parathyroid hormone (PTH), IL-1β, and TNF-α [127, 128]. It has also been demonstrated that TNF-α, directly or indirectly might activate RANκL, OPG and NFκB, leading to the up-modulation of osteoclastogenesis. Increased plasma TNF-α levels have been related to induce oxidative stress observed in postmenopausal women, which in turn, may lead to augmented bone resorption and changes cytokine production. It has been proposed that NFκB is essential for osteoclast formation as an oxidative stress-responsive transcription factor. Thus, free radicals may elevate bone resorption through activation of NFκB, which regulates the production of cytokines that induce the formation of osteoclasts [129].
Utilizing osteoclast precursors from bone marrow and osteoblast/pre-osteoclast co-culture, Chen et al. demonstrated that ethanol-induced RANκL expression by osteoblasts depended on elevated intracellular levels of superoxide anion and hydrogen peroxide through enhanced NADPH oxidase activity, implying that ROS promote osteoclast differentiation and subsequent bone resorption [130]. In addition to influencing the differentiation process, ROS may also affect the lifespan of osteoblasts. Glutaredoxin 5 (Grx5) is a highly expressed protein in bone that might be involved in the protection against ROS. Linares et al. showed that when over-expressed; Grx5 inhibits ROS generation and protects osteoblastic cells from ROS-induced apoptosis through a mechanism that relies on manganese superoxide dismutase activity (MnSOD) [131]. Altogether, these data illustrate that ROS not only directly promote RANκL-induced osteoclastogenesis, but also stimulate bone loss by promoting apoptosis and decreasing the differentiation and activities of osteoblasts. Therefore, by affecting both cell types as well as the bone cell communication, oxidative stress seems to play a significant role in bone cell function and the development of such bone disorders such as osteoporosis.

A general illustration demonstrating the role of oxidative stress and antioxidants in osteoporosis and other chronic diseases is shown in figure 2.3.

Figure 2.3: The role of oxidative stress in osteoporosis and how/where antioxidants play a role in mitigating ROS
III. Phytochemicals Antioxidants

IIIA. Overview

Although several micronutrients such as vitamins C, D, and B and calcium, magnesium, zinc, copper, manganese, and boron are known to have antioxidant properties, there is increased interest in other phytochemical antioxidants that are naturally present in plant derived foods. These phytochemical antioxidants belong to one of the two groups: 1) carotenoids, which are lipid soluble and 2) polyphenols, which are water soluble. greens+ bone builder\textsuperscript{TM}, the test material used in the thesis, is the major antioxidant phytochemicals present in this supplement.

IIIB. Structure and Food Sources

Polyphenols

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 12-16 phenolic hydroxy groups on five to seven aromatic rings per 1000 Da of relative molecular mass [132]. To date, over 8000 polyphenols have been identified [133]. Polyphenols can be divided into 2 main groups: flavonoids and non-flavonoids [134-136]. An organizational scheme of classifying polyphenols is shown in Figure 2.4.
Figure 2.4: Classification of polyphenols

**Flavonoids**
Flavonoids represent the most common group of polyphenols [137]. These consist of 2 phenyl rings connected by a chain of 3 carbon atoms forming a heterocyclic 6-membered ring with oxygen and 2 carbon atoms from an adjacent phenyl ring [138]. They can be further subclassified into anthocyanidins (e.g., cyanidin, delphinidin, malvidin), flavanols (e.g., catechin, epicatechin), flavonols (e.g., quercetin, fisetin), and flavones (e.g., luteolin), isoflavones, and anthrocyanidins. Many flavonoids are found as glycosides in nature, which makes it difficult to characterize [133]. The different flavonoids, their chemical structures and sources are shown in Table 2.2.
Table 2.2 Classification of flavonoids, general chemical structure and food sources

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Structure</th>
<th>Common flavonoid</th>
<th>Food examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanidins</td>
<td></td>
<td><img src="image1" alt="Structure" /></td>
<td>Cyanidin</td>
<td>berries, purple cabbage, beets, grape seed extract, red wine</td>
</tr>
<tr>
<td></td>
<td>Anthoxantins</td>
<td><img src="image2" alt="Structure" /></td>
<td>Catechins</td>
<td>white, green and black teas</td>
</tr>
<tr>
<td></td>
<td>Flavanols</td>
<td><img src="image3" alt="Structure" /></td>
<td>Proanthocyanidins</td>
<td>chocolate, fruits and vegetables, red wine, onion, apple skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Theaflavins</td>
<td>black teas</td>
</tr>
<tr>
<td></td>
<td>Flavonols</td>
<td><img src="image4" alt="Structure" /></td>
<td>Quercetin</td>
<td>red and yellow onions, tea, wine, apples, cranberries, buckwheat, beans</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Narigenin</td>
<td>citrus fruits</td>
</tr>
<tr>
<td></td>
<td>Flavanones</td>
<td><img src="image5" alt="Structure" /></td>
<td>Hesperidin</td>
<td>citrus fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Silybin</td>
<td>blessed milk thistle</td>
</tr>
<tr>
<td></td>
<td>Isoflavones</td>
<td><img src="image6" alt="Structure" /></td>
<td>Genistein</td>
<td>soy, alfalfa sprouts, red clover, chickpeas, peanuts, other legumes.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diadzein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavones</td>
<td><img src="image7" alt="Structure" /></td>
<td>Tangeritin</td>
<td>tangerine and other citrus peels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Luteolin</td>
<td>celery, thyme, green peppers,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Apigenin</td>
<td>chamomile, celery, parsley</td>
</tr>
</tbody>
</table>
Non-Flavonoids
The non-flavonoid group account for approximately one-third of the polyphenols and consists of phenolic acids, stilbenes (e.g. resveratrol), coumarin, and lignans [132, 139]. Phenolic acids can be subclassified into 2 groups: hydroxybenzoic acids (e.g. gallic, ellagic, salicylic and vanillic acid) and hydroxycinnamic acids (e.g. caffeic and ferulic acid) [140, 141]. The different non-flavonoids, their chemical structures and sources are shown in Table 2.3.

Table 2.3 Classification of non-flavonoids, general chemical structure and food sources

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Structure</th>
<th>Common flavonoid</th>
<th>Food examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic Acids</td>
<td>Hydroxycinnamic acids</td>
<td><img src="image" alt="Caffeic acid" /></td>
<td>Caffeic acid</td>
<td>coffee beans</td>
</tr>
<tr>
<td></td>
<td>Hydroxybenzoic acids</td>
<td><img src="image" alt="Gallic acid" /></td>
<td>Gallic acid</td>
<td>gallnuts, sumac, witch hazel, tea leaves, oak bark,</td>
</tr>
<tr>
<td>Stilbenes</td>
<td></td>
<td><img src="image" alt="Resveratrol" /></td>
<td>Resveratrol</td>
<td>gapes skins, red wine</td>
</tr>
<tr>
<td>Courmarin</td>
<td></td>
<td><img src="image" alt="Aesculetin and scopoletin" /></td>
<td>aesculetin and scopoletin</td>
<td>citrus fruits, strawberries, apricots, cherries and cinnamon</td>
</tr>
<tr>
<td>Lignans</td>
<td></td>
<td><img src="image" alt=" Secoisolaiciresinol" /></td>
<td>Secoisolaiciresinol</td>
<td>flaxseeds</td>
</tr>
</tbody>
</table>
The polyphenol content in plant foods and beverages has differed among many scientific publications. There are many factors that influence the value of the polyphenol content and profiles in plants, including: plant variety or cultivar, growth conditions (climate and soil), crop management (irrigation, fertilization, pest management), state of maturity at harvest, postharvest handling, storage, and processing [138, 142, 143]. With this in mind, it is understandable as to why it has been difficult to determine the exact value of the content of a given phenolic compound in a given food. Furthermore, specific polyphenols may only be present in some varieties and absent in others. For instance, blue potatoes or black rice contain anthocyanins, but are not present in their non-colored varieties (white potatoes or white rice).

Recently, the United States Department of Agriculture (USDA) created a detailed food database based on a compilation of literature sources [144]. This table contains values for individual flavonoid compounds for 500 foods. In addition to the USDA database, Institut National de la Recherche Agronomique in France (INRA) has developed a new Web-based database called Phenol-Explorer, a compilation of the scientific literature of 502 polyphenols found in foods [145]. This seems to be a more user-friendly database as it categorizes into phenolic acids, lignans and stilbenes in addition to flavonoids.

Extracted from the either database, there are foods that contain very high amounts of polyphenols [145, 146]. However, since certain foods and beverages that are rich in polyphenols such as sage (flavones), capers (flavanols), oregano (hydroxycinnamic acids), and wine (resveratrol) are consumed in very low amounts; they contribute very little to the total intake [138]. On the contrary, some foods and beverages that contain small amounts of polyphenols such as potatoes (chlorogenic acid) or tea (quercetin) are consumed in large amounts, which can contribute significantly to polyphenol intake. Other polyphenols such as stilbenes do not appear in the database because there are no known food sources that have 20 mg/100 g or greater [138].
IIIC. Dietary Intake

The daily intake of polyphenols has been estimated to be approximately 1g/day [147]. This is more than that of all other known dietary antioxidants, which is 10 times greater than that of vitamin C and 100 times higher than those of vitamin E and carotenoids [148]. However, several factors have been addressed that explains the challenge in estimating polyphenol intake: [138] 1) Polyphenols have a vast amount of chemical structures. 2) Polyphenols are present in a large variety of foods. For instance, quercetin can be present in several foods, whereas flavanones are predominately present only in citrus foods. 3) Their content in a given food can vary to a wide extent, as mentioned earlier, and 4) There have been no standardized methods to estimate the amount of polyphenols in foods and methods of analysis can vary between publications.

To date, there are no detailed intake values for all polyphenols. Having said that, catechins and proanthocyanidins make up for approximately 75% of the total flavonoids, the largest group of polyphenols, ingested. However, phenolic acids represent a majority of the polyphenols ingested among coffee consumers. There are numerous factors that may influence the polyphenol intake in a population. The major factors that influence the type and quantity of polyphenols consumed include cultural habits (soy consumption in Asian countries) and food preferences (wine, berries). Furthermore, polyphenol intake has been shown to vary with age, sex and ethnicity, all which have been known to affect food choices [149].

In addition to the dietary sources of polyphenols, nutritional supplements also contribute towards their intake. Several nutritional supplements including greens+ bone builder™ are now being marketed as sources of polyphenols.
CHAPTER 3
RATIONALE, HYPOTHESIS AND OBJECTIVES
I. Rationale

Evidence suggests that in postmenopausal women, the delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts is tipped towards increased resorption [150], thus leading to net loss of bone over time. Although the mechanisms underlying osteoporosis are not completely known, there is evidence suggesting that oxidative stress caused by reactive oxygen species (ROS) is associated with its pathogenesis [113, 116, 124, 151]. As other mechanisms exist in the development of osteoporosis, ROS has shown to mediate several signalling pathways affecting bone remodelling. There is renewed interest in the use of natural antioxidant components of foods and nutritional supplements as complimentary strategies due to the adverse effects of pharmaceutical drugs for the prevention and treatment of osteoporosis.

Nutritional supplements such as greens+™ (Genuine Health Inc., Toronto), a blend of several botanical products, have been shown recently to contain a combination of water-soluble antioxidant polyphenols naturally present in food. Previous research in our laboratory has shown that polyphenols from greens+™ stimulated the formation of mineralized bone nodules in human osteoblasts, raising the possibility that it may be effective in the management of osteoporosis [152]. Another nutritional supplement, the bone builder™ (Genuine Health Inc., Toronto), containing antioxidants, vitamins, minerals, trace minerals, and amino acids, all of which have been shown individually to be beneficial to bone health have also been shown in our laboratory to stimulate bone formation [153]. The third nutritional supplement, greens+ bone builder™ (Genuine Health Inc., Toronto) has been shown to be more effective in stimulating bone formation than either greens+™ or bone builder™ alone [154]. As greens+ bone builder™ has been shown to be the more effective stimulator of bone formation in vitro [154], we rationalized that this supplement will reduce the risk for development of osteoporosis in postmenopausal women.
II. Hypothesis

The overall hypothesis is that intervention with greens+ bone builder™, a good source of polyphenols and micronutrients will increase the serum antioxidant capacity and urinary total polyphenol content and decrease serum markers for oxidative stress in postmenopausal females. Supplementation with greens+ bone builder™ will also decrease the bone resorption marker, CTX and increase bone formation marker, PINP, thus reducing the risk of osteoporosis (fig. 3.1).

Figure 3.1: Illustration of hypothesis

III. Objectives

The objectives are to investigate whether greens+ bone builder™ is effective in decreasing the risk of osteoporosis in postmenopausal women (50-60 years of age) by:

1. Increasing serum antioxidant capacity and decreasing lipid peroxidation and protein oxidation, and
2. Increasing urinary polyphenol content and if there is a negative correlation with serum bone turnover markers, CTX and PINP.
IV. Subjects and Methods

IVA. Participant Recruitment and Sample Collection

Participant recruitment was conducted from the years 2008-2011. Female participants between the ages of 50-60 years, who were at least one year postmenopausal, were recruited by telephone and advertisements and were asked to sign an informed consent in conformation with the guidelines of the St Michael’s Hospital Research Ethics Board (REB) (Appendix II). Exclusion criteria included participants who smoked cigarettes or were on medications affecting bone metabolism, including those for heart disease, high blood pressure, diabetes and/or osteoporosis. Based on these criteria, a total of 48 subjects were selected to participate in the study. All participants were required to submit 7-day dietary records (Appendix III) outlining foods, beverages and nutritional supplements they consumed as well as baseline 12-hour fasting blood and 2nd void urine samples at the first visit. Another set of dietary records as well as fasting blood and urine samples were collected following a one-week washout period during which patients were asked to refrain from drinking or eating foods rich in polyphenols and herbal/vitamin-supplements. The participants were then assigned randomly to two groups: 1) greens+ bone builder™ (N=24), or 2) the placebo diet (rice flour), (N=24), for a period of 8 weeks. Fasting blood and urine samples were collected after 4 and 8 weeks following their respective treatment periods (Fig. 3.2). Blood samples were processed to obtain serum. Both the serum and urine samples were labeled, kept frozen and stored at -80°C until the time of analysis.
Figure 3.2: Study design

IVB. Serum Analyses

Processing of Blood Samples
Unless otherwise specified, all materials were obtained from Sigma Aldrich Canada, Oakville, ON, Canada. Fasting blood samples collected from participants were centrifuged at 2,500 RPM within 1 hour of collection. The serum was then collected, aliquoted equally into 4 eppendorf tubes (minimum of 250 µl each) and keep frozen at -80°C. The frozen samples were thawed for analysis of total antioxidant capacity (TAC) [155], protein oxidation (thiols) [156] and lipid peroxidation (TBARS) [157].

Total Antioxidant Capacity
Total antioxidant capacity (TAC) was measured, using the Trolox-equivalent antioxidant capacity assay (TEAC) [155], to determine the overall capacity of antioxidants in the serum using 2,2, azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), a blue-green chromophore. The absorbance was read at 734 nm to determine decolourization (Milton Roy Spectronic 1001 Plus, PA, USA), and compared to the decolourization produced by Trolox, a vitamin E analogue as a standard.

Oxidative Stress Parameters
Protein oxidation was determined by estimating the content of protein-sulphhydryl groups (thiols) in serum [156]. The optical density was read at 412 nm (Milton Roy Spectronic 1001 Plus, PA, USA) and protein thiol concentrations were calculated using an optical dentistry reading at a wavelength of 13600 cm⁻¹ M⁻¹. A high concentration of protein thiols corresponds to reduced levels of protein oxidation.

Lipid peroxidation in the serum was measured using the thiobarbituric acid-malondialdehyde (TBA-MDA) assay and was reported as thiobarbituric acid reactive substances (TBARS). The optical density was read at 535 nm (Milton Roy Spectronic 1001 Plus, PA, USA) and the concentration of TBARS was calculated using an absorptivity of 156 mM⁻¹cm⁻¹.

**Bone Turnover Markers**

Both serum crosslinks (CTX), a marker of bone resorption, and procollagen type I N-terminal propeptide (PINP), a marker of bone formation, were measured using an electrochemiluminescent immunoassay (ECLIA) technique, which were performed automatically by the Cobas® e601 (Roche Diagnostics, Germany).

**IVC. Urine Analysis**

**Total Polyphenol Content**

Total urinary phenolic compounds were measured in 2nd void urine samples by using the methodology described by Medina-Remón *et al.*[158]. This method was improved from the Roura *et al.* protocol to measure a broader spectrum of phenolic compounds contained in different foods [159]. Oasis® MAX96-well plate SPE cartridges were selected because they provided the best recovery. Absorbance was measured at 765 nm in the SpectraMax M5e Microplate Reader (Molecular Devices, LLC., US). This spectrophotometer allowed the absorbance in solutions contained in all 96 wells of this type of plate in to be determined in approximately in 10 s. To measure the creatinine levels in the urine samples, the modified Jaffé alkaline picrate method was used in, also with microtiter 96-well plates. Total polyphenols were expressed as mg gallic acid equivalent (GAE) per g creatinine.
Ethics

This protocol was approved by the Research Ethics Board at St. Michael's Hospital, Toronto, Ontario, Canada (approved June 11, 2008) (Appendix II).
CHAPTER 4

Antioxidant effects of a nutritional supplement containing polyphenols in postmenopausal women: a randomized controlled study

This chapter has been accepted for publication to the Journal of Aging: Research and Clinical Practice as a research paper entitled: “Antioxidant effects of a nutritional supplement containing polyphenols in postmenopausal women: a randomized controlled study” by N.N Kang, A.V. Rao, K. De Asis, L.A. Chan, L.G. Rao
I. Abstract

**Background:** Oxidative stress is an important factor in the development of osteoporosis. Antioxidants counteract the damaging effect of oxidative stress, which may reduce the risk of osteoporosis. Nutritional supplements, such as greens+™ and greens+ bone builder™ that contain water-soluble polyphenols and other micronutrients beneficial for bone health, are of recent interest as complementary strategies in the management of osteoporosis.

**Objective:** Clinically evaluate the antioxidant properties of greens+ bone builder™ in postmenopausal women.

**Design:** Forty-seven postmenopausal women, 50-60 years old were recruited for a ten-week clinical study. During week 1, participants recorded their baseline food intake. During week 2, the participants refrained from consuming polyphenol-rich foods, beverages and supplements. The participants were then 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:** Statistical analysis showed that after 4 and 8 weeks, the Treatment group significantly increased their serum total antioxidant capacity and decreased in lipid peroxidation and protein oxidation while the Placebo group showed no significant changes. These were also significantly different from those of the Placebo group.

**Conclusions:** Results suggest that a daily supplementation with greens+ bone builder™ may be important in reducing oxidative damage, thus reducing the risk of osteoporosis in postmenopausal women.
II. Introduction

Osteoporosis is a disease characterized by low bone mass and deterioration of the microarchitecture of bone tissue, leading to fragility fractures primarily in the hip, spine and wrist [160]. Approximately one in three women and up to one in five men over the age of 50 will develop osteoporosis world-wide [3]. Although several factors have now been identified as increasing the risk of osteoporosis, oxidative stress has now emerged as one of the most important life style risk factor associated with loss of bone mass [161-163].

Antioxidants capable of counteracting this effect have demonstrated to be important in decreasing the risk of osteoporosis [126, 164, 165]. There has been an increased interest in the water-soluble antioxidant polyphenols as a result of in vitro and in vivo evidence demonstrating that they may protect against oxidative damage, mainly due to their antioxidative and free radical quenching properties, thus limiting the risk of various degenerative diseases associated with oxidative stress, including osteoporosis [166-170].

Nutritional supplements such as greens+ bone builder™ contain not only polyphenol rich plant constituents, but also other nutrients that were shown to be individually good for bone health. The composition of bone builder™ includes the following: vitamins B6, B12, C, D3, and folic acid; minerals such as calcium, magnesium, zinc, chromium, selenium, manganese, boron, copper; and antioxidant lycopene. Previous in vitro results from our laboratory have shown that greens+™, a nutritional supplement rich in a variety of polyphenols is effective in stimulating osteoblasts to form bone nodules in a dose-dependent manner [171]. Another nutritional supplement, bone builder™, which is rich in minerals, vitamins and nutrients, also had a significant dose-dependent stimulatory effect on bone nodule formation [154]. When greens+™ and bone builder™ were tested as combination, the effects were six times more effective than either one alone [154].

This led us to believe that additive effects of greens+™ and bone builder™ may have a beneficial effect in reducing oxidative stress and reducing the risk of osteoporosis. The aim
of this study, therefore, was to investigate the effects of the nutritional supplement commercially known as greens+ bone builder™, which combines the total polyphenols in greens+™ with various vitamins, minerals and antioxidants present in bone builder™, on biomarkers of oxidative stress in postmenopausal women who are at risk for osteoporosis.
III. Methods

Statistics
All statistical analyses were performed using the latest version of SAS system (version 9.2; SAS Institute, Cary, NC, USA). Summary statistics of participant demographics such as age and BMI were generated and presented as means ± standard errors of the mean (SEM). Repeated-measures one-way analysis of variance (ANOVA), with Tukey’s multiple comparison test was used to test for significant differences in oxidative stress parameters and antioxidant status from the start of the treatment period to 4 and 8 weeks on the treatment. In cases where data were not normally distributed, the Friedman test with Dunn’s multiple comparison test was substituted. Percent change in oxidative stress parameters and antioxidant status were calculated using an unpaired t-test, or the Mann-Whitney test for data that were not normally distributed to analyze for significant differences between the Placebo and Treatment groups. Significance was considered p<0.05.
IV. Results

A total of 47 postmenopausal women, 24 in the Placebo group and 23 in the Treatment group (one participant withdrew due to personal reasons), completed the study. Table 4.1 describes the participant baseline characteristics. There were no significant differences among groups with respect to age, BMI, years since menopause, or blood pressure (Table 4.1). Similarly, there were no significant differences at baseline for average serum TAC (Treatment group, 1.45 ± 0.05 mM and Placebo group, 1.50 ± 0.05 mM) and protein oxidation (Treatment group, 469.7 ± 18.1 µM and Placebo group, 490.1 ± 12.5 µM). However, there was a significant difference between the Treatment group and Placebo group for TBARS at baseline with the Treatment group showing higher levels of TBARS at 7.1 ± 0.3 nmol/mL and 6.4 ± 0.2 nmol/mL, respectively (Table 4.1).

An interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) was detected (p<0.05) for TAC such that TAC was greater in the Treatment group than in the Placebo group (Figure 4.1a) after treatment. Significant increases of 3.8 ± 1.4% and 7.1 ± 1.9% from baseline to 4 and 8 weeks among this group was also significantly different from the Placebo group (p<0.01, p<0.0001, Figure 4.1b).

There was an overall interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) (p<0.0001) on lipid peroxidation, such that consuming the supplement rich in polyphenols and other micronutrients was lower than those who consumed the placebo (Figure 4.2a) after treatment. The Treatment group also resulted in a decrease of 6.6 ± 1.0% after 4 weeks, and to 10.0 ± 1.3% after 8 weeks in lipid peroxidation, which was significantly opposite to the 2.6 ± 1.7% and 4.3 ± 2.9% increase at 4 and 8 weeks of treatment seen among the Placebo group, respectively (p<0.0001, Figure 4.2b).

An interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) was detected (p<0.05) on protein thiols, such that the Treatment group was higher in protein thiols, indicating a decrease in protein oxidation, compared to the Placebo group.
(Figure 4.3a) after treatment. There was a 3.2 ± 1.1% and 5.3 ± 1.1% increase in protein thiols from baseline to 4 weeks, and to 8 weeks among the Treatment group, which was significantly different from the decrease in protein thiols of 3.5 ± 2.9% after 4 weeks and by 2.5 ± 1.8% after 8 weeks seen within the Placebo group (p<0.05, p<0.001, Figure 4.3b).
Table 4.1: Participant characteristics and baseline values for oxidative stress parameters and antioxidant capacity for each group.

<table>
<thead>
<tr>
<th>Parameters Measured</th>
<th>Placebo</th>
<th>greens+ bone builder&lt;sup&gt;TM&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>55.5 ± 0.3</td>
<td>56.2 ± 0.6</td>
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<tr>
<td>Weight (lbs)</td>
<td>144.4 ± 5.1</td>
<td>143.7 ± 5.4</td>
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<tr>
<td>Height (inches)</td>
<td>63.7 ± 0.5</td>
<td>64.6 ± 0.6</td>
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<tr>
<td>Pulse Rate (beats/min)</td>
<td>66.8 ± 1.8</td>
<td>65.9 ± 1.8</td>
</tr>
<tr>
<td>Blood Pressure - systolic (mmHg)</td>
<td>115.6 ± 3.2</td>
<td>118.9 ± 3.2</td>
</tr>
<tr>
<td>Blood Pressure - diastolic (mmHg)</td>
<td>75.1 ± 2.0</td>
<td>72.5 ± 2.3</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>25.0 ± 1.0</td>
<td>23.8 ± 0.8</td>
</tr>
<tr>
<td>Years since Menopause</td>
<td>5.7 ± 0.6</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Total Antioxidant Capacity (TEAC)(mM)</td>
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<td>1.45 ± 0.05</td>
</tr>
<tr>
<td>TBARS (nmol/mL)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.4 ± 0.2</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Protein Thiols (µM)</td>
<td>490.1 ± 12.5</td>
<td>469.7 ± 18.1</td>
</tr>
</tbody>
</table>

<sup>1</sup> Placebo values had significantly lower TBARS than the supplement group (unpaired t-test, p<0.05)
**Figure 4.1:** a) Total antioxidant capacity (TAC) in the serum over 8 weeks, as determined using the TEAC assay. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using a repeated-measures ANOVA with Tukey's Multiple Comparison test (p<0.05) b) The change in TAC was measured relative to baseline concentration and expressed as mean % change ± SEM for each supplement group. Significant differences between supplement groups at each time point were compared using an unpaired t-test (*p<0.01, **p<0.0001)
Figure 4.2: a) Concentration of lipid peroxidation in the serum over 8 weeks, as determined by TBARS. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using repeated-measures ANOVA with Tukey's Multiple Comparison test (p<0.0001). b) Change in lipid peroxidation was measured relative to baseline concentration and expressed as mean % change ± SEM for each supplement group. Significant differences between supplement groups at each time point were compared using an unpaired t-test (*p<0.0001)
Figure 4.3: a) Concentration of protein oxidation in the serum over 8 weeks as determined by thiols. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using the Friedman’s test with Dunn’s Multiple Comparison test (p<0.01). b) Change in protein oxidation was measured relative to baseline concentration and expressed as mean % change ± SEM for each supplement group. Significant differences between supplement groups at each time point were compared using an unpaired t-test (*p<0.05, **p<0.001)
V. Discussion

Previous studies showing the antioxidant properties of polyphenols were based mainly on *in vitro* and animal studies. Our laboratory is the first to evaluate the efficacy of the nutritional supplement containing polyphenols and other micronutrients administered to postmenopausal women who are at risk for osteoporosis. The Treatment group showed increased TAC after 8 weeks of treatment. Furthermore, there was a significant change at 4 and 8 weeks between the Placebo and Treatment group. The TEAC method has been validated to measure the antioxidant properties of both the lipid- and the water-soluble antioxidants *in vivo* \[172\]. This assay is used primarily for its ability to assess many endogenous antioxidants in addition to those found in the supplement and its capability to quench reactive oxygen species (ROS).

Although the polyphenols in greens+\(^{TM}\) may have been the principle component of the nutritional supplement contributing to the observed antioxidant effect, the combined effect with other micronutrients and antioxidants in the bone builder\(^{TM}\) may also have contributed to this effect \[173, 174\]. Similar results obtained in our *in vitro* study showed that greens+ bone builder\(^{TM}\) to be more effective than greens+\(^{TM}\) or bone builder\(^{TM}\) alone in stimulating bone nodule formation. Our study was not designed to study the interactive mechanisms between the various components of greens+\(^{TM}\) and bone builder\(^{TM}\). It is therefore, not possible to associate the antioxidant affects observed in the present study to any one component of green+ bone builder\(^{TM}\).

In addition to measuring TAC, other more sensitive biomarkers such as lipid peroxidation and protein oxidation to measure the effectiveness of the nutritional supplement in reducing oxidative stress. Greens+ bone builder\(^{TM}\) significantly reduced oxidative stress compared to placebo groups after both 4 and 8 weeks. Previous *in vitro*, *in vivo* and clinical studies \[166-168, 170, 171\] have also shown similar results where there was a decrease in oxidative stress parameters after the consumption of polyphenols. In a recent study, the effectiveness of lycopene, a fat-soluble antioxidant, on oxidative stress parameters in humans was investigated. The results showed decrease in oxidized proteins and lipid peroxidation at 4
weeks of treatment [113]. This is similar to our finding where there was a significant decrease in both protein oxidation and lipid peroxidation within the treatment group when compared to placebo at 4 and 8 weeks of treatment.

A variety of dietary antioxidants have been shown to be capable of scavenging ROS directly. Previous studies demonstrated that a diet high intake of foods rich in polyphenols and other micronutrients, increasing the antioxidant capacity, has been linked to lowered risks of many chronic diseases involving oxidative stress [175-177]. As well, an in-depth review suggesting that the combination effect of carotenoids found in foods and supplements and may have a greater reduction in the risk of chronic diseases such as osteoporosis when compared to a single nutrient [178].

Since polyphenols are quickly metabolism in the human body, its concentration found in blood is low (<1 µmol/L) [179-182]. This is such a low concentration to show any significant and direct antioxidant activities, thus some researchers believe that it is unlikely that polyphenols act as antioxidants in vivo [183, 184]. Thus, attention should be brought to the additive effect of micronutrients and polyphenols. It has also been noted that polyphenols may function as a co-antioxidant, and are involved in the regeneration of essential vitamins [184].

The relationship between the antioxidant property of the nutritional supplement and its effect on bone health was recently reported [171]. They demonstrated that supplementation with greens+TM containing polyphenols such as quercetin, apigenin, kaempferol and luteolin was shown to increase the proliferation of human osteoblast-like cells at early time points of addition as well as to stimulate bone nodule formation in a dose- and time-dependent manner [171]. These observations suggest a positive effect of ingesting polyphenol-rich foods and supplements in reducing oxidative stress, stimulating the activity of osteoblast cells, which may reduce the risk of osteoporosis.

In conclusion, our study has shown that daily supplementation of a combination of polyphenols and other trace nutrients and minerals in the form of the nutritional supplement,
greens+ bone builder\textsuperscript{TM}, can significantly increase the antioxidant capacity and decrease the extent of oxidative damage in postmenopausal women at risk of osteoporosis. The beneficial effects can be due to the additive effect of the polyphenols with other vitamins and minerals found within the supplement. This observation along with previously observed bone-protective effects of the individual polyphenols, nutrients and trace minerals, has generated increased interest into further exploring them as a nutritional supplement that is beneficial to bone health. Although there is evidence that implicate oxidative stress is an important mediator of bone loss aiding in the development of osteoporosis, our laboratory is the first to investigate and show the combined effect of a variety of polyphenols alongside other micronutrients in healthy postmenopausal women, suggesting that this may be a good alternative for the prevention or treatment of osteoporosis.
CHAPTER 5

Dietary polyphenols and nutritional supplements significantly decreased oxidative stress parameters and the bone resorption marker collagen type 1 cross-linked C-telopeptide in postmenopausal women

This chapter has been submitted for publication to Osteoporosis International as a research paper entitled: “Dietary polyphenols and nutritional supplements significantly decreased oxidative stress parameters and the bone resorption marker collagen type 1 cross-linked C-telopeptide in postmenopausal women” by N.N. Kang, A.V. Rao, R. Josse, H. Vandenberghe, K. De Asis, L.A. Chan, L.G. Rao. It has been reformatted for this thesis.
I. Abstract

Purpose: Oxidative stress caused by reactive oxygen species has been associated with the development of osteoporosis, which can be ameliorated via dietary antioxidants. Polyphenols are water-soluble phytochemical antioxidants known to decrease the risk of many age-related chronic diseases. However, the role of a variety of polyphenols in combination with micronutrients in osteoporosis has not been studied.

Methods: Forty-seven postmenopausal women, 50-60 years old were recruited for a ten-week clinical study. Week 1, the participants consumed a regular diet. Week 2, they refrained from consuming polyphenol-rich foods, beverages and supplements. They were then randomized to either Supplement group (N=23) or Placebo group (N=24) for 8 weeks. Fasting blood and urine samples were collected and measured at baseline and 8 weeks for serum total antioxidant capacity (TAC); the oxidative stress parameters, lipid peroxidation and protein oxidation, and the bone turnover markers, C-terminal telopeptide of type I collagen (CTX) and procollagen type I N-terminal propeptide (PINP). Urine samples were measured for total polyphenol content.

Results: Statistical analysis showed that at 8 weeks, the serum total antioxidant capacity and total urinary polyphenol content was increased while lipid peroxidation, protein oxidation and CTX were decreased significantly in the Supplement group, none of which was seen in the Placebo group. In fact, all measured parameters were altered in the Supplement group as compared to Placebo.
Conclusions: Results suggest that daily supplementation with polyphenols and micronutrients may be important in reducing oxidative damage by reducing bone resorption, thus the risk for development of osteoporosis in postmenopausal women.
II. Introduction

Osteoporosis is a disease characterized by low bone mass and deterioration of the microarchitecture of bone tissue and predisposition to fractures [160]. Postmenopausal women, in particular, experience an accelerated phase of bone turnover and loss of bone mass at the perimenopausal within the first few years after menopause, which can result in increased fragility of bone, potentially leading to bone fractures [185, 186]. Nutritional recommendations of a daily dietary intake of 1200 mg of calcium and 800-2000 IU of vitamin D have been suggested to promote bone health and perhaps help to mitigate the rapid bone loss at menopause [187]. Today, a vast amount of literature is focused on other important micronutrients and their effects on bone metabolism [188-191]. However, further assessments of other nutritional supplements, which may influence bone turnover, are necessary to assess their benefit for the prevention of osteoporosis.

Although several factors are known to increase the risk of osteoporosis, oxidative stress has been recognized as one of the most important risk factors associated with the loss of bone mass, due to increased bone resorption [162, 163, 192]. Antioxidants have been shown to counteract this increased bone resorption [126, 164, 165]. Specifically, there has been increased interest in the water-soluble antioxidant polyphenols as a result of evidence developed in studies done in vitro and in vivo, which shows that they may protect against oxidative damage. This is due mainly to their antioxidative properties which quench free radical formation. Polyphenols, therefore, have been suggested as agents to reduce the risk of various diseases associated with oxidative stress, including osteoporosis [166-168, 193, 194].
Nutritional supplements such as greens+ bone builder\textsuperscript{TM} contain not only polyphenol-rich plant constituents, but also other nutrients that are considered beneficial for bone health. Previous \textit{in vitro} results from our laboratory have shown that greens+\textsuperscript{TM}, a nutritional supplement rich in a variety of polyphenols, is effective in stimulating osteoblasts to form bone nodules in a dose-dependent manner \textit{in vitro} [171]. Another nutritional supplement, bone builder\textsuperscript{TM}, which is rich in minerals, vitamins and other nutrients, also had a significant dose-dependent stimulatory effect on bone nodule formation (Snyder \textit{et al.}, 2010). When greens+\textsuperscript{TM} and bone builder\textsuperscript{TM} were tested as a combination, the effects were six times more effective than either one alone [154]. This led us to believe that the combination effects of polyphenols with minerals, vitamins and other nutrients may be beneficial in reducing oxidative stress and may potentially decrease the risk of osteoporosis. The aim of this study, therefore, was to investigate the effects of the nutritional supplement, which combines the variety of polyphenols with various vitamins, minerals and antioxidants on biomarkers of oxidative stress and bone turnover markers in postmenopausal women who are at risk for osteoporosis.
III. Methods

Statistics
All statistical analyses were performed using GraphPad PRISM 5.00 for Windows (GraphPad Software, California). Summary statistics of participant demographics such as age and BMI were generated and presented as means ± standard errors of the mean (SEM). A paired t-test was used to determine the overall effect of the supplement or placebo at 8 weeks of treatment on urinary polyphenol content, biomarkers of bone turnover, parameters of oxidative stress parameters, and antioxidant status. Percent change on the mentioned clinical end-point markers and parameters were in the Supplement group was calculated and compared to the change in the Placebo group using an unpaired t-test. Significance was set at p<0.05.
IV. Results

A total of 47 postmenopausal women, 24 in the Placebo group and 23 in the Supplement group (one participant withdrew for personal reasons), completed the study. Baseline characteristics of the participants are described in Table 5.1. There were no significant differences among groups with respect to age, BMI, years since menopause, or blood pressure (Table 5.1). Similarly, there were no significant differences at baseline for average serum TAC, total urinary polyphenol content, protein oxidation, and bone turnover markers. However, there was a significant difference between the Supplement group and Placebo group for TBARS at baseline with the Supplement group showing higher levels of TBARS than the Placebo group at 7.1 ± 0.3 µM and 6.4 ± 0.2 µM, respectively (Table 5.1).

The Supplemented group had significantly decreased CTX over time. After 8 weeks of supplementation, CTX decreased from the baseline concentration of 521.4 ± 51.7 µg/L to 462.1 ± 47.5 µg/L (p<0.05, Fig. 5.1a) or 11.6 ± 3.8% (Fig. 5.1b). There was no significant change in participants consuming placebo from baseline (452.7 ± 42.9 µg/L) or 3.6 ± 4.4% (Fig. 5.1a) to after the eight week supplement period (456.1 ± 41.5 µg/L) (Fig. 5.1b). There were no changes in serum concentrations of PINP, a marker of bone formation, in either group (Fig 5.1c,d).

The TPC significantly increased after 8 weeks of treatment in the Supplement group (p<0.05, Fig. 5.2a), but was not shown in the Placebo group. The TPC measurements increased up to
almost 56% in the Supplement group, but only up to about 15% in the Placebo group (p<0.05, Fig. 5.2b)

A significant increase in the TAC from the baseline concentration of 1.45 ± 0.05 mM to a concentration of 1.54 ± 0.04 mM at 8 weeks (p<0.05, Fig. 5.3a) was observed for the Supplement group. This increased approximately 7% after 8 weeks in the Supplement group, which was significantly different from the Placebo group (p<0.0001, Fig. 5.3b). There were no changes in TAC in the Placebo group (Fig. 5.3a).

Consuming the supplement significantly decreased lipid peroxidation from the baseline concentration of 7.1 ± 0.3 µM to a concentration of 6.4 ± 0.2 µM at 8 weeks, respectively (p<0.0001, Fig. 5.4a). This is equivalent to a decrease of approximately 10% after 8 weeks, which was significantly opposite to the increase of approximately 4% seen in the Placebo group (p<0.0001, Fig. 5.4b).

The Supplement group showed a significant increase in protein thiols, indicating a decrease in protein oxidation, from the baseline concentration of 469.7 ± 18.1 µM to 495.0 ± 19.2 µM after 8 weeks of treatment (p<0.0001, Fig. 5.5a). Protein thiols increased roughly 5% from baseline among the Supplement group, which was significantly different from the decrease of roughly 3% in the Placebo group (p<0.001, Fig. 5.5b).
Table 5.1 Participant characteristics and baseline values for oxidative stress parameters and antioxidant capacity for each group

<table>
<thead>
<tr>
<th>Parameters Measured</th>
<th>Summary statistics (mean ± SEM)</th>
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<td></td>
<td>Greens+ bone builder(\textsuperscript{TM}) (N=23)</td>
</tr>
<tr>
<td>Age (yrs)</td>
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<td>Pulse rate (beats/min)</td>
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<td>Blood pressure-systolic (mmHg)</td>
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<td>Years since menopause</td>
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<tr>
<td>Total urinary polyphenol content</td>
<td>39.0 ± 4.3</td>
</tr>
<tr>
<td>Total antioxidant capacity (TAC)(mM)</td>
<td>1.45 ± 0.05</td>
</tr>
<tr>
<td>Bone Turnover Markers</td>
<td>CTX (µg/L)</td>
</tr>
<tr>
<td></td>
<td>PINP (µg/L)</td>
</tr>
<tr>
<td>Oxidative Stress Parameters</td>
<td>TBARS (µM)(^1)</td>
</tr>
<tr>
<td></td>
<td>Protein thiols (µM)</td>
</tr>
</tbody>
</table>

\(^1\) Significant difference between groups (p<0.05)
Figure 5.1: Concentration of bone resorption marker CTX in the serum for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test (*p<0.05) b Change in CTX for a period of 8 weeks and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*p<0.05) c Concentration of bone formation marker PINP in the serum for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test b Change in PINP for a period of 8 weeks and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test
Fig. 5.2: a Concentration of total polyphenol content in the urine over 8 weeks. 47 postmenopausal women between 50-60 years old were in the Supplement group (N=23) or Placebo group (N=24) for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test (*p<0.05). b Change in total polyphenol content over the 8 week supplement period and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*p<0.05)
Figure 5.3: a Total antioxidant capacity in the serum over 8 weeks, as determined using the TEAC assay. 47 postmenopausal women between 50-60 years old were in the Supplement group (N=23) or Placebo group (N=24) for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test (*p<0.05). b Change in total antioxidant capacity over the 8 week supplement period and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*p<0.0001)
Figure 5.4: a Concentration of lipid peroxidation in the serum over 8 weeks, as determined by TBARS. 47 postmenopausal women between 50-60 years old were in the Supplement group (N=23) or Placebo group (N=24) for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test (*p<0.0001). b Change in lipid peroxidation over the 8 week supplement period and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*p<0.0001)
Figure 4.5: 

a) Concentration of protein oxidation in the serum over 8 weeks as determined by thiols. 47 postmenopausal women between 50-60 years old were in the Supplement group (N=23) or Placebo group (N=24) for a period of 8 weeks. Values are mean ± SEM and were compared within supplement group using a paired t-test (*p<0.0001).

b) Change in protein oxidation over the 8 week supplement period and was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*p<0.001)
V. Discussion

We have shown that intervention with a supplement containing polyphenols and other nutritional components may decrease biomarkers for bone turnover meaning that the risk for development of osteoporosis might be reduced as a consequence of antioxidant treatment. This was tested by comparing bone turnover markers (CTX and PINP), oxidative stress parameters (TBARS and protein thiols), TAC, and TPC in early postmenopausal women who consumed a supplement rich in polyphenols and other micronutrients or a placebo. The findings reported here demonstrate a decline in CTX, oxidative stress parameters as well as an increase in TAC and total polyphenol content after 8 weeks of treatment among women who were supplemented with the combination of polyphenols and micronutrients. The changes were significantly different when comparing the two groups to one another. These findings are important because no differences were observed in any of the outcome measurements made in women who were on placebo for 8 weeks.

Previous studies showing the antioxidant properties of polyphenols were based mainly on in vitro and animal studies. Our laboratory is the first to evaluate the efficacy of the nutritional supplement comprised of polyphenols, vitamins, minerals, and antioxidants administered to postmenopausal women who are at risk for osteoporosis. Here, the Supplement group demonstrated increased levels of TAC and total urinary polyphenols at 8 weeks of treatment, which was significantly different when compared to the Placebo group. Although the polyphenols in greens+™ may have been the principal component of the nutritional supplement contributing to the observed antioxidant effect, the combined effect with other
micronutrients and antioxidants in the bone builder$^TM$ may also have contributed to this effect [173, 174] but it was not possible to actually prove this one way or the other in this study.

The TEAC method has been validated to measure the antioxidant properties of both the lipid- and the water-soluble antioxidants in vivo. This assay is used primarily for its ability to assess many endogenous antioxidants in addition to those found in the supplement and its capability to quench reactive oxygen species (ROS) [172]. Thus, the polyphenol-rich supplements consumed by the participants may have been absorbed and metabolites excreted in the urine as shown by the increase in the urinary concentrations of total polyphenols. This increased absorption may have resulted in an increased ability to suppress oxidative stress. This provides further credibility to the idea that it is the potent antioxidant capacity of the supplement, which is responsible for the decreased oxidative stress and CTX shown in this study.

Previous in vitro, in vivo and clinical studies [166-168, 171] on both lipid- and water-soluble antioxidants done in our laboratory have also demonstrated similar results. For example, in a recent study, the effectiveness of lycopene (a fat-soluble antioxidant) on oxidative stress parameters in humans was investigated. Decreased oxidization of proteins and lipid peroxidation following T2 months of treatment was shown [114]. This is similar to our previous findings in our laboratory where a significant decrease in both protein oxidation and lipid peroxidation was demonstrated within the Supplement group when compared to Placebo following 8 weeks of treatment. The relationship between the antioxidant property of the nutritional supplement and its effect on bone cells in vitro was recently reported.
Supplementation with greens+\textsuperscript{TM} containing polyphenols such as quercetin, apigenin, kaempferol and luteolin was shown to increase the proliferation of human osteoblast-like cells at early time points of addition as well as to stimulate the formation of bone nodules \textit{in vitro} in both a dose- and time-dependent manner [171]. These observations might suggest a positive effect of ingesting foods containing polyphenol and other supplements such as greens+ bone builder\textsuperscript{TM} in reducing oxidative stress, which may stimulate the activity of osteoblast cells, thus may possibly be beneficial in bone formation. However, our study was not able to show an effect on PINP, and longer treatment with greens+ bone builder\textsuperscript{TM} may be required.

The present intervention study demonstrated an association between increased urinary polyphenols and decreased CTX in postmenopausal women. Studies have shown that high bone turnover is correlated with a low BMD [195] and that increased bone turnover correlates with increased bone fragility and deterioration of bone microarchitecture [196]. Our findings showed that with supplementation, there was a significant decrease in CTX after 8 weeks of treatment. This is in keeping with another dietary intervention study, in which supplementation with calcium and vitamin D resulted in a significant decrease in CTX among postmenopausal women at risk of osteoporosis at 6 and 12 months of treatment [197].

More importantly, these changes in bone resorption markers are comparable to those seen in postmenopausal women supplemented with calcium [198, 199], which together with adequate vitamin D [55, 200], are currently recommended for maintenance of bone health and the prevention of osteoporosis. A study by Meunier \textit{et al.} showed that supplementation
with 596 mg/day of calcium significantly decreased serum CTX by 13% after 6 months in postmenopausal women [198]. Furthermore, data from another study involving a combined treatment of potassium citrate and calcium citrate are in line with this study [201]. They showed that when compared to placebo, the combined treatment reduced all of the bone resorption markers, thus suggesting bone protective properties through a synergistic effect.

There was no significant change found in PINP, a notable marker of bone formation, in these participants. Although serum PINP gives an accurate description of changes in bone turnover, particularly in postmenopausal osteoporosis [79, 202, 203], it is evident from other studies on osteoporosis medications that changes in PINP tend to be smaller in magnitude compared to those seen in CTX, and can take up to 6 months to be detected [72]. This intervention study was carried out for only 2 months, and perhaps a longer period of intervention would have resulted in significant changes in bone formation markers. Nevertheless, in the present study the effect on CTX is still biologically important, regardless of the lack of effect on PINP. Furthermore, a possible explanation for this result can be the coupling effect of bone resorption and formation. When there is a decrease in the rate of bone turnover, the bone first experiences decreases in bone resorption and subsequently bone formation. Bone resorption at any given site takes approximately one month, followed by bone formation in the same place the bone has been resorbed (coupling). Therefore, if the rate of resorption is slowed down, bone formation may also be slowed down. Many current therapies for osteoporosis target the increase in bone turnover markers by attempting to decrease bone resorption [75, 80, 204]. Bone resorption markers tend to decrease rapidly in response to potent anti-resorptive treatment, typically a 50-70% reduction in the telopeptides
within the first 12 weeks of treatment [71, 205]. Studies have shown that early antiresorptive-induced reductions in biochemical markers have been correlated with subsequent increases in BMD [206-209]. Notably, a smaller change of a 20% decrease in serum CTX within the first 6 months of therapy predicted significant increases in BMD at the total hip, greater trochanter, intertrochanteric region, and spine after 2.5 years of therapy (p<0.05) [209]. In this study, a similar anti-resorptive effect of supplementation for 8 weeks resulted in an average decrease in CTX of 11.6 ± 3.8%. Despite the short study duration preventing us from assessing the BMD of our participants, the present results on CTX suggest that long-term supplementation with a variety of polyphenols and micronutrients may result in increased BMD as long as the decreased CTX effect is sustained. Based on these findings, the results support the hypothesis that supplements such as greens+ bone builder™ may decrease the risk of osteoporosis as seen by the reduction of CTX. The effects of polyphenols on bone resorption looks promising, and a longer-term study with BMD measurements may confirm the bone-protective effects of total polyphenols in postmenopausal women at risk of osteoporosis.

Limitations of this study are acknowledged. First, the present study was not specifically designed to identify and quantify the individual types of polyphenols, minerals and other micronutrients absorbed in the body. Secondly, we did not control for intake of all foods and beverages that were rich in polyphenols, which could have also contributed to the changes in antioxidant capacity. Third, based on previous studies, our a priori sample size was originally intended to include 60 participants to show statistical power; however, with 47 participants, we were able to see a significant difference in most outcome measures. Lastly, as mentioned
before, this study was short-term. A study showing the long-term effects of the supplement on bone health should be considered along with measurements of BMD to document the benefit to bone of the additive effect of the polyphenols and other nutrients.

In conclusion, our study has shown that daily supplementation of a combination of polyphenols and other trace nutrients and minerals in the form of the nutritional supplement, greens+ bone builder\textsuperscript{TM}, can significantly increase the antioxidant capacity which results in decreased bone resorption, thus decreasing the extent of oxidative damage in postmenopausal women at risk of osteoporosis. The beneficial effects can be due to the combination of the polyphenols in greens+ with the other nutrients and minerals contained in the bone builder. This observation, along with previously observed bone-protective effects of the individual polyphenols, nutrients and trace minerals, should generate an increased interest into exploring further the potentially beneficial effects on bone of nutritional supplements such as those studied here. Although there is evidence that implicates oxidative stress as an important mediator of bone loss potentially enhancing the development of osteoporosis, our laboratory was the first to investigate the combined effects of a variety of polyphenols alongside other nutrients and minerals in healthy postmenopausal women, suggesting that greens+ bone builder\textsuperscript{TM} may prove to be a good alternative or complementary agent with other established therapeutics, in this case, actual drugs approved for the prevention or treatment of osteoporosis.
CHAPTER 6
GENERAL DISCUSSION AND CONCLUSIONS
I. Discussion of Results

Polyphenols are water-soluble phytochemical antioxidants that may protect bone against oxidative stress-induced osteoporosis. In the past few years, polyphenols and their antioxidant capabilities have been studied extensively. Studies have focused primarily on the effects of individual polyphenols on various chronic diseases associated with oxidative stress [166, 210-212]. Furthermore, there is convincing scientific evidence to support the important role that oxidative stress plays in bone metabolism. There are several lines of evidence that indicate oxidative stress increases differentiation and function of osteoclasts [85, 104, 119, 213] as well as inhibiting differentiation of osteoblasts [214, 215]. Therefore, based on these findings, we hypothesized that consumption of polyphenols may be beneficial and could prevent the development of osteoporosis due to antioxidant effects.

Previous in vitro studies in our laboratory have demonstrated the beneficial effects of greens+TM, bone builderTM and greens+ bone builderTM using systems of in vitro osteogenesis. greens+TM is a nutritional supplement rich in polyphenols whereas greens+bone builderTM is rich in minerals, vitamins and micronutrients. greens+ bone builderTM contains all the components of the first two supplements. greens+TM and bone builderTM were shown separately to have a favourable effect on osteoblasts by increasing the number of bone nodules formed in vitro in a dose-dependent manner. Conversely, when greens+TM and bone builderTM were tested together, the effects were greater than either one alone suggesting either additive or synergistic effects. This finding suggested that there might be a use for combining the contents of these agents into a single supplement for improvement of bone-health. Therefore, a clinical study was conducted to determine whether greens+ bone builderTM could reduce the risk indicators for osteoporosis in postmenopausal women as a consequence of the supplement’s antioxidant properties.
Results obtained in this study are presented in two manuscripts (Chapters 4 and 5). The key findings are as follows:

1. Postmenopausal women supplemented with greens+ bone builder\textsuperscript{TM} for a period of 8 weeks had a significant interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) for total antioxidant capacity with a concomitant interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) effect in the oxidative stress parameters for protein oxidation and lipid peroxidation.

2. Supplementation of greens+ bone builder\textsuperscript{TM} for a period of 8 weeks resulted in an increase in total urinary polyphenols and total antioxidant capacity as well as a decrease in oxidative stress biomarkers in postmenopausal women. This decrease in oxidative stress corresponded to a significant decrease in the bone resorption marker, CTX. It is possible that such corresponding decreases may subsequently reduce the risk of osteoporosis. However, due to the duration of the study period, intervention with greens+ bone builder\textsuperscript{TM} did not lead to any statistically significant differences for the bone formation marker between the two test groups.

There is sufficient evidence to indicate that antioxidants provide defense against oxidative stress in the body, and thereby protect the body against chronic diseases including osteoporosis. A study has revealed that plasma total oxidative status (TOS) and oxidative stress index (OSI) values were significantly higher, and plasma total antioxidant status (TAS) levels were lower in postmenopausal females who were also osteoporotic, as compared to healthy controls [126]. The health benefits of antioxidants depend on the amount consumed and on their bioavailability [216]. In this thesis, the Supplemented group had an increase of 7.1 ± 1.9% from baseline to 8 weeks, which was significantly different from the Placebo group, suggesting that the polyphenols and other micronutrients contributed to improving the antioxidant status of individuals and consequently in mitigating the damaging effects of oxidative stress. These results concur with the results of a study by Cao et al. wherein TEAC values increased in elderly women after they consumed antioxidant-rich foods such as strawberries, spinach or red wine [217].
Previous studies showing the antioxidant properties of polyphenols were based mainly on *in vitro* and animal studies. Our laboratory is the first to evaluate the efficacy of the nutritional supplement that comprised of polyphenols, vitamins, minerals, and antioxidants administered to postmenopausal women who are at risk for osteoporosis. Here, the Supplement group showed increased TAC and total urinary polyphenols by 8 weeks of treatment. This differed significantly from the Placebo group. Although the polyphenols in greens+™ may have been the principal component of the nutritional supplement contributing to the observed antioxidant effect, the combined effect with other micronutrients and antioxidants in the bone builder™ may also have contributed to this effect [173, 174].

Although polyphenols are the most abundant class of antioxidant phytochemicals in fruits and vegetables, they also contain different vitamins and minerals. Many studies have shown the advantageous role of these polyphenols, vitamins and minerals found in fruits and vegetables against adverse effect of oxidative stress and related diseases [218, 219]. An excellent review by Bouayed and Bohn suggested that the beneficial effects on human health of antioxidants derived from fruits and vegetables can be explained by the additive or synergistic effect of all of the ingredients. However, these findings have shown to be controversial with the theory that individual compounds may be the major contributors associated with proposed beneficial effects on health as previously postulated following epidemiological studies and associated meta-analyses [220, 221].

Nevertheless, the most common polyphenols in the human diet are not inevitably the most active *in vivo*, either because they have less intrinsic activity than others or because they are inadequately absorbed from the intestine, highly metabolized, or rapidly eliminated [222]. The chemical nature of individual polyphenols influences their biological properties including their bioavailability, antioxidant activity, specific interaction with cell receptors and enzymes and other properties [148]. It is important to note that only a mix of polyphenols was examined here and not individual polyphenols. Yet, an indirect evidence for polyphenol absorption through the gut barrier is suggested by the rise seen in the antioxidant capacity of the blood following consumption of polyphenol-rich foods [148]. The results showed that the total antioxidant capacity of the supplemented group (i.e. with a variety of polyphenols and other micronutrients) was greater than that shown in the Placebo group.
More direct evidence on the bioavailability of polyphenols has been acquired by determining their concentrations in plasma and urine after the ingestion of polyphenol-rich products [135]. The total concentration of polyphenol metabolites excreted in urine is roughly proportional to maximum plasma concentrations, which are reached approximately 1.5 to 5.5 hours after ingestion of polyphenol-rich foods [148]. Typically, once the polyphenols are absorbed, they are conjugated to glucuronide, sulphate and methyl groups in the gut mucosa and inner tissues [223]. These reactions facilitate their excretion and prevent accumulation of unsure levels [223]. Therefore, recovery of polyphenols and their metabolites in urine indicate that polyphenols are being absorbed into the small intestine and then go to the liver and other tissues to be metabolized, and are then excreted by the kidneys into the urine. Thus, higher urinary total polyphenol levels indicate greater levels of serum polyphenols which can be explained by absorption from the gut.

In this thesis, total polyphenol levels in the urine were used to estimate polyphenol intake and absorption. Gallic acid was used as a standard because studies have shown that it is far better absorbed than other polyphenols [135]. Total plasma polyphenols levels were not measured because, in terms of the elimination half-lives, gallic acid appears to be one of the polyphenols with a minimal chance of accumulating in plasma despite repeated ingestion.

The TEAC method has been validated to measure the antioxidant properties of both the lipid- and the water-soluble antioxidants in vivo. This assay is used primarily for its ability to assess many endogenous antioxidants in addition to those found in the supplement and its capability to quench reactive oxygen species (ROS) [172]. Thus, the polyphenol-rich supplements consumed by the participants may have been absorbed and metabolites excreted in the urine as shown by the increase in the urinary concentrations of total polyphenols. This increased absorption may have resulted in an increased ability to suppress oxidative stress. This provides further credibility to the idea that it is the potent antioxidant capacity of the supplement that is responsible for the decreased oxidative stress and CTX shown in this study.
Previous *in vitro, in vivo* and clinical studies [166-168, 171] from our laboratory on both lipid- and water-soluble antioxidant have also shown similar results. Thus, a recent study investigated the effectiveness of lycopene, a fat-soluble antioxidant, on oxidative stress parameters in humans. The results showed decreased oxidization of proteins and lipid peroxidation at 2 months of treatment [114]. This is similar to our finding where there was a significant decrease in both protein oxidation and lipid peroxidation within the Supplement group when compared to Placebo at 8 weeks of treatment.

Furthermore, this thesis demonstrated an association between increased urinary polyphenols and decreased CTX in postmenopausal women. Studies have shown that a high bone turnover is correlated with a low BMD [195] and that an increased bone turnover correlates with increased bone fragility and deterioration of bone microarchitecture [196]. Our findings showed that with supplementation, there was a significant decrease in CTX after 8 weeks. This is in keeping with another dietary intervention study, in which supplementation with calcium and vitamin D resulted in a significant decrease in CTX at 6 and 12 months of treatment among postmenopausal women at risk of osteoporosis [197].

More importantly, these changes in bone resorption markers are comparable to those seen in postmenopausal women supplemented with calcium [198, 199], which together with adequate vitamin D [55, 200], are currently recommended for maintenance of bone health and the prevention of osteoporosis. A study by Meunier *et al.* showed that supplementation with 596 mg/day of calcium significantly decreased serum CTX by 13% after 6 months in postmenopausal women [198]. Furthermore, data from another study involving a combined treatment of potassium citrate and calcium citrate are in line with this study [201]. They showed that when compared to placebo, the combined treatment reduced all of the bone resorption markers, thus suggesting bone protective properties through a synergistic effect.

The relationship between the antioxidant property of the nutritional supplement and its effect on bone cells *in vitro* was recently reported. Supplementation with greens+™ containing polyphenols such as quercetin, apigenin, kaempferol and luteolin was shown to increase the proliferation of human osteoblast-like cells at early time points of addition as well as to
stimulate bone nodule formation in a dose- and time-dependent manner [171]. These observations may suggest a positive effect of ingesting foods containing polyphenol and other supplements such as greens+ bone builder™ in reducing oxidative stress, which may stimulate the activity of osteoblast cells, thus may possibly be beneficial in bone formation. However, our study was not able to show an effect on PINP, a notable marker of bone formation, in these participants. Although serum PINP gives an accurate description of changes in bone turnover, particularly in postmenopausal osteoporosis [79, 202, 203], it is evident from other studies on osteoporosis medications that changes in PINP tend to be smaller in magnitude compared to those seen in CTX, and can take up to 6 months to be detected [72]. This intervention study was carried out for only 2 months, and perhaps a longer period of intervention would have resulted in significant changes in bone formation markers. Nevertheless, in the present study the effect on CTX is still biologically important, regardless of the lack of effect on PINP.

Furthermore, a possible explanation for this result can be the coupling effect of bone resorption and formation. When there is a decrease in the rate of bone turnover, the bone first experiences decreases in bone resorption and subsequently bone formation. Bone resorption at any given site takes approximately one month, followed by bone formation in the same place the bone has been resorbed (coupling). Therefore, if the rate of resorption is slowed down, bone formation may also be slowed down. Many current therapies for osteoporosis target the increase in bone turnover markers by attempting to decrease bone resorption [75, 80, 204]. Bone resorption markers tend to decrease rapidly in response to potent antiresorptive treatment, typically a 50-70% reduction in the telopeptides within the first 12 weeks of treatment [71, 205]. Studies have shown that early antiresorptive-induced reductions in biochemical markers have been correlated with subsequent increases in BMD [206-209]. Notably, a smaller but significant change of a 20% decrease in serum CTX within the first 6 months of therapy predicted significant increases in BMD at the total hip, greater trochanter, intertrochanteric region, and spine after 2.5 years of therapy [209]. In this study, a similar anti-resorptive effect of supplementation for 8 weeks resulted in an average decrease in CTX of 11.6 ± 3.8%.
Despite the short study duration preventing us from assessing the BMD of our participants, the present results on CTX suggest that long-term supplementation with a variety of polyphenols and micronutrients may result in increased BMD as long as the decreased CTX effect is sustained. Based on these findings, the results support the hypothesis that supplements such as greens+ bone builderTM may decrease the risk of osteoporosis as seen by the reduction of CTX. The effects of polyphenols on bone resorption looks promising, and a longer-term study with BMD measurements may confirm the bone-protective effects of total polyphenols in postmenopausal women at risk of osteoporosis. As well, since the nutritional supplement contained minerals and vitamins known to be beneficial for bone health, in addition to the phytochemical antioxidants, it is possible that mechanisms in addition to the antioxidant may also be involved. Future studies should be directed towards studying these interactions further.

An increase in serum antioxidant capacity following the consumption of greens+ bone builderTM was expected since it contains several polyphenols and other micronutrients. The combined effect of the supplement seen in this thesis could have contributed toward a decrease in the status of oxidative stress as indicated by the reductions in lipid peroxidation and protein oxidation. Previous studies have shown a causal relationship between oxidative stress and the risk of osteoporosis. Results obtained in this study, therefore, support the hypothesis that increased serum antioxidant capacity had a beneficial effect toward the reduction of oxidative stress, which may have an association to the decrease seen in bone resorption, thus reducing the risk of osteoporosis.
II. Implication of Findings

This thesis provides the rationale in that supplementation with a variety of polyphenols in combination with other micronutrients may decrease the risk of osteoporosis via decreasing oxidative stress parameters and bone resorption markers. The significant decrease seen in CTX was similar to previous studies among postmenopausal women who were supplemented with calcium and vitamin D [197-199]. Based on these findings, the consumption of diets and nutritional supplements that are rich in polyphenols and other minerals, vitamins and nutrients, by women at risk for osteoporosis, should be considered as a complementary strategy, as it may improve overall bone health.
III. Limitations

The major limitation, as with many randomized controlled trials, was participant recruitment. The sample of participants required had narrow inclusion criteria, thus limiting participant eligibility. For instance, recruiting women between the ages of 50 and 60 who were not taking any medications were able to refrain from consuming other vitamins and supplements, as well as restricting high-polyphenol foods and beverages for 8 weeks happened to be very challenging and difficult. Thus, many eligible participants did not enroll, which limited the sample size to 48. Our original aim was to recruit 60 participants to show statistical power. Nonetheless, we were able to show significant differences in most outcome measures with 47 participants.

Secondly, the present study was not specifically designed to identify and quantify the individual types of polyphenols, minerals and other micronutrients absorbed in the body. However, our previous *in vitro* study has shown the additive effect of the supplement on bone health. Furthermore, we did not control for the intake of all foods and beverages that were rich in polyphenols, which may have greatly contributed to the changes seen in the total antioxidant capacity and total urinary polyphenol content.

Lastly, this study illustrated the short-term effects of the supplement on bone metabolism. A study showing the long-term effects of the supplement should be considered along with periodic measurements of BMD for several years to strengthen the efficacy of the additive effects of polyphenols along with other micronutrients that are beneficial to the bone.
IV. Future Area of Research

A longitudinal prospective study where early postmenopausal women are given a supplement containing polyphenols and other micronutrients and monitored for BMD and fracture risk would further support the present findings. A strong correlation has been shown between BMD, fracture risk and BTMs, and measuring all three parameters would provide strong support for the findings shown in this thesis. Based on the paucity of various studies, further research should consider potential confounders such as dietary (i.e. consumption of other foods and nutrients) using the 7-day food records collected as well as lifestyle factors (i.e. prior fracture incidences, exercise). Also, future studies should also consider the dosage, type, duration, and frequency of consumption of polyphenols in addition to the micronutrients. Complying with many of these guidelines may ultimately lead to the development of evidence-based dietary recommendations for maintaining bone health.

As well, genomics is becoming an area of interest where future studies may consider the beneficial effect of polyphenols as well as micronutrients on bone health through genetics and the gene-environment interaction.

Finally, as this thesis has shown a favourable outcome of the consumption of a nutritional supplement rich in a variety of polyphenols and micronutrients in a randomized controlled study, further research investigating these effects in conjunction with FDA-approved medication to treat osteoporosis is warranted. If this additive product can provide a benefit to currently approved methods for preventing and treating osteoporosis, this would result in a more comprehensive understanding of bone health.
V. Conclusions

Osteoporosis is an enormous public health concern as fractures from this debilitating disease occur more often than a heart attack, stroke and breast cancer combined [56]. Dietary components are noteworthy aspects for the prevention of osteoporosis. Their incorporation into the daily lifestyle is rather straightforward and inexpensive. This thesis demonstrated that the additive effect of polyphenols with other micronutrients act as potent antioxidants to decrease oxidative stress parameters and bone resorption, which suggests that it may also decrease the risk of developing osteoporosis in postmenopausal women and may improve their overall bone health.
References


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APPENDIX I
GREENS+ BONE BUILDER+™ INGREDIENTS
### 1 SERVING (8.5g) OF GREENS+ CONTAINS:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl complex (28% phosphatidyl choline from 97% oil-free lecithin)</td>
<td>2171mg</td>
</tr>
<tr>
<td>Organic barley, alfalfa and wheat grass, and red beet powders</td>
<td>1543mg</td>
</tr>
<tr>
<td>Spirulina</td>
<td>1450mg</td>
</tr>
<tr>
<td>Apple fiber powder</td>
<td>1033mg</td>
</tr>
<tr>
<td>Japanese chlorella (cracked cell)</td>
<td>383mg</td>
</tr>
<tr>
<td>Soy sprout powder</td>
<td>383mg</td>
</tr>
<tr>
<td>Organic whole brown rice powder</td>
<td>383mg</td>
</tr>
<tr>
<td>Stevia leaf powder</td>
<td>225mg</td>
</tr>
<tr>
<td>Eight non-dairy bacterial cultures containing:</td>
<td></td>
</tr>
<tr>
<td>Lactobacilli and bifidobacteria (2.5 billion per serving)</td>
<td></td>
</tr>
<tr>
<td>In a special base of fructo-oligosaccharides (FOS)</td>
<td>200mg</td>
</tr>
<tr>
<td>Royal Jelly</td>
<td>150mg</td>
</tr>
<tr>
<td>Bee pollen powder</td>
<td>150mg</td>
</tr>
<tr>
<td>Licorice root extract (std. to 10% glycyrrhizin)</td>
<td>116mg</td>
</tr>
<tr>
<td>Acerola berry extract (std. to 18% Vitamin C)</td>
<td>115mg</td>
</tr>
<tr>
<td>Siberian ginseng extract (std. to 0.8% eleutherosides)</td>
<td>60mg</td>
</tr>
<tr>
<td>Milk thistle extract (std. to 60% silymarin)</td>
<td>60mg</td>
</tr>
<tr>
<td>Organic Atlantic clover powder</td>
<td>33mg</td>
</tr>
<tr>
<td>Ginkgo biloba extract (std. to 24% ginkgo flavonglycosides and 6% terpene lactones)</td>
<td>20mg</td>
</tr>
<tr>
<td>Japanese green tea extract (std to 90% polyphenols)</td>
<td>15mg</td>
</tr>
<tr>
<td>European Bilberry extract (std. to 25% anthocyanidins)</td>
<td>10mg</td>
</tr>
<tr>
<td>Full Spectrum Grape extract (std. to 95% proanthocyanidins and 500ppm Resveratrol)</td>
<td>5mg</td>
</tr>
<tr>
<td><strong>Bone builder blend:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin C (calcium and magnesium ascorbate)</td>
<td>100mg</td>
</tr>
<tr>
<td>Vitamin D3 (cholecalciferol)</td>
<td>800IU</td>
</tr>
<tr>
<td>Vitamin B6 (pyridoxine hydrochloride)</td>
<td>10mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>400mcg</td>
</tr>
<tr>
<td>Vitamin B12 (cyanocobalamin)</td>
<td>10mcg</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>200mg; citrate-malate 150mg; biglycinate 150mg</td>
</tr>
<tr>
<td>Magnesium (asparate)</td>
<td>300mg</td>
</tr>
<tr>
<td>Zinc (Bioactive proteinate)</td>
<td>10mg</td>
</tr>
<tr>
<td>Selenium (Bioactive proteinate)</td>
<td>100mcg</td>
</tr>
<tr>
<td>Copper (HVP chelate = Hydrolyzed Vegetable Protein [rice])</td>
<td>1mg</td>
</tr>
<tr>
<td>Manganese (Bioactive proteinate)</td>
<td>3mg</td>
</tr>
<tr>
<td>Boron (FruiteXB Osteoberon)</td>
<td>3mg</td>
</tr>
<tr>
<td>Equisetum arvense, Horsetail stem and leaf extract supplying 3mg of Silicon</td>
<td>43mg</td>
</tr>
<tr>
<td><strong>Other ingredients</strong></td>
<td></td>
</tr>
<tr>
<td>L-Lysine</td>
<td>400mg</td>
</tr>
<tr>
<td>Lycopene fruit extract</td>
<td>7mg</td>
</tr>
<tr>
<td><strong>Non-medicinal ingredients:</strong></td>
<td></td>
</tr>
<tr>
<td>Natural flavour blend with Stevia</td>
<td>1.33g</td>
</tr>
</tbody>
</table>
APPENDIX II
INFORMED CONSENT PACKAGE
Form A
Selection, Inclusion and Exclusion Criteria

Oxidative stress and the risk of osteoporosis: the role of dietary polyphenols and nutritional supplements (Genuine Health Inc, Toronto, Ontario)

Selection and Inclusion Criteria:

- Healthy women whose menses have ceased at least one year prior to entry
- Aged 50-60

The women who are willing to participate should agree to provide fasting blood and urine samples and maintain their dietary records when needed.

Exclusion Criteria:

- Those who smoke
- Those who are on hormone replacement therapy
- Those who take polyphenols, multivitamins or other supplements containing antioxidants (i.e. vitamin C, or vitamin E)
- Those who take medications for osteoporosis, coronary heart disease, high blood pressure, diabetes and cancer
- Those with other metabolic bone diseases
- Those who are allergic to bees and/or pollen or ingredients listed on Appendix to Consent Form 1
- Those who may still become/plan to be pregnant
- Those who have a body mass index of $\geq 30$
- Those who have a systolic blood pressure of $\geq 140$ mm Hg or a diastolic blood pressure of $\geq 90$ mm Hg
INFORMED CONSENT DOCUMENTS – FORMS B & C

FORM B
Consent to Participate in a Research Study

TITLE OF STUDY: Oxidative Stress And The Risk Of Osteoporosis: The Role Of Dietary Polyphenols And Nutritional Supplements

Introduction: Before agreeing to take part in this research study, it is important that you read the information in this research consent form. It includes details we think you need to know in order to decide if you wish to take part in the study. If you have any questions, ask a study doctor or study staff. You should not sign this form until you are sure you understand the information. All research is voluntary. You may also wish to discuss the study with your family doctor, a family member or close friend. If you decide to take part in the study, it is important that you are completely truthful about your health history and any medications you are taking. This will help prevent unnecessary harm to you.

Investigators:

The following investigators are involved in this study:

1. Principal Investigator: Dr. Leticia G. Rao, Associate Professor of Medicine and Co-Director, Calcium Research Laboratory, Division of Endocrinology and Metabolism, St. Michael’s Hospital, 38 Shuter St. Annex, Toronto, Ontario M5B 1A6. Tel #: (416) 864-5838, E-mail: leticia.rao@utoronto.ca Mon – Fri, 9:00 AM – 5:00 PM.

2. Co-investigator: Dr. A.V. Rao, Professor of Nutrition and Director, Program in Food Safety, Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Tel #: (416) 978-3621, Email: rao.v@utoronto.ca Tue – Thurs, 9:00 AM – 12:00 PM.

3. Co-investigator: Dr. Alan C. Logan, ND, FRSH, 50 Yonkers Terrace #8-J, Yonkers, NY 10704.

4. MSc student: Ms Nancy Kang, MSc student at the Department of Nutritional Sciences, University of Toronto. Supervisor: Drs. A.V. Rao and L.G. Rao, Tel #: (416) 864-5838, Email: n.kang@utoronto.ca Mon – Fri, 9 AM - 5 PM.

5. Collaborator and Qualified Investigator: Dr. R.G. Josse, Professor and Director of the Osteoporosis Centre and Associate Physician-In-Chief, St Michael’s Hospital and Department of Medicine, University of Toronto. 61 Queen St East. Toronto, Ontario.

Study Sponsor: Genuine Health, Inc., Canada
Conflicts of Interest:
Dr. L.G. Rao is the principal investigator for the project. She gives educational talks to the public, and scientific conferences and is often invited to give talks at various Universities. The sponsor, Genuine Health, sometimes compensates her for related expenses. Drs. A.V. Rao and A. Logan provide advice on research-related matters to the company and are compensated for their time. However, they are not being financially compensated to conduct this research study.

PURPOSE OF THE RESEARCH:
St. Michael’s Hospital and the University of Toronto are carrying out research in order to understand the role of oxidative stress, the water-soluble antioxidant polyphenols, and nutritional supplements in osteoporosis (weakening of the bones). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals (molecules), which start chain reactions that damage cells. Oxidative stress is the harmful condition that occurs when there is an excess of free radicals. Free radicals are molecules that produced when your body breaks down food, or by environmental exposures to tobacco smoke and radiation. Free radicals are also produced as a result of aging, poor nutrition or lifestyle, and a decrease in antioxidant levels such as polyphenols, or both.

Known antioxidants include a number of enzymes and other substances such as vitamin C, vitamin E, lycopene and beta carotene (which is converted to vitamin A) that are capable of counteracting the damaging effects of oxidation. Studies suggest that oxidative stress may play a role in the development of osteoporosis. Although these studies suggest that the antioxidants vitamin C, E and beta-carotene may improve bone health, little is known of the role played by polyphenols. Polyphenols are water-soluble antioxidants found in green tea and a variety of fruits and vegetables, as well as in nutritional supplements. One such nutritional supplement is the greens+ bone builder™. This is a new formulation which contains the original greens+ nutritional supplement combined with other supplements beneficial to bone, including folic acid, vitamins C, B and D; minerals calcium, zinc, selenium, silicon, boron, copper and magnanese; amino acid L-lysine and the antioxidant lycopene. Recent studies have shown that high dietary intake of polyphenols may reduce the risk of several chronic diseases, and several studies suggest that green tea, a good source of polyphenols may also help in the reduction of the risk for developing osteoporosis. It is for this reason that our laboratory is studying whether polyphenols and nutritional supplements are beneficial to postmenopausal women who are at risk of osteoporosis.

We are seeking postmenopausal, female participants aged 50-60 to take part in a 2-month study, in which we will supplement your diet with the nutritional supplement greens+ bone builder (Genuine Health, Inc.) or placebo (an inactive substance).

Our major objectives are: (1) To determine whether the intake of greens+ bone-builder™ will increase with the antioxidant capacity or the total antioxidants present in the blood using vitamin E as standard, and decrease oxidative stress parameters and bone turnover markers (the blood parameters that will show whether the treatment is helping in the formation of bone and
decreasing the destruction of bone (2) To determine whether polymorphism, the genetic differences in enzymes that protect against oxidative stress, can explain any of the associations that are observed.

This preliminary study should provide initial clinical data on which to base future studies of the effects of dietary antioxidants and nutritional supplements on the development of osteoporosis. Approximately 60 participants will be recruited from the greater Toronto area, each of whom will be enrolled in the study at St. Michael’s Hospital.

DESCRIPTION OF THE RESEARCH:
There will be 5 visits in total:

On your first visit, the study will be explained to you and you will be given forms A to F to take with you to read and study. Once you agree to participate, the following schedules will be followed:

**Week 1**
You will be asked to record your daily diet for one-week on Form E-2 of the package given to you. After 1 week, you will be asked to come to the hospital after 12 hours without food to give a fasting blood and urine sample.

**Week 2** (wash-out period)
From week 2 until the end of the study (week 10) you will be asked to refrain from consuming green tea and nutritional supplements containing polyphenols and/or other antioxidants and vitamins. You will also be required to record your daily diet for one week, and at the end of Week 2, you will be asked to come to the hospital and give fasting blood and urine samples and hand in your food records.

**Week 3 to Week 6**
For week 3, you will be randomized, that is selected by chance to receive either the greens+ nutritional supplement or placebo. The chance of receiving the placebo is 50:50. The section below will explain how the supplement is to be taken. You will be required to take your supplement every day. Record your daily food intake at start of Week 6, then come to the hospital to give a fasting blood and urine sample and hand in your food records.

**Weeks 7 to Week 10:**
Take your supplement every day. Record your daily food intake at the start of Week 10, then come to the hospital to give a fasting blood and urine sample and hand in your food records.

At each of the clinic appointments we will take your height, weight and blood pressure. For the duration of the study we ask that you maintain your usual habits, including diet, exercise and lifestyle, as best as possible. Please remember to avoid consuming the foods listed on Form F from Weeks 2-10 of the study.
Total time commitment: Participation in this study requires 2 trips to our office (first visit and end of week 1) and 4 trips to the outpatient blood clinic of St. Michael’s Hospital; appointments will be scheduled to your convenience, between Monday-Friday from 7:30 AM to 9 AM. Each visit should take approximately half an hour. Additionally, participants will be required to record their diet for 7 days prior to each appointment. Participants will be contacted by telephone to confirm these appointments and to monitor progress during weeks 5 and 9 of the study. Additionally, if any new information relevant to the study is revealed during the study, participants will be contacted immediately by telephone.

Supplement: Participants will be randomly assigned to take either greens+ bone builder supplement or placebo. The supplement or placebo is a powder which will be mixed with water and consumed daily with breakfast for a period of 8 weeks. If you forget or are not able to consume the supplement with your breakfast, please consume later in the day with a meal or a snack. If you do not remember until the following day that the supplement was missed please do not take extra supplement, however, please record the day that the supplement was missed, and advise the study coordinator at your next clinic appointment. Please return any unused study product to the study coordinator at the end of the study during the final clinic appointment (end of study week 10).

Blood and urine samples: Blood samples will be taken at the outpatient lab of St. Michael’s Hospital by experienced nurses. A study coordinator will be present as well. Blood and urine samples are fasting samples; we ask that for 12 hours prior to the appointment you consume no food or beverages; however you may drink as much water as you like. Approximately 40mL (approximately 2.5 tablespoons) of blood will be taken. The blood and urine samples (urine is to be collected in the vial that will be provided to you) will be processed and stored for the analyses of antioxidant capacity, antioxidant polymorphisms, oxidative stress parameters and bone turnover markers. Samples will be frozen in a −80°C freezer at St. Michael’s Hospital for analyses as described above, all samples will be disposed of after 5 years.

Availability of supplement: This supplement is currently available in health food stores and those wishing to continue taking it after the study period may purchase it quite easily.

Placebo: As a participant in this study you may be randomly assigned to take a placebo; the chance of receiving a placebo is 50:50. A placebo is an inactive substance, and in this study it looks and tastes the same as the greens+ bone builder supplement, however it does not contain any polyphenols or other nutritional substances. We use a placebo in this study to ensure that any effects we see are due to the polyphenols and nutritional supplements contained within the greens+ bone builder supplement and not a result of the powder itself or any changes in your diet.
POTENTIAL HARS (INJURY, DISCOMFORTS OR INCONVENIENCES):

There are no known physical risks or injuries associated with consumption of greens+ bone builder supplement or its placebo as given in this study. However, first time users may experience temporary symptoms. These symptoms can include headaches, constipation, diarrhea, nausea and skin blemishes. There is no rescue medication required to relieve these symptoms, however you may treat these symptoms as you normally would. Please call the study coordinator if the symptoms persist, and for your safety you may be asked to withdraw from the study. There are no known drug interactions with greens+ bone builder supplement or its placebo; however those on prescription medication should consult their physician before taking this product. Those who are allergic to bees or bee pollen may experience a reaction to the supplement, which is why they are excluded from the study; there are no other known allergic reactions to this supplement.

When blood is taken from the vein, a slight discomfort or redness may be experienced which will usually disappear in a few days. If a more severe reaction occurs, such as infection, please consult a physician and inform the study coordinator.

We ask participants to record their diet - everything they eat and drink as well as quantities for 7 days prior to each appointment (weeks 1, 2, 6 and 10 of the study) - and submit the completed records to the study coordinator at each appointment. We will provide you with blank diet records (Form E-2) on which to record this information. Some participants may find this process to be inconvenient as it may be time consuming.

Withdrawal from the Study: Those wishing to withdraw from the study may do so at any time. For your safety, you may be asked to withdraw from the study if you experience persistent symptoms include headaches, constipation, diarrhea, nausea and skin blemishes. Any personal health information, dietary records, and blood and urine samples will be destroyed.

POTENTIAL BENEFITS:

You may receive no direct benefits from being in this study. However, results from this study may further medical and scientific knowledge on the role of nutritional supplements in bone health.
PROTECTING YOUR HEALTH INFORMATION:

The study investigators, sponsor (Genuine Health, Inc.), coordinators, nurses and delegates (hereby referred to as “study personnel”) are committed to respecting your privacy. No other persons will have access to your personal health information or identifying information without your consent, unless required by law. Any medical records, documentation, study samples or information related to you will be coded by study numbers to ensure that persons outside of the study (i.e., sponsors) will not be able to identify you. No identifying information about you will be allowed off site. All information that identifies you will be kept confidential and stored and locked in a secure place that only the study personnel will have access to. In addition, electronic files will be stored on a secure hospital or institutional network and will be password protected. It is important to understand that despite these protections being in place, experience in similar studies indicates that there is the risk of unintentional release of information. The principal investigator will protect your records and keep all the information in your study file confidential to the greatest extent possible. The chance that this information will accidentally be given to someone else is small.

By signing this form, you are authorizing access to your medical records by the study personnel, authorized representatives of the sponsoring company, Genuine Health, Inc., the St. Michael’s Hospital Research Ethics Board and by government regulatory authorities (i.e., Health Canada, the US Food and Drug Administration (FDA) and/or regulatory agencies from other countries). Such access will be used only for purposes of verifying the authenticity of the information collected for the study, without violating your confidentiality, to the extent permitted by applicable laws and regulations.

National and Provincial Data Protection regulations, including the Personal Information Protection and Electronic Documents Act (of Canada) or PIPEDA and the Personal Health Information Protection Act (PHIPA) of Ontario, protect your personal information. They also give you the right to control the use of your personal information, including personal health information, and require your written permission for your personal information (including personal health information) to be collected, used or disclosed for the purposes of this study, as described in this consent form. You have the right to review and copy your personal information. However, if you decide to be in this study or chose to withdraw from it, your right to look at or copy your personal information related to this study will be delayed until after the research is completed.

It is possible that a commercial product (device or pharmaceutical) may be developed as a result of this study. Neither the sponsor (Genuine Health, Inc.) nor the principal investigator (Dr. L. Rao) will compensate you if this happens, and you will have no rights nor receive royalties to any products that may be created as a result of this study or any future research studies.

All study records will be kept confidential for 25 years. De-identified samples will be frozen in a – 80°C freezer at St. Michael’s Hospital for analyses as described above, all samples will be disposed off after 5 years.

Osteoporosis, Antioxidants and Nutritional Supplements, Form B&C
Ver 14 Sept 2010
ALTERNATIVES TO PARTICIPATION:
The alternative to participation in the study is non-treatment. There is no likely consequence to you if you decide not to participate in the study. Recruitment will be continued until the required number of participants is attained.

STUDY RESULTS:
Participants may contact the Study Coordinator or Principal Investigator by telephone for the results of the study, both individual and overall, once the study has been completed. Additionally, if requested, the participants will be given the information on any published manuscript related to the study.

The results of this study will be published in peer-reviewed scientific journals and/or presented at conferences, seminars or other public forums without breaking the confidentiality and privacy as stated above. Your identity will not be disclosed in any presentations or publications of the results of the study.

POTENTIAL COSTS OF PARTICIPATION AND REIMBURSEMENT TO PARTICIPANT:
Those who participate will be provided $150.00 compensation for time and travel costs. The participant will be provided with $37.50 per visit to cover these costs (4 visits in total for the duration of the study), which will be given at one time, either at premature withdrawal or upon completion of the study. The participant will be asked to sign a receipt indicating the sum to be reimbursed and the hospital will issue a check within 2 months of study completion.

COMPENSATION FOR INJURY:
If you suffer a physical injury from taking the nutritional supplement and/or from participation in this study, medical care will be provided to you in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form waive your legal rights nor release the study doctor(s), Genuine Health, Inc., the study sponsor or St. Michael’s Hospital from their legal and professional responsibilities.

PARTICIPATION AND WITHDRAWAL:
Participation in research is voluntary. If you choose not to participate, you and your family will continue to have access to customary care at St Michael’s Hospital. If you choose to participate in this study you can withdraw from the study at any time without any effect on the care you or your family will receive at St Michael’s Hospital.

NEW FINDINGS OR INFORMATION:
We may learn new things during the study that you may need to know. We can also learn about things that might make you want to stop participating in the study. If so, you will be notified about any new information in a timely manner. You may also be asked to sign a new consent form discussing these new findings if you decide to continue in the research study.
RESEARCH ETHICS BOARD CONTACT:
The study protocol and consent form have been reviewed by a committee called the Research Ethics Board at St. Michael’s Hospital. The Research Ethics Board is a group of scientists, medical staff, and individuals from other backgrounds (including law and ethics) as well as members from the community. The committee is established by the hospital to review studies for their scientific and ethical merit. The Board pays special attention to the potential harms and benefits involved in participation to the research participant, as well as the potential benefit to society. This committee is also required to do periodic review of ongoing research studies. As part of this review, someone may contact you from the Research Ethics Board to discuss your experience in the research study.

If you have any questions regarding your rights as a research participant, you may contact Dr. Julie Spence, Chair, and Research Ethics Board at 416-864-6060 ext. 2557 during business hours.

STUDY CONTACTS:
In case of questions or emergency please contact Dr. L.G. Rao at (416) 864-5838, Dr. A.V. Rao at (416) 978-3621 or Nancy Kang at (416) 864-5838.
Signatures of Participants, Person Obtaining the Consent and Investigator

TITLE OF STUDY: Oxidative Stress and The Risk of Osteoporosis: The Role of Dietary Polyphenols and Nutritional Supplements

Consent: The research study has been explained to me, and my questions have been answered to my satisfaction. I have been informed of the alternatives to participation in this study. I have the right not to participate and the right to withdraw without affecting the quality of medical care at St. Michael’s Hospital for me and for other members of my family. As well, the potential harms and benefits (if any) of participating in this research study have been explained to me.

I have been told that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional responsibilities. I know that I may ask now, or in the future, any questions I have about the study. I have been told that records relating to me and my care will be kept confidential and that no information will be disclosed without my permission unless required by law. I have been given sufficient time to read the above information. I consent to participate. I have been told I will be given a signed copy of this consent form.

I hereby consent to participate as shown below:

I agree to participate □ Yes □ No (initial) (initial)
I agree to be contacted for other future studies related to bone health (initial):
□ Yes □ No (initial) (initial)

<table>
<thead>
<tr>
<th>Name of Participant (Print)</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

<table>
<thead>
<tr>
<th>Name of Person obtaining the consent</th>
<th>Signature</th>
<th>Date</th>
</tr>
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Research Coordinator

Osteoporosis, Antioxidants and Nutritional Supplements, Form B&C
Ver 14 Sept 2010
FORM D Participants Source Document

Oxidative stress and the risk of osteoporosis: the role of dietary polyphenols and nutritional supplements (Genuine Health, Inc, Toronto, Ontario)

Date ________

I.D. CODE __________________

Health Status

Age _____ Weight _____ Height _____ Pulse Rate _____ Blood Pressure _____

Record of consumption or use of the following items:

<table>
<thead>
<tr>
<th>Please check the appropriate line</th>
</tr>
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<tbody>
<tr>
<td><strong>Caffeine</strong> (coffee/tea/soda consumption in cups/day):</td>
</tr>
<tr>
<td>______ 0</td>
</tr>
<tr>
<td>______ 1-2</td>
</tr>
<tr>
<td>______ 3-4</td>
</tr>
<tr>
<td>______ 5+</td>
</tr>
</tbody>
</table>

| **Supplements:** vitamins, herbals, polyphenols: |
| ______ No |
| ______ Yes |

if yes, please include the amount, frequency and brand in the 7-day food record

| **Smoking:** |
| Non-smoker |
| Ex-smoker. When did you stop smoking? ________ |
| Smoker |
| ______ ½ pack/day |
| ______ 1 pack/day |
| ______ 2+ packs/day |

| **Occasional. When did you last smoke? ________** |

| **Alcohol consumption:** |
| Beer ______ glass/week |
| Wine ______ glass/week |
| Hard liquor ______ oz/week |

| **Hormone Replacement Therapy** |
| ______ Never ______ Stopped, when ______ Yes ______ date started |

Other Estrogen users:

| date started |

6. Notes

__________________________________________________________________________
APPENDIX III
FOOD RECORD
Form E-2 (day 1)
Food Record

Oxidative stress and the risk of osteoporosis: the role of dietary polyphenols and nutritional supplements (Genuine Health Inc, Toronto, Ontario)

<table>
<thead>
<tr>
<th>Time eaten</th>
<th>Quantity (cups, ml, g, tsp, etc)</th>
<th>Food/Beverage and Description Include vitamin supplements when applicable</th>
<th>For Official Use Only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Is this a usual day? Yes __________ No _______
If “No”, please explain________________________________________________________
Form F
Foods to Avoid by Participants

Oxidative stress and the risk of osteoporosis: the role of dietary polyphenols and nutritional supplements (Genuine Health, Inc, Toronto, Ontario)

Please avoid consuming the following during the wash-out and treatment phases of the study:

Green Tea
Herbal Products containing polyphenols (i.e. Greens+ herbal supplements) Multivitamins or other supplements containing antioxidants (i.e. vitamin C, lycopene and vitamin E)