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OSTEOPOROSIS

A MAJOR HEALTH PROBLEM IN VIETNAM

lifestyle factors and determinants of bone mass

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In memory of my Father

To my Mother, my Husband and my Children

ABSTRACT

While the prevalence of osteoporosis and risk factors for low bone mineral density (BMD) has been well documented in Caucasian populations, there is a lack of data from Asia. This work was designed to clarify to what extent osteoporosis could be regarded as a major public health problem in Vietnam. Furthermore, to elucidate the prevalence of certain risk factors, such as vitamin D deficiency and other determinants of bone mass as a basis to identify high-risk individuals among the Vietnamese women and men.

The **clinical studies** were designed as cross-sectional investigations using a multistage sampling scheme. Within the setting of northern Vietnam (latitude 21°N), districts were selected to represent urban and rural areas. In total 612 healthy women and 222 men aged 13-83 years were investigated. BMD was measured at the lumbar spine, femoral neck and total hip in all qualified subjects with dual energy X-ray absorptiometry. Serum concentrations of 25(OH)D, parathyroid hormone, estrogen and testosterone were quantified by electrochemiluminescence immunoassay. Data on clinical history and lifestyle were collected by individual face-to-face interviews.

Reference values for peak BMD were defined. These data allowed the calculation of T-scores and thus for the first time, an accurate identification of osteoporosis in a Vietnamese population. As determined at the femoral neck, the **prevalence of osteoporosis** was 17-23% in women and 9% in men. The results clearly suggest that osteoporosis is an important public health problem. Postmenopausal women living in **urban** areas experienced osteoporosis more than **rural** residents. Serum levels of 25(OH)D and estrogen were significantly associated with bone mass in both women and men. The **prevalence of vitamin D deficiency** (<20 ng/mL) was very high, 30% in women and 16% in men.

An **experimental study** on the isoflavone content of different soymilk preparations was performed by HPLC (high pressure liquid chromatography). Values of isoflavones were very low, around 60-80 mg/L, and there were only 10-20% of *bioactive aglycones*. This is far below the reported threshold levels to exert significant effects on bone.

In the future these data will be useful as a valuable reference base to diagnose osteoporosis and for the clinical management of its consequences. The high prevalence of vitamin D deficiency should raise the awareness of potentially important health issues such as osteoporosis but also about other serious diseases within the Vietnamese society.

Key words: Vietnamese men and women, peak bone mineral density, osteoporosis, vitamin D deficiency, estrogen, testosterone, soymilk, aglycone content.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

H.T.T. Nguyen, B. von Schoultz, D.M.T. Pham, D.B. Nguyen, Q.H. Le, D.V. Nguyen, A.L. Hirschberg, T.V. Nguyen. Peak bone mineral density in Vietnamese women. Arch Osteoporos, 2009, 4(1-2):9-15.

Huong TT. Nguyen, Bo von Schoultz, Tuan V. Nguyen, Dang N. Dzung, Pham TM. Duc, Vu T. Thuy, Angelica L. Hirschberg. Vitamin D deficiency in northern Vietnam: prevalence, risk factors and associations with bone mineral density. Submitted.

Huong TT. Nguyen, Bo von Schoultz, Tuan V. Nguyen, Trinh X. Thang, Tran T. Chau, Pham TM. Duc, Angelica L. Hirschberg. Sex hormone levels as determinants of bone mineral density and osteoporosis in Vietnamese women and men. Manuscript.

Huong TT. Nguyen, Mo Pourian, Birgitta Byström, Ingrid Dahlin, Pham TM. Duc, Tuan V. Nguyen, Bo von Schoultz, Angelica L. Hirschberg. Low aglycone content in commercial soy drink products. Asia Pac J Clin Nutr 2012, 21 (1):52-56.

LIST OF ABBREVIATIONS

25(OH)D	25-hydroxychole-calciferol or calcidiol
1,25 (OH)D	1,25- hydroxychole-calciferol or calcidiol
1 α -OH-vit D ₃	Alfacalcidol = 1 α -hydroxycholecalciferol =, Calcitriol
7-DHC	7-dehydrocholesterol
AIC	Akaike information Criterion
BMD	Bone mineral density
BMI	Body mass index
CaBP	Calcium binding protein
CI	Confidence interval
CV	Coefficient of variation
DXA	Dual-energy xray absorptiometry
D ₂	Ergocalciferol
D ₃	Cholecalciferol
ECaC	Epilethial channel calcium
ECLIA	Electrochemiluminescence immunoassay
ER	Estrogen receptor
FGF23	Fibroblast growth factor 23
Fig	Figure
FN	Femoral neck
FSH	Friendship and Science for Health
HMU	Hanoi Medical University
HPLC	High pressure liquid chromatography
HRT	Hormone replacement therapy
ISCD	Intenational Society of Clinical Densitometry
KI	Karolinska Institutet
KIRT	Karolinska International Research Training
LS	Lumbar spine
M-CSF	Macrophase-colony stimulating factor
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOF	National Osteoporosis Foundation
OJ	Orange juice
OPG	Osteoprotegerin
OR	Odd ratio
Pi	Inorganic phosphate
pBMD	Peak bone mineral density
PTH	Parathyroid hormone
R-1, 2, 3, 4	Home-made recipe-1, 2, 3, 4
RANKL	Receptor activation of nuclear factor kappa B ligand
S-1, 2, 3	Sample from Sweden-1, 2, 3
SD	Standard deviation

SERM	Selective estrogen receptor modulator
TGF- β	Transforming growth factor
TH	Total hip
UK	United kingdom
US	United state
USA	United state of America
UV	Ultraviolet
UVB	Ultraviolet B radiation
VDBP	Vitamin D-binding protein
VDR	Vitamin D receptor
VN-1, 2, 3	Sample from Vietnam-1, 2, 3

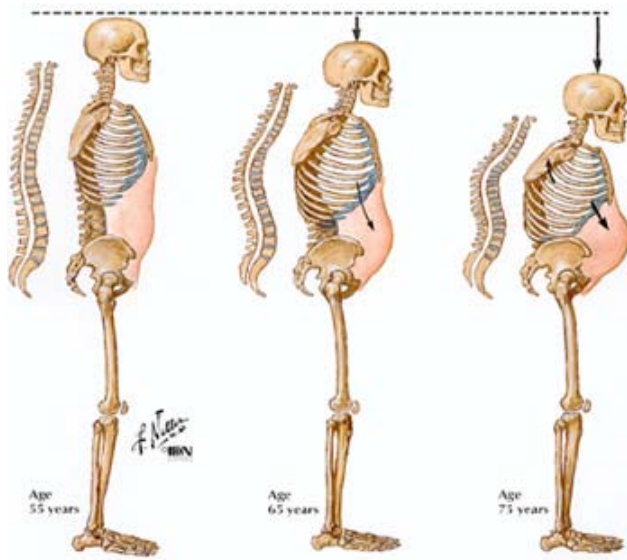
1 BACKGROUND

1.1 OSTEOPOROSIS

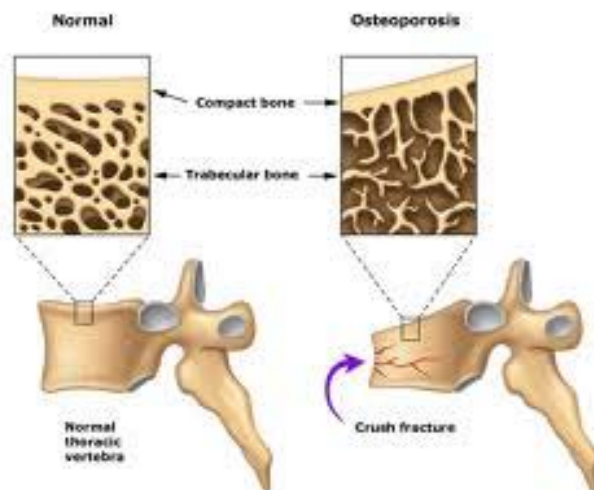
Osteoporosis, and fracture as its consequence, is recognized as an important global public health problem. Fracture is associated with increased mortality (Center et al 1999), concomitant morbidity, and reduced quality of life (Randell et al 2000). In the year of 2000 alone, there were an estimated 9 million osteoporotic fractures worldwide, resulting in a loss of 5.8 million disability-adjusted life-years (Johnell & Kanis 2006). It is projected that approximately half of all hip fractures in the world occur in Asia (Cooper et al 1992; Lau et al 2001). Despite the expected magnitude of the problem, the prevalence of and risk factors for osteoporosis in Asian countries are not well documented due to lack of epidemiologic data.

Osteoporosis is generally thought as a “woman’s disease” because the prevalence of osteoporosis and the rate of fractures are much higher in postmenopausal women than in older men (National Osteoporosis Foundation, 2002). However, older men still suffer from poor health outcomes related to osteoporosis and fractures. Aging men lose bone mineral density (BMD) at a rate of approximately 1% per year (Hannan et al 2000). It is estimated that one in five men over the age of 50 will suffer from an osteoporotic fracture during his lifetime (Melton et al 1992). Additionally, a lower proportion of men at high risk of fracture are treated than women at high risk (Kiebzak et al 2002). Among many different sites of osteoporotic fracture, the hip fracture contributes to the greatest morbidity and mortality (Johnell & Kanis 2006). Men also tend to have worse outcomes after fracture than women; they are twice as likely to die after a hip fracture than women (Haentjens et al 2010). Thus, it is important to pay attention not only to women but also to men as regards osteoporosis, its diagnosis, prevention and treatment.

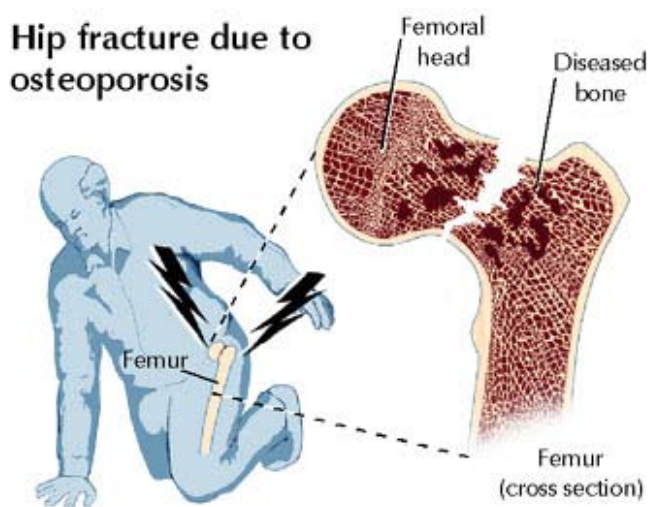
Because bone loss occurs insidiously and is initially asymptomatic, osteoporosis is often diagnosed only after the first clinical fracture has occurred (**Fig 1**) (Unnanuntana et al 2010; Vestergaard et al 2005). Osteoporosis and fracture are preventable if we could have early diagnosis or indentify the high-risk factors and individuals.



(a)



(b)



(c)

Figure 1. Osteoporosis at a glance

Osteoporosis is often asymptomatic. A decrease in height may be the only first sign of the disease.

A gradual loss of trabecular bone may cause a vertebral crush fracture.

A hip fracture may occur after only a minor trauma.

1.2 BONE MINERAL DENSITY

Osteoporosis is a disease characterized by a systemic skeletal impairment of bone mass and micro-architecture that lead to an increase in bone fragility and susceptibility to fracture (2001).

At present, the best method for screening and diagnosis of osteoporosis in both men and women is measurement of bone mineral density by dual-energy xray absorptiometry (DXA). The bone mineral content is given as gram within the area measured (g/cm^2). For the diagnosis of osteoporosis, the BMD of an individual is compared to the mean value of a young population (peak bone mass). A T-score value is calculated from an individual's areal BMD levels and reference values. The T-score is the number of standard deviations (SDs) for an individual's BMD from the mean of the young reference group (Baim et al 2008). A person is diagnosed with osteoporosis if the T-score is -2.5 or lower, or osteopenia/low bone mass if the T-score is between -2.5 and -1.0; or normal if the T-score is -1.0 or higher. A low BMD is the most important risk factor of fracture. A decrease of 1 SD will increase the risk of fracture about 2-3 fold (Kung et al 2007b) (**Fig 2**). Therefore, it is essential to obtain accurate BMD data measured by DXA.

The World Health Organization recommends the femoral neck as the most important anatomic region for the diagnosis of osteoporosis. However, the International Society of Clinical Densitometry (ISCD) and the National Osteoporosis Foundation (NOF) guidelines recommend that a diagnosis of osteoporosis should be based on three sites i.e. the femoral neck, the total hip and the lumbar spine. Measurement on these different sites could come up with different results.

The ISCD recommends that a young female reference group should be used to calculate the T-score for both men and women (Baim et al 2008). However, the NOF suggests that a young reference group value of the same sex should be used to diagnose osteoporosis (NOF, 2010). Women have lower levels of BMD than men (NOF, 2010); and BMD is influenced by genetic and environmental factors (Krall & Dawson-Hughes 1993). Therefore, it should be important to have ethnic- and sex-specific reference values in order to obtain accurate diagnosis.

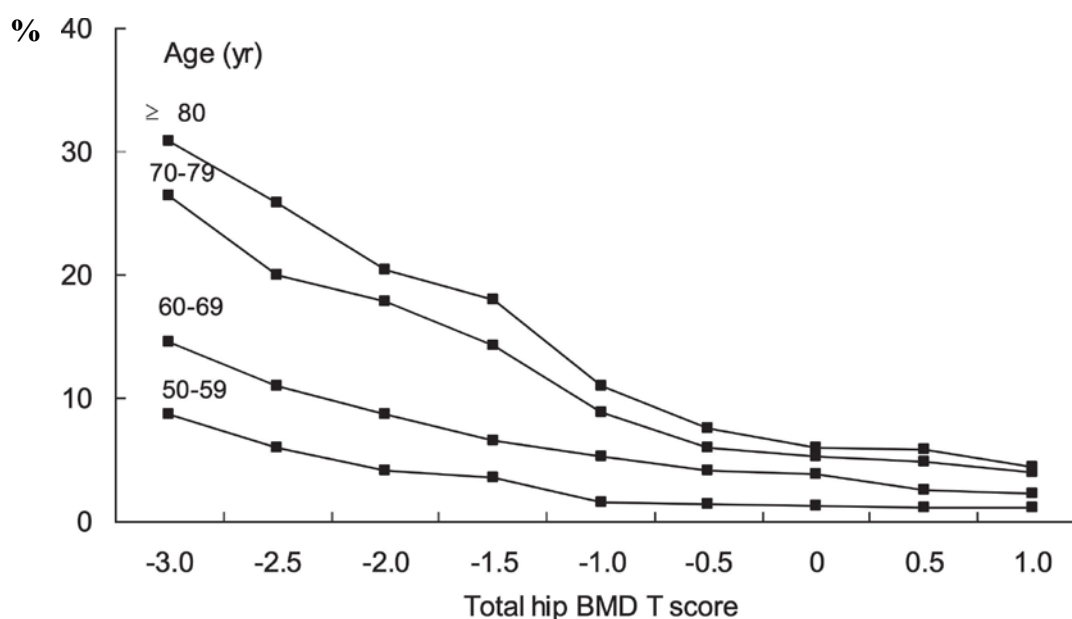


Figure 2. Ten-year risk of osteoporotic fracture in southern Chinese women according to age and BMD T-score (Kung et al 2007b). Reproduced with the permission from the publisher.

1.3 LIFE STYLE FACTORS AND DETERMINANTS OF BONE MASS

1.3.1 Bone biology

Bone is composed of support cells (osteoblasts and osteocytes), remodeling cells (osteoclasts), osteoid (non-mineral matrix of collagen and glycosaminoglycans) and inorganic mineral salts (hydroxyapatite, a complex of calcium and phosphate). Osteoblasts are responsible for synthesis of the osteoid and its mineralization, which makes bone rigid and hard. Resorption of mineralized bone is done by osteoclasts.

Bone remodeling is the coupled process of bone formation and bone resorption to maintain bone mass in adults (**Fig. 3**). This process consists of five phases. In the *activation phase* the resting bone surface becomes a remodeling surface. Precursors of osteoclasts differentiate into functional osteoclasts due to stimulation by cytokines and growth factors. In the next phase (*resorption phase*), osteoclasts digest mineralized bone, making scalloped erosions in the bone surface. After the resorption phase there is a *reversal phase*, coupling formation to resorption. This requires proliferation and differentiation of osteoblasts precursor cells and accumulation of the new osteoblasts in the resorption cavity. This phase is followed by the synthesis of osteoid and its subsequent mineralization (*formation phase*) by osteoblasts. Finally, most

of the osteoblasts become inactive bone lining cells on the bone surface (*quiescence phase*). Some osteoblasts however become incorporated in the mineralized bone. These cells, then called osteocytes, are thought to communicate with the osteoblasts and to initiate the next cycle of bone resorption and formation (Parfitt 1984).

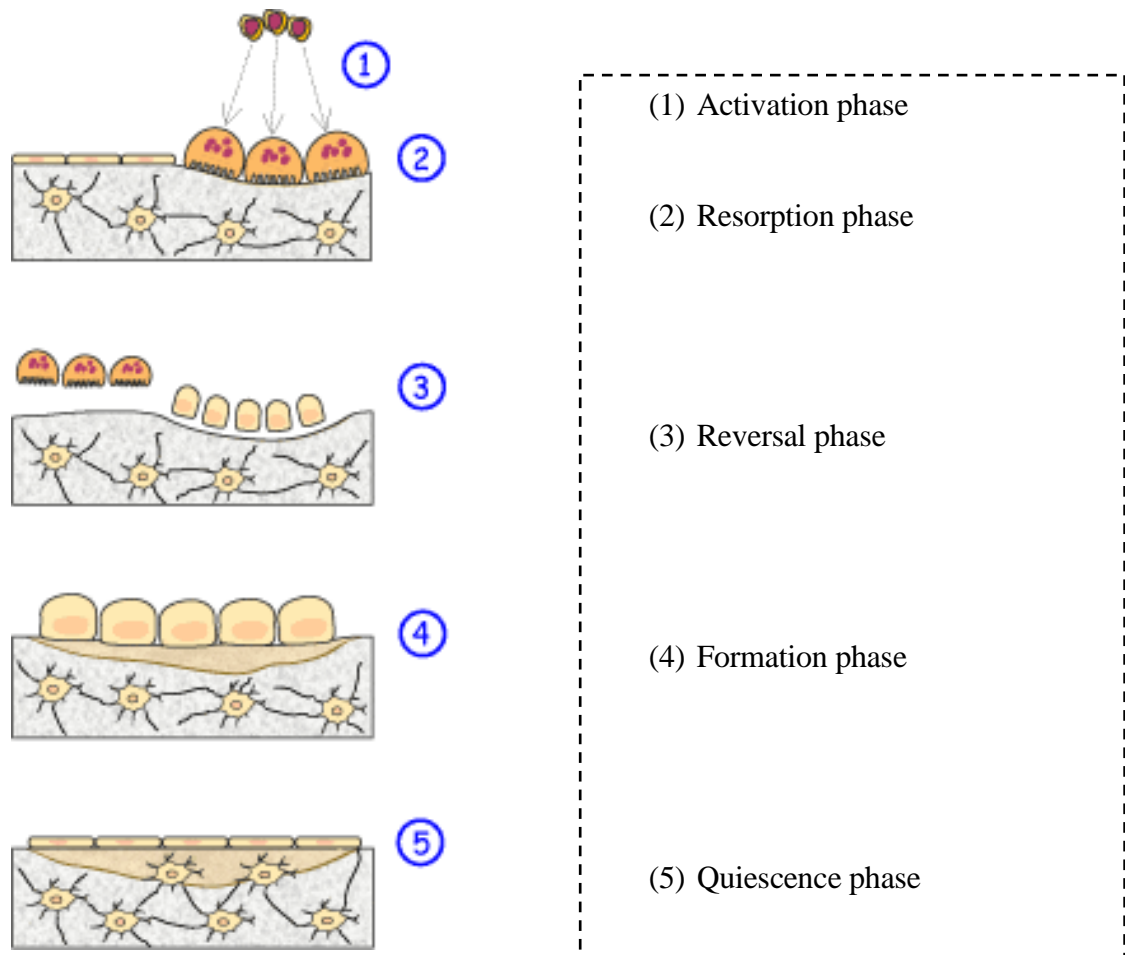


Figure 3. Five phases of bone remodeling

1.3.2 Sex steroid hormones and bone

Sex steroids i.e. estrogen and testosterone are major determinants of bone metabolism in both women and men (Kuchuk et al 2007). *In vitro* and *in vivo* studies indicate estrogens and androgens to act via different cellular mechanisms (Clarke & Khosla 2009).

The bone sparing effect of estrogen is anti-resorption by inhibition of osteoclast activity. The cellular and molecular mechanism by which estrogen deficiency leads to bone loss are increasingly well understood (**Fig 4**). Estrogen deficiency increases receptor activation of nuclear factor kappa B ligand (RANKL),

leading to increased osteoclast recruitment and activation and decreased osteoclast apoptosis. RANKL is the final key molecule required for osteoclast development and is normally expressed by bone marrow stromal/osteoblast precursor cells, T and B lymphocytes. RANKL binds to its receptor RANK on osteoclast lineage cells and is neutralized in the bone microenvironment by its soluble decoy receptor osteoprotegerin (OPG), which is produced and secreted by osteoblast lineage cells.

Combined *in vitro* and *in vivo* studies have shown that estrogen normally suppresses RANKL production by osteoblastic cells and T and B lymphocytes and increases OPG production by osteoblastic cells. Thus, estrogen deficiency leads to an alteration in the RANKL/OPG ratio that favors bone resorption (Clarke & Khosla 2010).

Estrogen normally also increases the production of transforming growth factor (TGF)- β by osteoblast precursor cells. TGF- β induces apoptosis of osteoclasts. Estrogen also directly stimulates apoptosis of osteoclast precursor cells and decreases osteoclast precursor differentiation by blocking RANKL/macrophase-colony stimulating factor (M-CSF)-induced activator protein-1-dependent transcription by reducing c-jun activity. C-jun activity is reduced by decreasing c-jun transcription and decreasing phosphorylation. Estrogen is also capable of inhibiting the activity of mature osteoclasts by direct, receptor-mediated mechanisms (Clarke & Khosla 2010).

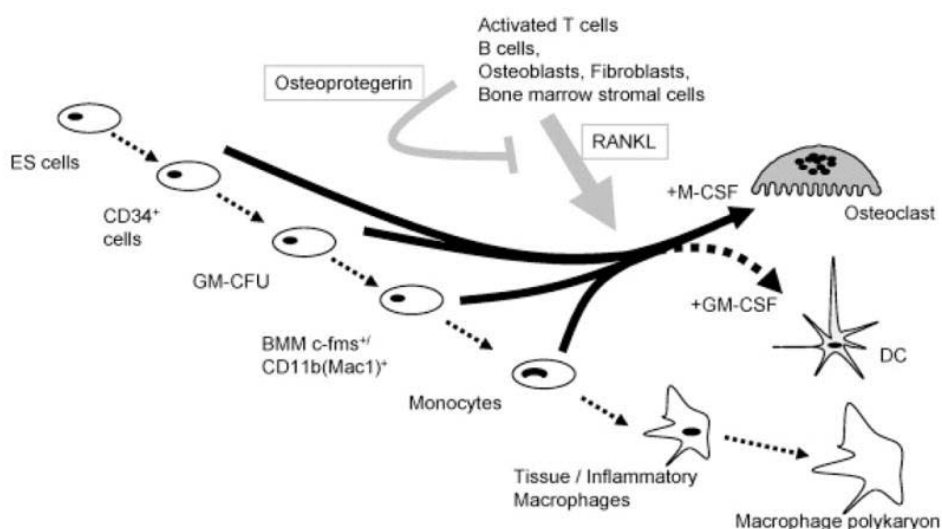


Figure 4. Summary of stimulatory and inhibitory factors involved in osteoclast development and apoptosis. (Reproduced from Quinn & Saleh 2009 with permission).

The skeletal effect of androgens may be partly mediated from local aromatization to estrogen. However, there are also data to support a direct androgen action on bone. The presence of specific androgen receptors in cultured osteoblasts has been reported (Huber et al 2001). Androgens were shown to stimulate proliferation and differentiation of osteoblasts and to inhibit apoptosis. In animal studies, treatment with dihydrotestosterone which cannot be aromatized to estrogen, stimulated bone formation and thus supported a direct stimulatory effect of androgens on bone (Vanderschueren et al 2004).

In women, reduced estrogen levels e.g. following oophorectomy or natural menopause are well-known to increase bone loss, increase the risk of osteoporosis and fracture (Clarke & Khosla 2010; Riggs et al 2002). Oophorectomy also leads to a decline in testosterone levels by one-half, whereas the levels in postmenopausal women with intact ovaries are variable (Hughes et al 1991). Bone resorption increases by 90% after menopause, whereas bone formation also increases but only by 45%, as assessed by markers of bone formation (Garnero et al 1996). This difference favors greater bone resorption, which leads to accelerated bone loss during the first 8-10 years after menopause. Increased bone resorption leads to an efflux of calcium from the skeleton into the extracellular pool. Compensatory increased renal calcium excretion (Young & Nordin 1967), decreased intestinal calcium absorption (Gennari et al 1990), and partially suppressed parathyroid hormone (PTH) secretion (Riggs et al 1998) prevent development of hypercalcemia.

In men, the relation between sex hormone levels, bone loss and risk of osteoporosis is more uncertain. Also, the relative importance of testosterone versus estrogen for the maintenance of BMD in men is unclear. Men with gonadal insufficiency have lower BMD than healthy subjects of the same age and testosterone replacement therapy has been suggested to reverse this condition (Khosla 2006). However, the role of testosterone for BMD in men has also been questioned (Clarke & Khosla 2009; Khosla et al 2001).

Risk factors for low BMD and for osteoporosis in Asian populations have not been well documented, particularly with respect to men. Genetic variations between Asian and Caucasian populations in sex steroid hormone metabolism regulating bone mass have been demonstrated (Jakobsson et al 2006). Furthermore, there may be ethnical differences in gene polymorphisms involved in bone metabolism (Gorai et al 2007).

For decades now, hormone replacement therapy (HRT) has been employed to relieve menopausal symptoms such as hot flashing, sweating and sleep disturbances, as well as for prevention of osteoporosis and related bone fractures in women. However, more recent randomized as well as epidemiological investigations have revealed that HRT of postmenopausal women with estrogen and progestin increases the risk for breast cancer and thrombosis (Santen et al 2010). Consequently, the benefits versus risks of such treatment have been discussed extensively and new guideline proposed (Santen et al 2010). Although the use of HRT has never been widespread in Vietnamese postmenopausal women, alternative therapy for treatment and prevention of osteoporosis and fracture is strongly needed.

1.3.3 Phytoestrogens and bone

Currently there is a growing interest in plant-derived phytoestrogens which may offer a cheap and non-pharmacological alternative for the prevention and treatment of bone loss in postmenopausal women (Albertazzi & Purdie 2002). It is often assumed that Asian populations have a lower rate of osteoporosis because of a high phytoestrogen content in their traditional diet (Adlercreutz & Mazur 1997; Eden 1998). Soy bean based food is an important source of plant protein in Asia including Vietnam. It has been estimated that between 200-300 thousand tons of local soy bean are consumed every year in different soy based products e.g. soymilk, tofu and soy sauce (Huong 2004). While soymilk and tofu are consumed daily by most Vietnamese individuals, the prevalence of osteoporosis in Vietnam is nevertheless still high and comparable to that of many Western populations (I).

Bisphosphonates and selective estrogen receptor modulators (SERMs) e.g. raloxifene or tamoxifen offer alternative treatments. However, these drugs are relatively expensive and their affordability in developing countries, where 1.5 billion elderly (60 years and more) are estimated to be living by 2050 (Mirkin 2001), is limited.

The most important biologically active phytoestrogens (aglycones) recognized are daidzein, glycitein and genistein and their inactive isomers (glycosides) daidzin, glycitin and genistin (Cassidy et al 2006; Song et al 1998). The chemical structure of these isoflavones is similar to that of estrogen (**Fig 5**). They have a higher affinity to estrogen receptor (ER) beta (Kuiper et al 1997) than ER alpha (Makela et al 1999).

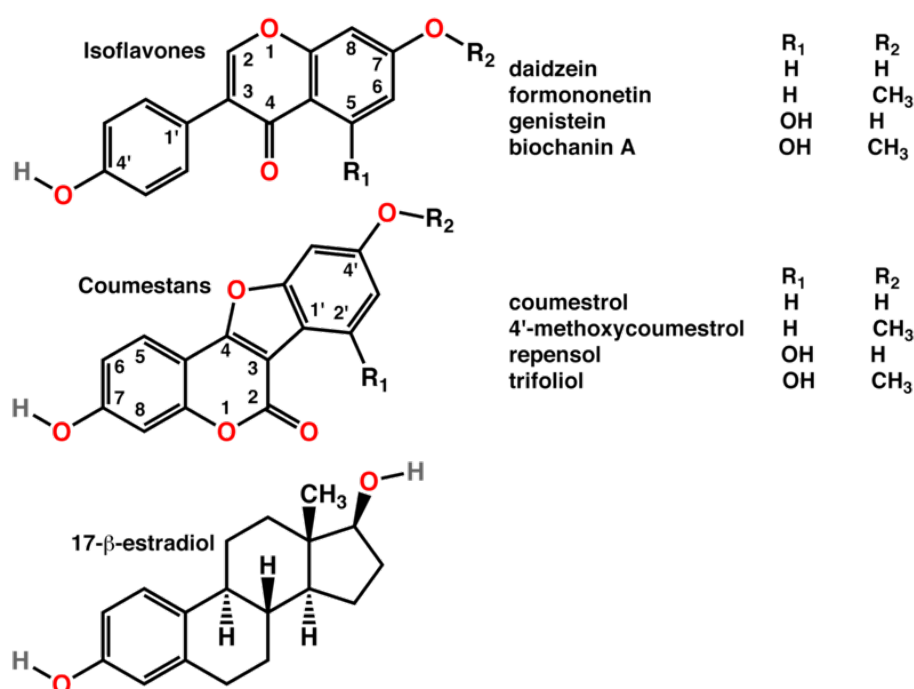


Figure 5. Chemical structures of the most common phytoestrogens found in plants (top and middle) compared with estradiol (bottom).

While many scientific panels have now reached a consensus on the effect of phytoestrogens on cardiovascular disease prevention (Erdman 2000; J.H.C.I 2002; U.S 1999), the same conclusions have not been reached for prevention and treatment of osteoporosis (Branca 2003; Weaver & Cheong 2005).

The effectiveness of soy isoflavones on bone metabolism in postmenopausal women is controversial. In a recent meta-analysis from 9 randomized clinical trials, significant inhibition of bone resorption and stimulation of bone formation were observed (Ma et al 2008b). In contrast, Cheong et al. found no significant effects of isoflavone treatment (Cheong et al 2007).

The minimal threshold intake of dietary phytoestrogen to yield significant effects on bone has been suggested to be between 90 and 120 mg/day of aglycones (Ma et al 2008a; Wong et al 2009). Soymilk in general seems to provide a better pharmacokinetic profile of isoflavones than more solid matrix consumables, because its absorption is rapid with easily achievable high peak concentrations (Cassidy et al 2006). Previous studies found marked differences in isoflavone content among different commercially available soy foods in Australia and in the US (Setchell & Cole 2003). Therefore, it is important to quantify the isoflavone content in different commercial soy products.

1.3.4 Vitamin D and bone

Vitamin D is the name of a group of fat-soluble compounds that are essential for maintaining the appropriate mineral balance in the body. The most important forms of vitamin D are D₂ (ergocalciferol) and D₃ (cholecalciferol) (**Fig 6**). These two forms differ chemically only in their side-chain structure, vitamin D₂ has a side chain that contains a double bond between carbon 22 and 23 and a carbon 24 methyl group.

Vitamin D₃ is produced from 7-dehydrocholesterol in the skin as a result of ultraviolet (UV) irradiation or by digestion of animal products. Vitamin D₂ is formed by UV radiation from the plant sterol ergosterol and humans can obtain vitamin D₂ only from plant products. Calciferol-vitamin D refers to both vitamin D₂ and D₃. Vitamin D is biologically inactive and requires metabolism in the liver on carbon 25 to form the main circulating form of vitamin D, 25-hydroxychole-calciferol or calcidiol [25(OH)D]. The compound 25(OH)D is used to measure vitamin D status. To be activated, 25(OH)D must be converted into 1,25-dihydroxychole-calciferol or calcitriol [1,25(OH)D] (**Fig 6**). Vitamin D-binding protein (VDBP) is the major carrier of vitamin D and its metabolites, 25(OH)D and 1,25(OH)D. The vitamin D receptor (VDR) is the mediator of the biological actions of 1,25(OH)D. Receptor polymorphisms, hormonal status and several other factors influence the possible effects of vitamin D (Holick & Chen 2008).

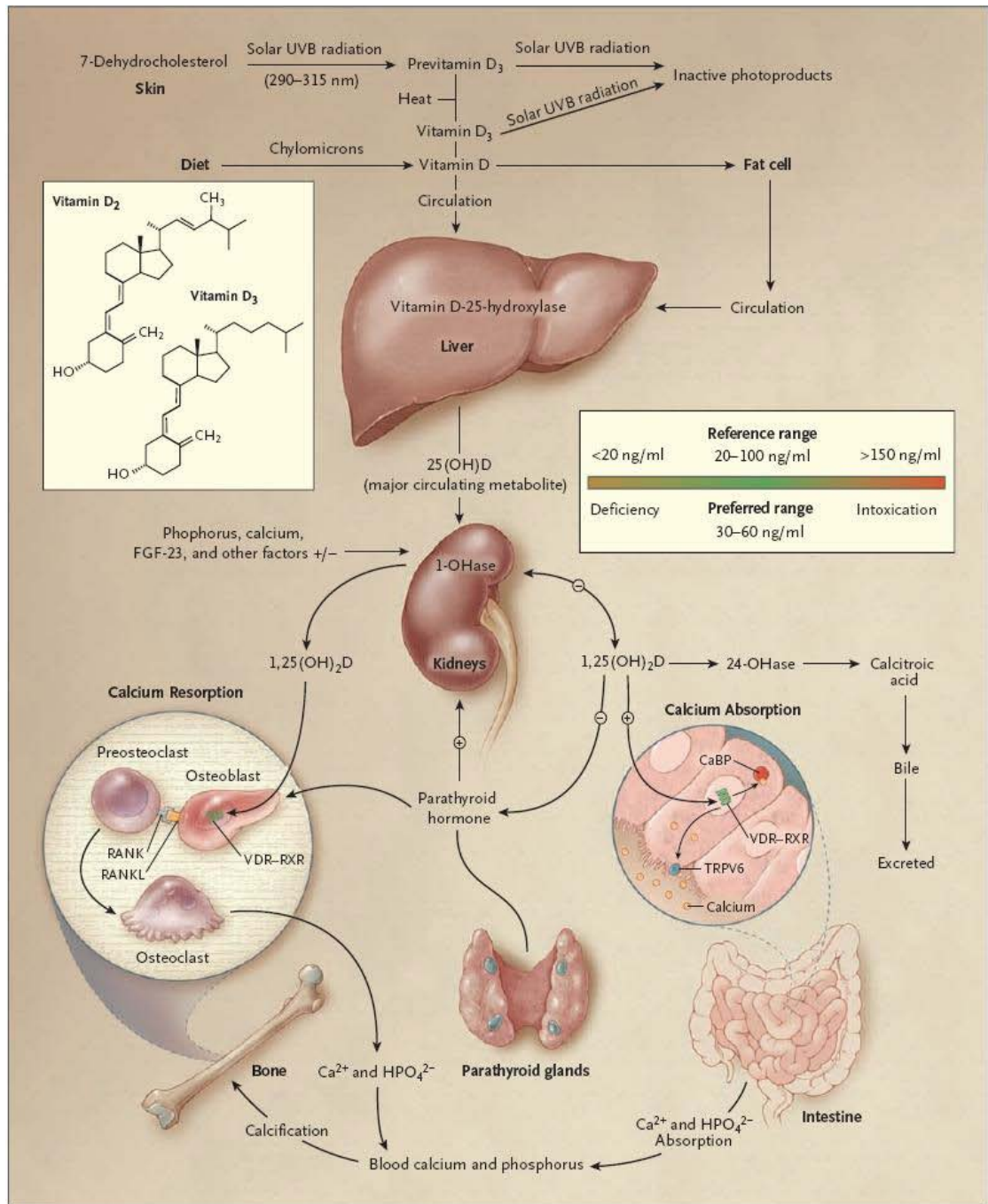


Figure 6. Schematic representation of the synthesis and metabolism of vitamin D for regulating calcium, phosphorus and bone metabolism. (During exposure to sunlight, 7-dehydrocholesterol (7-DHC) in the skin is converted to pre-vitamin D₃ and then by a heat-dependent process to vitamin D₃. Vitamin D made in the skin or ingested from the diet is converted by the vitamin D-25-hydroxylase to [1,25(OH)₂D]. This will increase the 25-hydroxyvitamin D-24-hydroxylase to catabolize 1,25(OH)₂D and 25(OH)D into the water-soluble biologically inactive calcitroic acid. 1,25(OH)₂D will enhance intestinal calcium absorption and it is recognized by its receptor in osteoblasts. After ligand binding, there is an increase in the expression of the activator NF_κB (RANKL).

Vitamin D plays an important role in the regulation of calcium and bone metabolism (Bischoff-Ferrari; Lips 2006; Mason et al). Lack of 25(OH)D is a cause of rickets due to abnormality in bone remodeling. Supplementation of 25(OH)D will reverse the abnormal bone formation (Holick & Chen 2008). Recent studies have also demonstrated the presence of specific receptors in a wide variety of tissues (Haussler et al; Holick 2008; Mason et al), and indicated many important effects of vitamin D besides bone health (Bischoff-Ferrari; Holick 2008; Mason et al).

Indeed, vitamin D deficiency has been associated with certain forms of cancer (Ahearn et al; Holick 2004; 2008; Norton & O'Connell), type II diabetes, cardiovascular disorders (Forman et al 2007; Giovannucci et al 2008; Lee et al 2008; Witham et al), autoimmune and infectious diseases (Cutolo & Otsa 2008; Liu et al 2006; Martineau et al; Munger et al 2004). A recent meta-analysis found a marked increase in the risk of mortality among vitamin D deficient individuals as compared to those with normal 25(OH)D levels (Zittermann et al).

Although there is no consensus on a definition of vitamin D deficiency, it has been generally agreed that measurement of 25(OH)D should be used as an indicator of an individual's vitamin D status (Holick & Chen 2008; Lips). Serum 25(OH)D levels below 20 ng/mL are considered to be "deficiency" (Sai et al 2011). Using this criterion, studies on vitamin D status in different populations have shown considerable variation in the prevalence of vitamin D deficiency (Mithal et al 2009). The common trend in all studies is that populations in temperate regions have higher prevalence of vitamin D deficiency than populations in tropical regions (Lips; Mithal et al 2009), which indicates an effect of sunlight exposure on vitamin D variation (**Fig 7**).

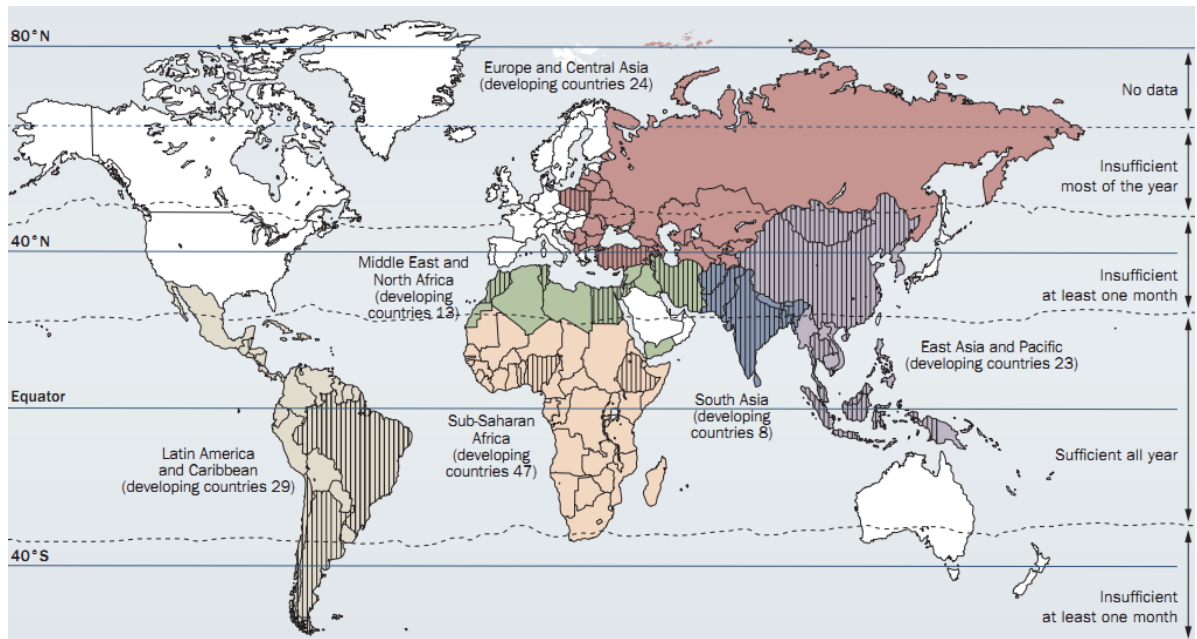


Figure 7. World map of geographic regions to skin ability to synthesize vitamin

D. Developed countries are shown in white. Information on the prevalence of hypovitaminosis D, risk factors and effect on health was available from 23 developing countries that are indicated as shaded areas on the map. Reproduced with permission from (Tavera-Mendoza & White 2007).

Approximately 90% of vitamin D is synthesized in the skin after sunlight exposure (Holick 2006; Holick & Chen 2008). A small amount of vitamin D can be absorbed through food intake (Holick 2007). It is therefore assumed that people living in countries with high amounts of sunlight may have a lower risk of vitamin D deficiency. However, recent studies in tropical countries have indicated that the prevalence of vitamin D deficiency still could be as high as that observed in Western populations (Mithal et al 2009). Melanin is extremely efficient in absorbing UVB radiation and thus, increased skin pigmentation markedly reduces vitamin D synthesis (Clemens et al 1982; Holick & Chen 2008; Springbett et al). Reports from Hong Kong (Wat et al 2007), Malaysia and Singapore (Hawkins 2009) have indicated that between 60% and 100% of the population had vitamin D levels below 30 ng/mL, a level that is considered “insufficient”. However, these studies were conducted on urban residents who may have a lower level of sunlight exposure than rural residents. Moreover, 25(OH)D production is known to be affected by seasonal variation (Karohl et al; Snellman et al 2009) which was not taken into account in these studies. Therefore, it is important to assess the

prevalence of and risk factors for vitamin D deficiency in a representative sample women and men in urban and rural settings.

1.3.5 Coffee, smoking, alcohol and bone

Caffeine is known to increase calcium excretion (Heaney & Recker 1982; Massey & Whiting 1993) and to reduce intestinal calcium absorption (Barger-Lux et al 1995), with a net loss of approximately 5 mg calcium per cup of coffee (Heaney & Recker 1982). Animal studies suggest a delay of the osteogenic process in rats treated with coffee (Lacerda et al 2010). In a recent study, Hallström et al. found high consumption of coffee to reduce BMD of the femoral neck in elderly men (Hallstrom et al 2010). However, results from epidemiological studies investigating the relation between coffee consumption and BMD in both men and women have been conflicting (Atalar et al 2009; Cauley et al 2005; Glynn et al 1995; Hannan et al 2000; Johansson et al 1992; Patel et al 2003; Reyes et al 2004; Ruffing et al 2006).

Cigarette smoking was identified as a risk factor for osteoporosis more than 20 years ago. Studies show a direct relationship between tobacco use and decreased bone density. In addition, most studies suggest that smoking increases the risk of having a fracture. However, the interpretation of data is complex as smoking is associated also with other life style factors e.g. alcohol and dietary habits and also with reduced estrogen levels and early menopause in women (Maurel et al).

The influence of *alcohol* on bone is controversial and the effect seems to be dose-dependent. Light alcohol consumption (1-10 g of ethanol per day) has been suggested to have positive effects whereas heavy consumption (30 g of ethanol per day) may reduce bone mass due to a bone remodeling imbalance, with a predominant decrease in bone formation. Alcohol seems to increase osteocyte apoptosis and oxidative stress, and also to modulate the Wnt signaling pathway. Moreover, in heavy drinkers reduced total fat mass, increased lipid content in the bone marrow and eventual hypo-leptinemia could be confounding factors.

1.4 VIETNAM – AN ASIAN COUNTRY IN RAPID DEVELOPMENT



Figure 8. Map of Vietnam

Vietnam is a developing country situated in the South-East Asia with a rapid increase in population. From 24 millions in 1945, it increased to 57 millions in 1975 (Vietnam historical demographic data of the whole country) and up to 90 million in 2011. Vietnam's population today is 9 times more than Sweden's, while the area of Vietnam is just about two-thirds in comparison to this Nordic country. The population of Vietnam is large enough to make it the second most populous country in the South-East Asia, the seventh in the Asia-Pacific region and the 12th most populous in the world (General Statistics Office of Vietnam, 2012).

Vietnam has had a long history of wars from a millennium of the Chinese dynasty era to many years under French colonization, and thereafter the Vietnam war against the USA. The French administration imposed significant political and cultural changes on the Vietnamese society. A Western-style system of modern education was developed and Roman Catholicism was widely propagated within the society. Vietnam became independent in 1945, and was really united in 1975 after decades of war hardship and poverty.

Since 1986, Vietnam follows the "Doi moi" policy which is a revolution strategy to promote the transition from a planned economy into a "socialist-oriented market economy". Private ownership was encouraged in industries, commerce and agriculture (Vuong et al, 2009). Thanks largely to these reforms, Vietnam achieved an about 8% annual GDP growth between 1990-1997 and the economy has continued to

grow at an annual rate of around 7% from 2000-2005, making Vietnam one of the world's fastest growing economies. According to a 2008 prognosis by PricewaterhouseCoopers, Vietnam may be the fastest growing of the world's emerging economies by 2025 (Vietfinancenews.com. 29 December 2010).

During the last 50 years, the inhabitants' average life span has increased by 33 years from only 40 years in 1960. In comparison the world average life span has increased by 21 years. The infant mortality has rapidly declined from 157 in 1950 to 70 in 1975 and down to 20 per 1,000 live births in 2011 (UN, 2011).

Vietnam is located between the latitudes 8° and 24°N, and the longitudes 102° and 110°E. The differences in latitude cause a marked variation of the climate. In northern Vietnam, the seasonal variations between winter/dry season (November to April) and summer/rainy season (May-October) are more pronounced than in the southern part of the country.

Osteoporosis is often considered as a consequence of industrialization, because the incidence of osteoporotic fractures is higher in industrialized countries than in developing countries (Lau et al 2001). Furthermore, within a country, the incidence of fractures is higher in urban than in rural communities (Chevalley et al 2002; Madhok et al 1993; Mannius et al 1987; Sanders et al 2002). Clearly, studies on osteoporosis in emerging developing countries are important to provide more information on the evolution of the disease.

Due to recent economic development, Vietnam has undergone rapid urbanization and there is a clear separation between urban and rural areas. During this process, differences in life styles, dietary and working habits and also exposure to polluted air and environment have gradually developed between citizens living in different settings. Although osteoporosis is increasingly recognized as a major public health problem, there is currently a lack of knowledge about its prevalence. Furthermore, reference data on peak bone mass and risk factors to allow screening in these specific populations are not available.

Capacity building through international collaborations is one of the key strategies to drive Vietnam to a better future

One of the important keys to drive Vietnam to a better future is to actively engage not only in international economic integration but also to expand international cooperation in other fields including research and education (Ministry of

Foreign Affairs, 2009). In line with national strategies, the KIRT (Karolinska International Research Training) program between Karolinska Institutet (KI) and Hanoi Medical University (HMU) started in the year 2000. This program was entitled “pathogenesis, diagnosis, epidemiology and treatment of common diseases in Vietnam”. The aims of the program were to increase the research capacity in Vietnam, to enhance knowledge transfer and to update biomedical and clinical research in some selected fields of public health importance and mutual interest. The program was financed by Sida, both in Vietnam and in Sweden.

This project within the KIRT program started in 2003. The research education has been carried out both in Vietnam and in Sweden through joint Vietnamese and Swedish supervision. All the field work was done in Vietnam. The experimental work was done in Sweden, where standardized methods and experienced supervisors would assure reliable results based on the data.

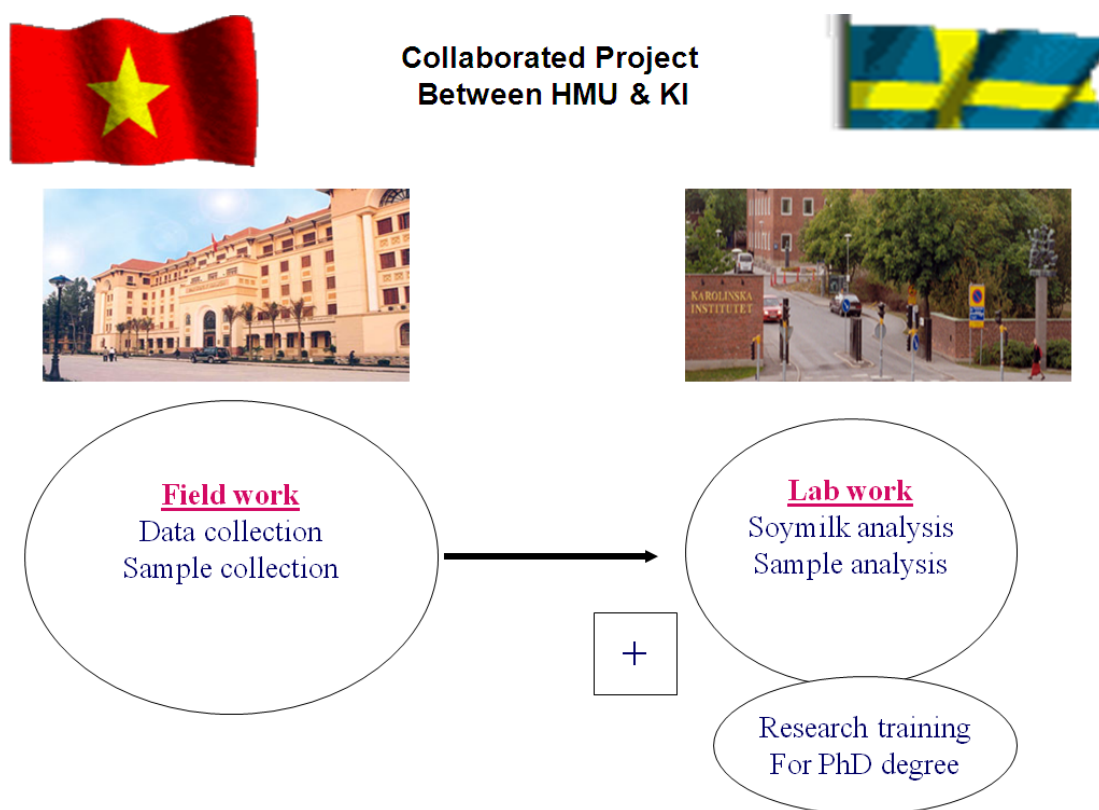


Figure 9. The KIRT collaboration started in 2000

The PhD student has been the key person in every step of the project development from the first design, planning to implementing and monitoring. The PhD student has learned not only in terms of knowledge and skills in the research field but also in other capacities such as to cooperate, coordinates and collaborate with other health care professionals and institutions in two different environments (Vietnam and Sweden). During the research project work, the PhD student has formed her own research student group. This team, named as “FSH” (Friendship and Science for Health), has over the years held regular scientific seminars and discussions of research theory, research methods and health problems for the society. Several of these “scientific children” of hers have now become master and PhD students of their own and some currently work with their own projects in e.g. France, Holland, USA and also here at KI in Sweden.



Figure 10. Friendship and Science for Health (FSH) research group

2 AIMS

The overall aims of this Thesis were to clarify to what extent osteoporosis could be regarded as a major public health problem in Vietnam. Furthermore, to elucidate the prevalence of certain risk factors and determinants of bone mass as a basis to indentify high-risk individuals among Vietnamese women and men.

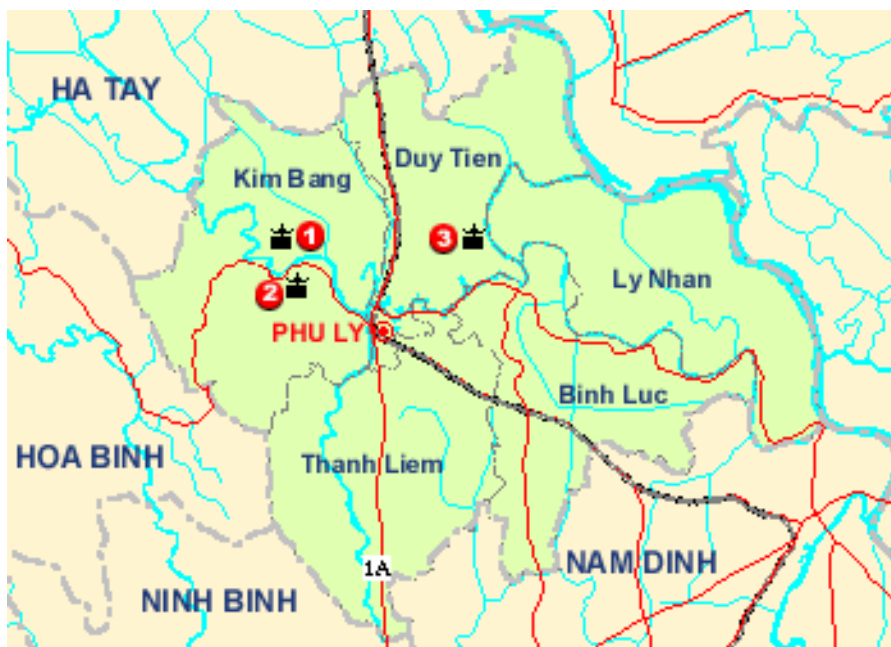
Specific aims

- To determine peak bone mass in order to estimate the prevalence of osteoporosis in Vietnamese women and men.
- To explore possible differences between urban and rural residents with respect to risk factors for low bone mineral density and osteoporosis.
- To investigate the prevalence of vitamin D deficiency and the associations of this risk factor with bone mineral density in samples of Vietnamese women and men.
- To measure sex steroid hormone levels in relation to bone mass among Vietnamese women and men.
- To quantify the aglycone content in different soymilk preparations and to evaluate its potential for osteoporosis prevention.

3 MATERIAL AND METHODS



Hanoi map



Hanam map

Figure 11. Studied sites: clinical materials were collected (**I**) from Dong Da and Soc Son in Hanoi (top panel) and (**II & III**) from Dong Da in Hanoi and Kim Bang in Hanam (bottom panel).

3.1 SUBJECTS AND SAMPLES (I, II, III)

The clinical studies were designed as cross-sectional investigations using a multistage sampling scheme. Within the setting of northern Vietnam (latitude 21°N), for study **I**, two districts in Hanoi (Dong Da & Soc Son) and for studies **II** and **III**, two districts (Dong Da in Hanoi and Kim Bang in Hanam) were selected to represent urban and rural areas, respectively. From each of these districts, 4 communes were randomly selected, and a full list of all inhabitants was obtained from the local government authority, which served as the sampling frame. The lists of inhabitants were then sorted by age in 10-year groups. For each age group, a total of 100 women (**I**) and a total of 140 individuals (70 women, 70 men) (**II** & **III**) were randomly selected by a computer-generated numbers and invited for screening interview.

In **paper I**, we assumed that the peak BMD was 1 g/cm² with a standard deviation of 0.1 g/cm², then the required sample size was about 120-150 women aged between 10 and 49 years. In order to estimate the prevalence of osteoporosis in women, we aimed to recruit at least 400 individuals. Among the 400 women randomly selected, 373 women (response rate of 93%) agreed to participate in the study. However, after excluding women who did not meet the study entry criteria, only 168 women were finally included in the study. A further random sample of women aged between 50 and 65 from the same communes were invited to participate in the study for estimating the prevalence of osteoporosis. In this sampling scheme, women aged 50 and 65 were randomly selected from all communes. Initially 386 women agreed to participate in the study; however, only 160 women met all the inclusion and exclusion criteria. In total, 343 women aged between 10 and 65 were eligible for participating in the study.

For **paper II**, based on published literature (Holick 2007; Mithal et al 2009), where the prevalence of vitamin D deficiency in the world populations ranged between 30-50%, we estimated that a sample size of 170 individuals would be adequate to calculate the prevalence within 8 percentage points of the true proportion with a 95% confidence.

For **paper III**, based on published literature (Melton et al 1992), where the prevalence of osteoporosis in men ranged between 10-20%, we estimated that a sample size of 170 individuals would be adequate to calculate the prevalence within 6 percentage points of the true proportion with a 95% confidence interval. In a subsample of at least 100 individuals (men ~~on~~ 50 years of age) the corresponding figure was 8 percent.

For **paper II & III**, a letter of invitation was sent to a total of 980 individuals, among whom 823 came for the screening. Screening interviews were performed at the local healthcare center by health professionals from Hanoi Medical University, and participants were offered a free health check-up. After screening, a total of 604 individuals fulfilled the inclusion criteria, and among them 559 ultimately participated in the study.

Exclusion criteria. Women and men were excluded from the study (**I, II and III**) if they had conditions that were deemed to affect bone metabolism such as chronic diseases (dysfunctions of liver, gut, kidney, endocrine system, respiratory or cardiovascular system, auto-immune diseases and cancer), or use of medications, or more than one month in bed, or premature menopause or hysterectomy/oophorectomy, or history of fracture.

3.2 ETHICAL ASPECTS

The research protocol and procedures were approved by the ethics council of Hanoi Medical University (No. 05/IRB & No.97/HMU IRB). All participants were provided with adequate information about the objectives of the study and had given their oral informed consent to participate, according to the principles of medical ethics of the World Health Organization.

3.3 DATA COLLECTION (I, II, III)

All participants were subject to individual interviews carried out at the National Cancer Hospital (**I**) or the Bach Mai hospital (**II & III**) by health professionals from Hanoi Medical University. Data were collected on age, clinical history, lifestyle, dietary habits, smoking, alcohol and coffee intake. Height without shoes (in centimeters) was measured by a wallmounted stadiometer. Weight, without shoes or clothing, was measured on an electronic scale. Body mass index (BMI) was then derived as the ratio of weight (kg) over height squared (in m²).

3.3.1 Bone mineral density measurement

BMD was measured at the lumbar spine (LS), left and right femoral neck (FN) and total hip (TH) in all qualified subjects. The measurements were done with a dual energy X-ray absorptiometry (DXA) densitometer (GE Lunar Prodigy advance (**I**) and Hologic Explore 4500 (**II & III**)). The precision error (%CV) in our laboratory for both

machines was 1.8% for lumbar spine and 1.5% for hips. The machines were standardized by standard phantom every time before measurement. In these analysis, BMD at the lumbar spine was estimated from L2 - L4 (I) or L1-L4 (II & III). Femoral neck and total hip BMD used in the analysis were estimated from the right side.



Figure 12. DXA densitometer (GE Lunar Prodigy Advance)

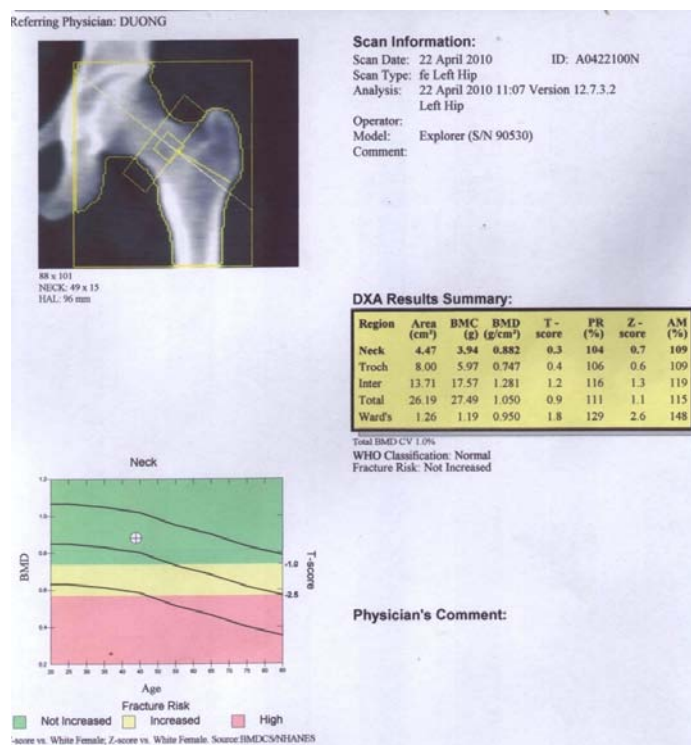


Figure 13. Result report of DXA measurement

In order to estimate **peak BMD**, a series of polynomial regression models (up to the third degree) were fitted to femoral neck, total hip and lumbar spine BMD as a function of age. In these models, the expected value of BMD at each skeletal site was expressed as follows: $BMD = \alpha + \beta_1(\text{age}) + \beta_2(\text{age})^2 + \beta_3(\text{age})^3$, where α is the intercept, β_1 , β_2 , and β_3 are regression parameters to be estimated from the observed data. Reduced models (i.e., quadratic and linear models) were also considered, and the “final” model was chosen based on the Akaike Information Criterion (AIC) (Akaike 1973). Peak BMD and age at peak BMD were estimated from the final model. The 95% confidence interval of peak BMD and age at peak BMD were determined by the bootstrap (resampling) method (Efron B & Tibshirani R 1986). In this method of analysis, 1000 repeated samples, each with 300 individuals, were drawn from the original material. The cubic equation was fitted to each sample, and the 95% confidence of the 1000 values for peak BMD and age at peak BMD were then determined. The analysis was performed with R environment (Carlberg & Molnar 2012) and the Design library (Harrell et al 1996).

To classify **osteoporosis**, from the peak BMD, a T-score, which is the number of standard deviations from the peak BMD, was calculated for each individual in the study sample. The prevalence of osteoporosis was estimated for women aged 50+ (**I**), menopausal women and men aged 50+ (**II & III**). An individual was classified as having osteoporosis if her/his femoral neck BMD T-score was equal or lower than -2.5 (Kanis & Gluer 2000). Standard error and 95% confidence intervals of the prevalence were estimated based on the assumption of binomial distribution.

3.3.2 Serum analyses

Blood samples were drawn in the fasting condition in the morning and centrifuged within 30 minutes after collection. Serum samples were frozen at -80°C until analysis. Serum concentrations of 25(OH)D, PTH, estradiol-17 β and testosterone were measured by electrochemiluminescence immunoassay (ECLIA, Roche diagnosis). The measuring range was from 4 to 100 ng/mL (10-250 nmol/L) and from 1.2 to 5000 pg/mL (0.127-530 pmol/L) for 25(OH)D and PTH, respectively. The intra-assay coefficient variation (CV) was 5.6% for 25(OH)D and 11.62% for PTH. The inter-assay CVs were 9.9% and 11.9%, respectively. The intra-assay coefficient variation (CV) was 5.2% for

estradiol and 5.7% for testosterone. The inter-assay CVs were 9.2% and 9.9%, respectively.

3.4 DATA ANALYSIS (I, II, III)

Characteristics of the participants were presented as mean and standard deviation or median and range. For categorical data frequency counts and percentages were used. In comparisons between men and women, with respect to the characteristics of the participants, the **t test** for independent samples was used for continuous data and the **Chi-square test** for data measured on a nominal scale (**II & III**).

For **paper II**, to find the most important factors predicting vitamin D deficiency (<20 ng/mL) both univariate and stepwise **logistic regression** analyses were performed. All regression analyses were done separately for men and women. The predictors were age classes, <30, 30-49, 50-59 and >60 years, height, weight, BMI, residency, season, smoking, alcohol- and coffee drinking and also for women contraceptive pill use. The results from the logistic regression were presented as odds ratio (OR) and 95% confidence intervals (CI). Furthermore, univariate and forward stepwise multiple **linear regression** analysis was used to evaluate to what extent the variation in different BMD measures could be explained by Vitamin D, PTH, age, height, weight, BMI, residency, smoking, alcohol- and coffee drinking and also for women contraceptive pill use. The results from the stepwise regression models were presented as unstandardized regression coefficient, 95% CI and R^2 . A p-value <0.05 was considered statistically significant.

For **paper III**, to find the most important factors predicting osteoporosis (T-score less than or equal to -2.5) both univariate and stepwise multinomial **logistic regression** analyses were performed (osteoporosis versus normal). The predictors were age, height, weight, BMI, residency, smoking, alcohol- and coffee drinking, levels of estradiol and testosterone. The results from the logistic regression were presented as odds ratio (OR) and 95% confidence interval (CI). Furthermore, univariate and forward stepwise multiple **linear regression** analysis was used to evaluate to what extent the variation in different BMD measures could be explained by estradiol, testosterone, age, height, weight, BMI, residency, smoking, alcohol- and coffee drinking. The results from the stepwise regression models were presented as the unstandardized regression coefficient, 95% confidence interval (CI) and R^2 . A p value <0.05 was considered statistically significant.

3.5 EXPERIMENTAL STUDY (IV)

3.5.1 Samples

Commercial soymilk products: Six different brands of soy-milk drinks commercially available in Vietnam (VN1-Vinamilk[®]; VN2- Vinasoy[®]; VN3-Unisoy[®]) and Sweden (S1-Provalmel[®]; S2-Gogreen[®]; S3-Alpro[®]) were purchased from local supermarkets. According to package labeling, all products were manufactured within the last 30 days. In Vietnamese soymilk, the protein and total fat content ranged 1.5-2.6 g/100 ml and 0.8-1.5 g/100 ml, respectively. The corresponding values for Swedish soymilk were 3.0-3.7 g/100 ml and 1.9-2.2 g/100 ml.

“Home-made” soymilk prepared at the laboratory: Soymilk was prepared from beans of different origin R1-2 (US); R3 (UK) and R4 (Vietnam). 100 g of whole dry soybeans and 1000 mL of tap water were mixed in a common soymilk homogenizer (Soja-queen, Miko, GmbH, Frankfurt, Germany). The original processing time for homogenizing and heating at 80°C was 18 min (R1). For preparations R2-4, heating was prolonged until 3 hours. Each soymilk preparation was freeze-dry in 10 ml aliquots until extraction.



Figure 14. 10 different preparations of soymilk were analyzed by HPLC

3.5.2 Methods

Isoflavone extraction from soymilk: Frozen dried samples from 20 ml soymilk were used for extraction as described by Griffith et al, (2001) and Apers et al, (2004); with modification. Isoflavone calculation was based on 10 ml of soymilk. Samples were extracted in 50 mL of 80% methanol heated at 80⁰C and simultaneously shaken at 100 rpm in water bath (Heto, Denmark) for 60 minutes.

All samples were immediately filtered (Munktell's Swedish paper No 8) into disposable tubes, and centrifuged at 300 x g for 20 minutes. The mobile phase was evaporated under a steam of nitrogen at 55-60⁰C to complete dryness. Dried samples were kept at -20⁰C until analysis by reverse phase high-pressure liquid chromatography (HPLC).

Prior to injecting into the column, sample extracts were re-suspended in 1mL of acetonitril 16%, vortex-mix and centrifuged at 13000 x g for 20 min. 500 µl of supernatant of each sample was transferred into a clean HPLC vial and analysed for their aglycone and glycoside concentrations.

HPLC quantification of isoflavones: HPLC was carried out on a 4 mm x 150 mm C18 reverse phase column (Reposil-pur Column C18-AQ), 5 mm packing (150 mm x 4 mm ID, 70306-21004, R15.AQ. S1504, Ammerbuch) with an auto sampler (Basic Marathon type 816, Netherlands) to inject 50 µl of sample and flow rate of 0.8 mL/min. Mobile phase was prepared as follows: Solvent A, 0.1% (V/V) acetic acid in water; Solvent B, 0.1% (V/V) acetic acid in 90% HPLC-grade Acetonitrile. The solvents were filtered through a 0.45 µm filter. The gradient was started immediately upon injection. The gradient elution program was:

Time (min)	A%	B%
0	95	5
3	95	5
4	80	20
6	80	20
10	70	30
12	70	30
13	65	35
15	65	35
20	60	40
30	50	50
32	95	5
33	95	5
36	95	5

Total sample to sample time was 36 min for this method. Detection was by UV absorbance at 250 nm. The peak areas were integrated for quantization by CSW32 chromatographic station program for windows.

After injection of 50 μ L standards and extract samples, elution was monitored at 250 nm, and recorded over the time of run on all samples. The retention times (min) of our standards were as follow: Daidzin (12.18); Glycitin (12.44); Genistin (14.22); Daidzein (18.74); Glycitein (19.13); and Genistein (24.23) (Figure 1). Isoflavones were identified by comparing spectral data retention times to those of standard references.

Isoflavones standards: Daidzin ($C_{21}H_{20}O_9$) from Fluka; Daidzein ($C_{15}H_{10}O_4$), Genistin ($C_{21}H_{20}O_{10}$), Genistein ($C_{15}H_{10}O_5$), Glycitein ($C_{16}H_{12}O_5$) from Sigma; Glycitin ($C_{22}H_{22}O_{10}$) from Fisher; The internal standards Formononetin from Fuka. All these standards were purchased from Sigma-Aldrich Fine Chemicals, St.Louis, MO, USA.

Chemicals and Reagents: Acetonitrile, methanol, dimethylsulfoxid (DMSO), acetic acid (HPLC grade), and filters from Sigma-Aldrich Fine Chemicals, St.Louis, MO, USA, Milipore water were used for all mobile phases.

Calculation of isoflavone levels: The amount of isoflavone was calculated from the sample peak area calibrated against the peak area of standards (Figure. 1). Formononetin was used as an internal standard. The total isoflavone content was calculated as the sum of 6 isoflavones (daidzin, daidzein, glycitin, glycitein, genistin and genistein). All data were mean values from duplicate measurements. The intra-assay variation (n=35) was 2.1% and the inter-assay variation (n=35) was 3.1%.

The theoretical maximum yield of aglycone after total transformation of its glycoside was calculated as: amount of glycoside (mg/L) / glycoside MW x aglycone MW.

3.5.3 Statistical analysis

All data were expressed as mean from duplicate measurements. Differences between products from Vietnam, Sweden and “home-made” soy drink were analyzed by Kruskal-Wallis ANOVA followed by Mann-Whitney Utest. Correlations were assessed using Spearman's rankorder correlation. A p -value < 0.05 was considered statistically significant.

4 RESULTS

4.1 CHARACTERISTICS OF PARTICIPANTS (I, II & III)

In total, 328 women aged between 10 and 65 years were enrolled into study I. Out of these 328 women, 49% were aged 50 years or older. Mean values for weight and height of the different age groups (**Table 1**) were similar to the normal range as reported for Vietnamese women (Le 2003). BMD measurement at each site (LS, TH, FN) was significantly and independently correlated with height ($r = 0.42$; $p < 0.0001$) and weight ($r = 0.46$; $p < 0.0001$). Lumbar spine BMD was significantly correlated with total hip BMD ($r = 0.75$; $p < 0.0001$), which was in turn correlated with femoral neck BMD ($r = 0.94$; $p < 0.0001$).

Table 1. Characteristics of study subjects (I)

Age group	<20 (n=58)	20-29 (n=36)	30-39 (n=37)	40-49 (n=37)	50-59 (n=136)	60-65 (n=24)
Age (yr)	13.8±2.8	23.8 ± 2.9	34.1±2.4	32.6±2.8	55.4±2.5	60.8±1.4
Weight (kg)	38.8± 9.2	46.1±4.9	48.8±5.8	50.6±7.2	44.4±6.7	44.3±8.4
Height (cm)	146.6± 9.2	152.1±4.8	152.1±5.6	152.6± 5.8	148.7± 4.8	148.9±4.1
BMI (kg/cm ²)	17.8± 2.8	20.0 ±1.8	21.1±2.2	21.7±2.5	20.1±2.6	20.0±4.2
<u>BMD (g/cm²)</u>						
Femoral neck	0.81±0.13	0.9 ±0.11	0.89±0.12	0.89±0.12	0.73±0.10	0.69±0.06
Total hip	0.87±0.15	1.00±0.10	0.98±0.12	1.00± 0.13	0.84±0.11	0.78±0.08
Lumbar spine	0.93±0.16	1.11±0.12	1.12±0.17	1.08± 0.15	0.85±0.12	0.80±0.13

*Values shown are mean and standard deviation

Studies **II & III** involved 222 men and 269 women aged between 13 and 83 years (**Table 2**). There were no differences between men and women in terms of age and BMI. As expected, men had greater height and weight than women. The prevalence of smoking, alcohol and coffee consumption was several times higher in men than in women.

Table 2. Characteristics of participants (II & III)

Variables	Reproductive Women	Menopausal Women	Men
N	118	151	222
Age (yr)	34 (13-53)	59 (44-83)	49 (14-83)
Weight (kg)	48 (32-65)	49 (34-68)	53 (35-85)
Height (cm)	155 (143-166)	152 (134-168)	164 (148-181)
Body mass index (kg/m ²)	20 (14-26)	21 (15-30)	20 (15-31)
Current smoking*	0 (0%)	1 (1%)	96 (43%)
Alcohol drinking*	4 (3%)	7 (5%)	124 (56%)
Coffee drinking*	14 (12%)	7 (5%)	46 (21%)
Number of Children	2 (0-5)	4.0 (0-10)	na
Breast feeding*	81 (70%)	142 (94%)	na
Oral contraceptive use*	16 (14%)	-	na
Fracture history*	8 (7%)	10 (7%)	10 (5%)
Estradiol (pg/mL) ^{a,b}	128.2 (95.9)	45.1 (66.9)	42.8 (12.46)
Testosterone (ng/mL) ^{a,b,c}	0.45 (0.77)	0.24 (0.74)	6.2 (2.25)
BMD, femoral neck (g/cm ²) ^{a,c}	0.77 (0.09)	0.63 (0.09)	0.76 (0.12)
BMD, total hip (g/cm ²) ^{a,c}	0.84 (0.09)	0.73 (0.10)	0.85 (0.12)
BMD, lumbar spine (g/cm ²) ^{a,c}	0.93 (0.10)	0.75 (0.13)	0.90 (0.14)

For age, weight, height, and BMI, values are given as median and range (in brackets), for estradiol, testosterone, and BMD, values are given as mean and standard deviation (in brackets) and for *categorical variables, values are given as number and percentage (in brackets).

^a*p*<0.05 between reproductive and menopausal women. ^b*p*<0.05 between reproductive women and men. ^c*p*<0.05 between menopausal women and men

4.2 PEAK BONE MINERAL DENSITY IN VIETNAMESE WOMEN AND MEN

4.2.1 Reference curve for bone mineral density in Vietnamese women (I & II) and men (II)

In women, as expected, BMD at the lumbar spine, total hip and femoral neck rapidly increased during the adolescence and then gradually declined after the age of 40 (**Fig 15 & 16**). The equations that best described this trend were a third degree polynomial function of age as follows:

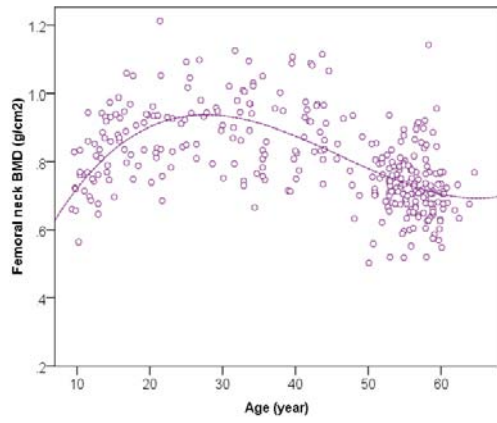
$$\text{Femoral neck BMD} = 0.3353 + 0.0508 \times \text{Age} - 0.0013 \times \text{Age}^2 + 0.000009 \times \text{Age}^3$$

$$\text{Total hip BMD} = 0.3582 + 0.0532 \times \text{Age} - 0.0013 \times \text{Age}^2 + 0.000009 \times \text{Age}^3$$

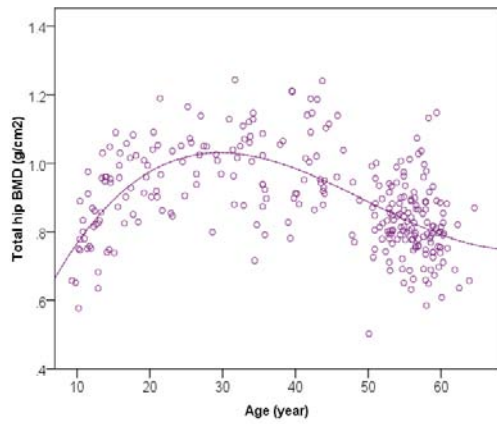
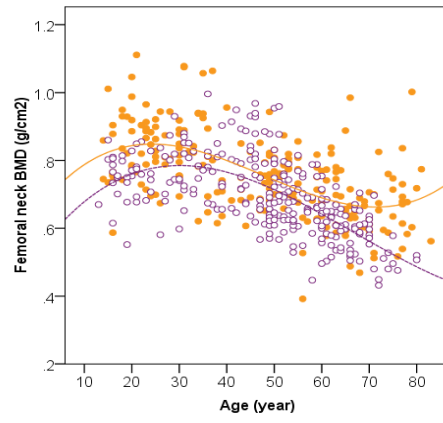
$$\text{Lumbar spine BMD} = 0.1236 + 0.0855 \times \text{Age} - 0.0022 \times \text{Age}^2 + 0.0000155 \times \text{Age}^3$$

These equations accounted for 36%, 30% and 47% of the variation at the femoral neck BMD, total hip and lumbar spine, respectively. The regression coefficients suggest that the increase in BMD during the adolescence was greater at the lumbar spine than at the hip (0.085 vs 0.05 g/cm²); however, the subsequent decrease in BMD was also greater at the lumbar spine than at the hip (0.0022 vs 0.0013 g/cm²). For any given site, BMD among those aged 50-65 years was reduced between 20% and 28% as compared to the peak BMD level.

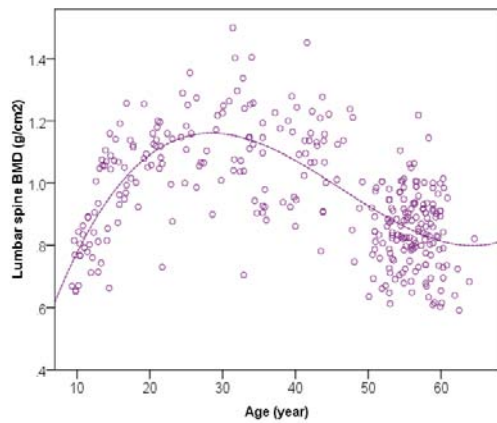
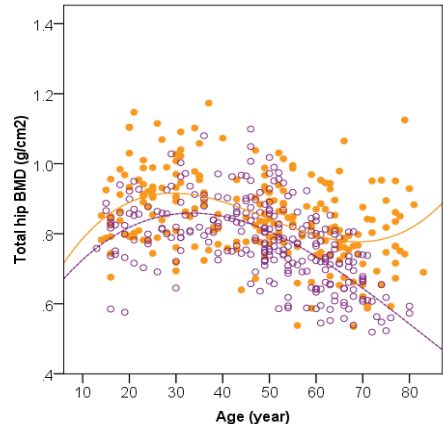
The age-related decline in BMD in women was greater than that in men. For example, for the lumbar spine among women aged 70+ years there was a decrease of about 30%; but in men, the corresponding rate of decrease was only around 15%. A similar sex-differentiated decline was also observed in femoral neck BMD.



(a)



(b)



(c)

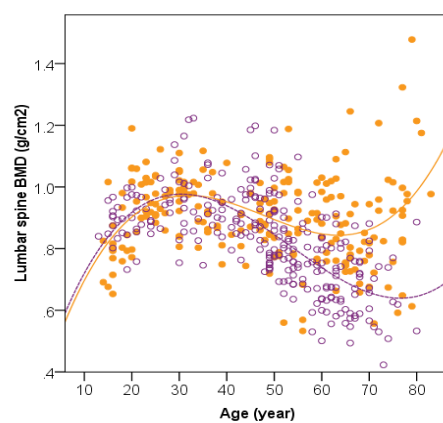


Figure 15. Relationship between age and BMD at femoral neck (a) total hip (b) and lumbar spine (c), in *Vietnamese women*. (I; Lunar Prodigy Advance)

Figure 16. Relationship between age and BMD at femoral neck (a), total hip (b) and lumbar spine (c) in *Vietnamese women* (violet circle and line) and *men* (orange dot and line). (II & III; Hologic Explorer)

4.2.2 Peak bone mineral density in Vietnamese women (I & II) and men (II)

Estimates of peak BMD (pBMD) and the age when pBMD was reached (**Table 3**) were based on a bootstrap analysis. In women, the pBMD values as measured by Lunar prodigy advance (**I**) for the lumbar spine and total hip were 1.16 g/cm² (SD 0.13) and 1.02 g/cm² (SD 0.12), respectively. The age at pBMD was around 29 years. The corresponding value for pBMD at femoral neck was slightly lower [0.94 g/cm² (SD 0.11)] and the age at pBMD was around 28 years.

A similar trend was also observed for BMD data measured by Hologic explorer (**II**). The absolute values of pBMD were highest for the lumbar spine, then for the total hip and lowest for the femoral neck. The age at the peak was higher for the total hip compared to the femoral neck and lumbar spine. The absolute values of BMD measured by Hologic explorer were lower than those measured by Lunar prodigy advance.

As expected, pBMDs at femoral neck and total hip were higher in men compared with women and approximately 0.05 g/cm² (about 5.8-6.3%). However, pBMD at the lumbar spine was quite similar between men and women. The age at pBMD for different bone sites also were similar for the two sexes.

Table 3. Peak bone mineral density (pBMD) and age at pBMD in women and men (**I**, **II**)

Bone site	Paper I		Paper IV	
	pBMD (with Lunar)	Age at pBMD	pBMD (with Hologic)	Age at pBMD
Women				
Femoral neck	0.94 (0.11)	27.7	0.80 (0.10)	25
Total hip	1.02 (0.12)	29.3	0.86 (0.10)	32
Lumbar spine	1.16 (0.13)	28.5	0.98 (0.11)	30
Men				
Femoral neck	-		0.85 (0.11)	25
Total hip	-		0.91 (0.12)	30
Lumbar spine	-		0.97 (0.11)	32

4.3 PREVALENCE OF OSTEOPOROSIS IN VIETNAMESE WOMEN AND MEN (I & III)

Using the estimated peak BMD and standard deviation, femoral neck T-scores were calculated for each woman and man, and the prevalence of osteoporosis (i.e., T-scores ≤ -2.5) (Kanis & Gluer 2000) was estimated as shown in **Table 4**.

For women aged 50-65 years, approximately 23% were classified to have osteoporosis at the femoral neck (I). The prevalence increased with advancing age such that among those aged between 50 and 59 it was estimated at 21% and increased to 33% in those aged 60-65 years or above. The calculation for osteoporosis at the lumbar spine was 49% for women aged 50-69.

For menopausal women (III), the prevalence of osteoporosis was around 17-18% for the femoral neck and total hip; and approximately 37% for the lumbar spine. As expected, the prevalence of osteoporosis among men ≥ 50 years of age was lower than in women. It ranged from 7 to 12% for the different sites measured.

There was discordance in the diagnosis of osteoporosis as based on T-scores between the femoral neck and total hip (I). When the total hip BMD T-score was used, the prevalence was 26%. However, when either femoral neck or total hip BMD was used, the prevalence increased to 34% in women aged 50-65 years. Although the proportion of concordance in the diagnosis between the two BMD sites was 80%, the kappa statistic was 0.49 (95% confidence interval: 0.34 – 0.65, data not shown) which was significantly different from 0 ($p < 0.0001$).

Table 4. Prevalence (%) of osteoporosis by site of BMD measurement in women and men

Bone site	Women (I & III)		Men (III)
	50+ (n=160)	menopausal (n=151)	50+ (n=106)
Femoral neck	23.1	17.9	8.5
Total hip	25.6	16.6	6.6
Lumbar spine	49.0	37.3	12.4

4.4 DETERMINANTS OF BONE MASS (II & III)

4.4.1 Vitamin D deficiency and association with bone mineral density in Vietnamese women and men (II)

4.4.1.1 Prevalence of vitamin D deficiency in women and men

As shown in **Table 5**, serum levels of 25(OH)D and PTH were significantly higher in men than in women. The prevalence of vitamin D deficiency (defined as 25(OH)D < 20 ng/mL) was twice as large in women and reached 30% in comparison with 16% in men.

Table 5. Levels of vitamin D and PTH (mean & SD); and number of individuals with vitamin D deficiency (%) in women and men (**paper II**)

Variable	Women	Men	P – value
N	269	222	
PTH (ng/L)	29.1 (11.2)	31.8 (13.5)	<0.05
25(OH)D (ng/mL)	23.2 (7.4)	28.6 (8.9)	<0.001
25 (OH) D < 20 ng/mL	82 (30.5%)	35 (15.8%)	<0.001

4.4.1.2 Levels of vitamin D in different age groups in women and men

Levels of vitamin D differed significantly between age groups in both women and men (**Fig 17**). In women the lower levels of vitamin D were found to be highest in the age groups younger than 30 years, as well as in women older than 60 years compared to those between 30-59 years ($p<0.01$). A similar trend was also observed in men, i.e. the lower levels of vitamin D were highest in the youngest age group (less than 30 years) compared to the older age groups ($p<0.05$).

4.4.1.3 Levels of vitamin D by season

The levels of vitamin D varied significantly by season (**Fig 18**). Levels of vitamin D were lower in the winter season (December and January) than in autumn (October and November) and summer (May and June) for both women and men ($p<0.001$). In the summer and autumn seasons, but not in the winter, the levels of vitamin D were significantly higher in women than in men ($p<0.01$, respectively).

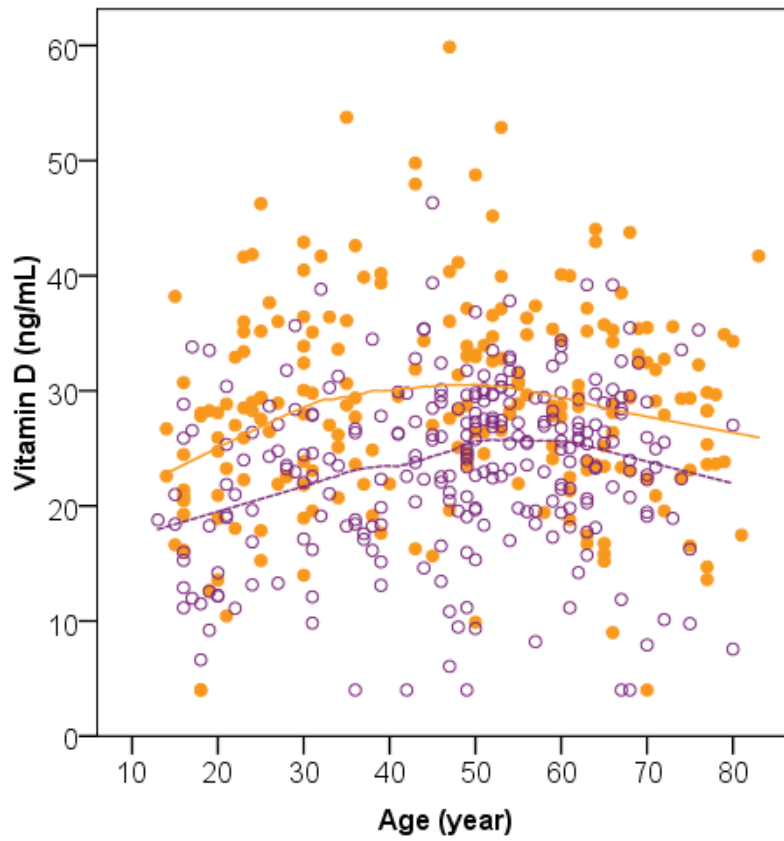


Figure 17. Vitamin D concentrations (ng/mL) by age and sex.
(women violet and men orange color)

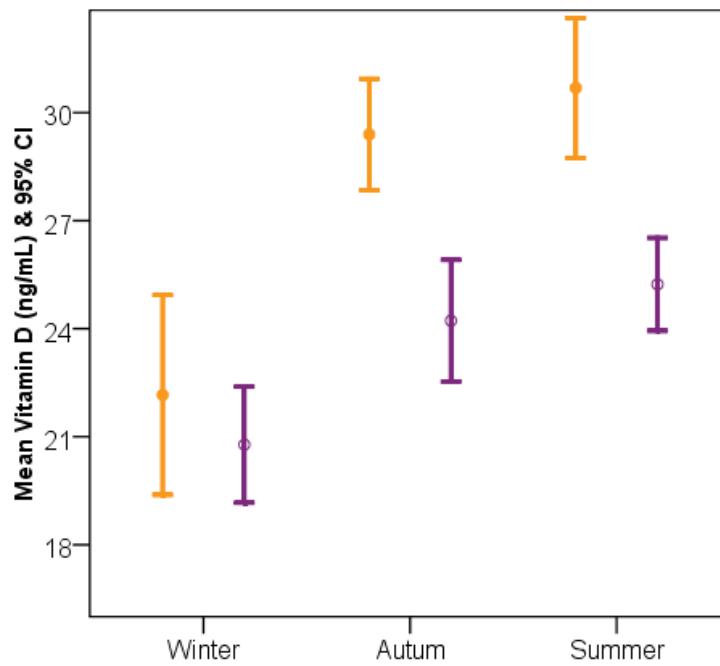


Figure 18. Vitamin D levels (ng/mL, 95% CI) by season and sex.
(women violet and men orange color)

4.4.1.4 Levels of vitamin D - urban versus rural residence

There were also markedly lower levels of vitamin D among women and men living in urban areas than in rural areas ($p<0.001$) (**Fig 19**). In urban areas the levels of vitamin D were similar for subjects of both sexes, while in rural areas they were significantly lower in women than in men ($p<0.001$).

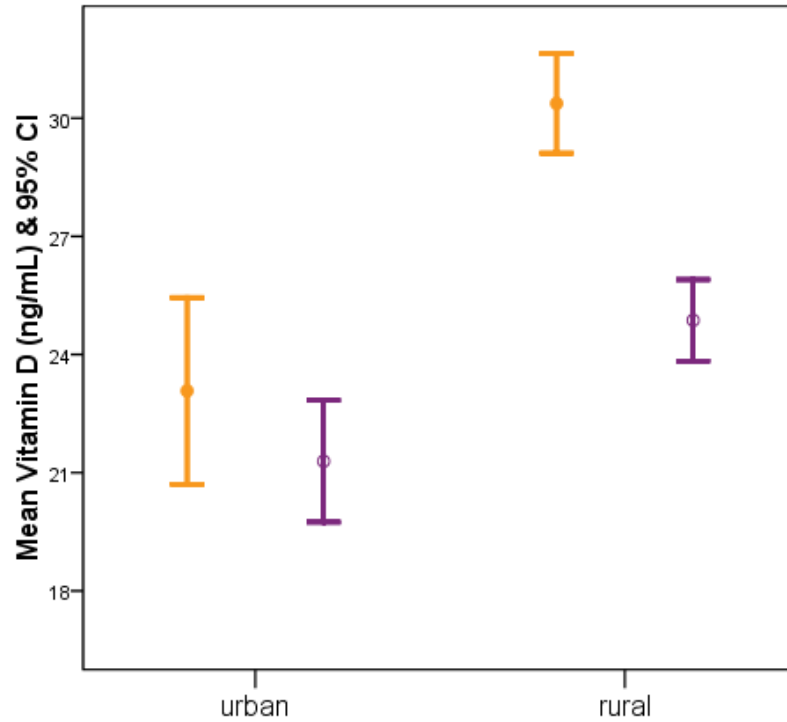


Figure 19. Vitamin D levels (ng/mL, 95% CI) by area and sex.
(women violet and men orange color)

Multiple linear regression analysis revealed that the strongest predictors of vitamin D deficiency in women were age less than 30 years ($p<0.01$) and living in an urban area ($p<0.01$), whereas the use of contraceptive pill was protective ($p<0.01$) (**II**). In univariate analyses, winter season ($p<0.001$) and coffee drinking ($p<0.05$) were also associated with vitamin D deficiency in women. In men, multiple regression analysis showed that winter season was the only significant predictor of vitamin D deficiency ($p<0.01$). In the univariate analyses, urban area ($p<0.001$) and younger age than 30 years compared to middle age (50-59 years) ($p<0.05$) were also associated with vitamin D deficiency in men (**II**).

4.4.1.5 Levels of vitamin D and bone mineral density in women and men

The relationship between serum levels of 25(OH)D, PTH and BMD was examined by multiple regression analysis in **paper II** and **Fig 20**.

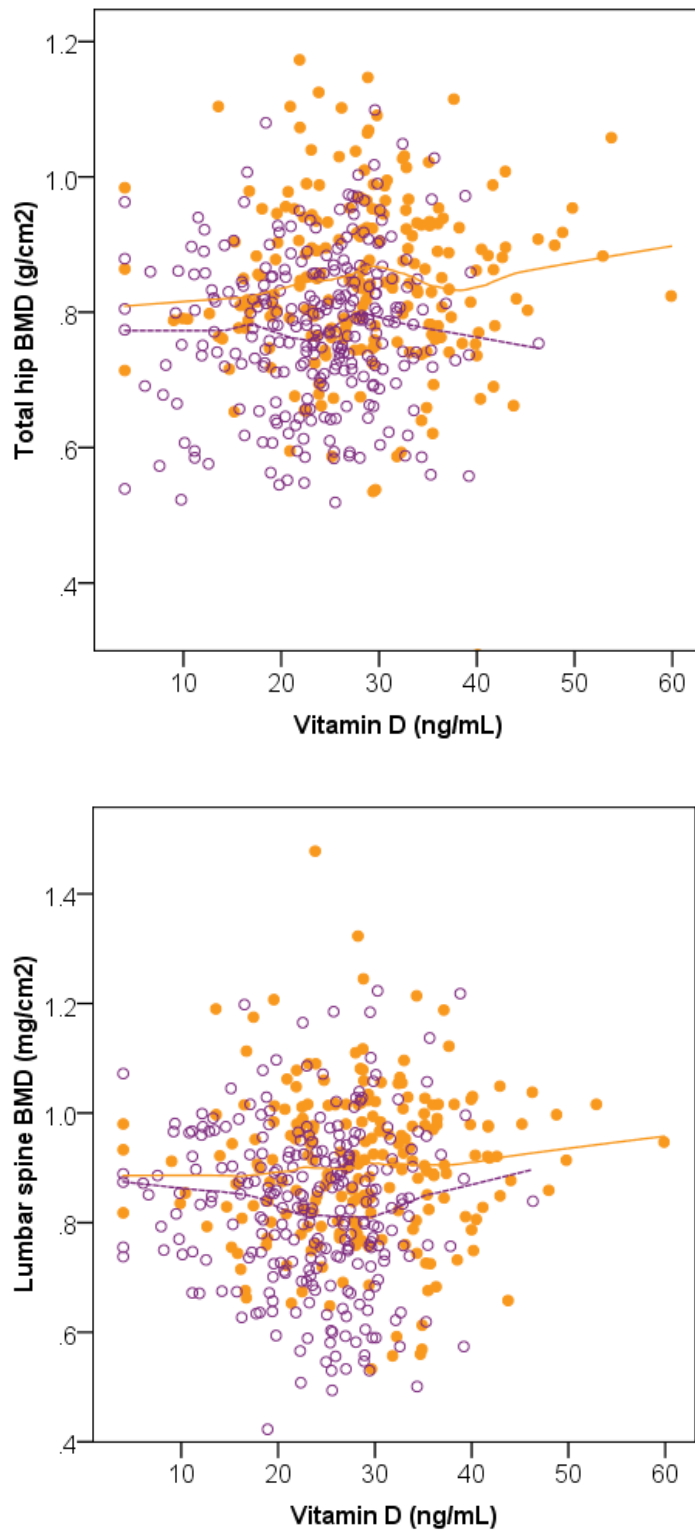


Figure 20. Levels of Vitamin D (ng/mL) and BMD (g/cm²) at femoral neck (upper panel) and lumbar spine (lower panel) in women and men (women violet and men orange color)

Among women, serum levels of 25(OH)D were significantly associated with BMD in total hip in multivariate analysis ($p<0.001$) (II). Also, serum levels of PTH were associated with BMD in the femoral neck and the lumbar spine ($p<0.001$) (II). In men, there were positive associations between vitamin D concentration and BMD in both total hip and lumbar spine ($p<0.001$, respectively). Among predictors for BMD in women, age, body weight, residence, coffee drinking, serum levels of PTH and 25(OH)D were estimated to explain around 40% of the variation in BMD at different sites. In men, significant predictors for BMD were age, BMI and vitamin D concentration.

4.4.2 Urban and rural differences in bone mineral density and prevalence of osteoporosis (II & III)

Overall the mean BMD values of urban women tended to be lower as compared with their rural counterparts. After menopause, this difference was significant ($p<0.01$) for BMD values at the femoral neck and total hip (Table 6).

Table 6. BMD and Osteoporosis by different groups of women and areas.

Variable	Reproductive Women			Menopausal Women		
	Urban	Rural	P	Urban	Rural	P
N	(51)	(67)	value	(55)	(96)	value
BMD, femoral neck (g/cm ²)	0.75	0.78	ns	0.61	0.65	<0.01
BMD, total hip (g/cm ²)	0.82	0.85	0.08	0.69	0.75	<0.01
BMD, lumbar spine (g/cm ²)	0.95	0.91	0.08	0.77	0.74	ns
Prevalance of osteoporosis at						
femoral neck (%)	-	-	-	20.0	17.0	<0.01
total hip (%)	-	-	-	20.0	15.0	<0.05
lumbar spine (%)	-	-	-	30.9	41.1	ns

Data shown as mean values; *categorical variables, values are given as percentage

Also, the prevalence of osteoporosis for these sites (III) was found to be higher among menopausal women from urban than rural areas (femoral neck 20% vs 17%, $p<0.01$; total hip 20% vs 15%; $p<0.05$).

In contrast, the mean BMD in men from urban areas and below 50 years of age was found to be somewhat higher than their rural counterparts at all three studied sites (femoral neck 0.87 mg/cm² vs 0.81 mg/cm², $p < 0.05$; total hip 0.93 mg/cm² vs 0.88 mg/cm², $p < 0.05$; 0.97 mg/cm² vs 0.91 mg/cm², $p < 0.05$). However, after 50+, only BMD values at lumbar spine of men in urban areas were significantly higher than the men living in rural areas (0.94 mg/cm² vs 0.85 mg/cm², $p < 0.01$). Furthermore, there was no difference in the prevalence of osteoporosis between men from urban and rural areas (III).

4.4.3 Sex hormone levels as determinants of bone mineral density in women and men (III)

In men, serum levels of testosterone averaged 6.2 ng/mL and no apparent change by age was recorded (III). Average estrogen levels were similar to those of postmenopausal women and there was a positive association with increasing age ($r_s = 0.26$; $p < 0.001$) (III). Values of BMD for femoral neck, total hip and lumbar spine in men were similar to those quantified in women at reproductive age but higher than in postmenopausal women (Table 2).

Among predictors for BMD in men (Fig 21 and III), multiple regression analysis revealed age, BMI and blood levels of estrogen to be the most important factors and estimated to explain 41% of BMD at the femoral neck and 27% at the total hip. For BMD of the lumbar spine, age, BMI and testosterone levels were the most important predictors. Similarly, age, weight, BMI, levels of estrogen and testosterone predicted osteoporosis at the different sites in men.

In women, as expected, sex hormone levels and BMD were lower in menopausal subjects as compared to those of reproductive age (Table 2). When the hormonal status for women was controlled by splitting into reproductive and menopausal groups, different predictors for BMD appeared (III). For reproductive women, weight and residence were the most significant predictors for BMD at all three sites, and these two variables contributed to 12-14% of the BMD variation. For the menopausal women, age, weight, and residence explained 40-46% of the variation (III). For the whole female population, multiple regressions revealed age, weight, residence, estrogen levels and oral contraceptive use as the most important predictors of BMD and estimated to explain around 50% of the variation at different sites (data not shown).

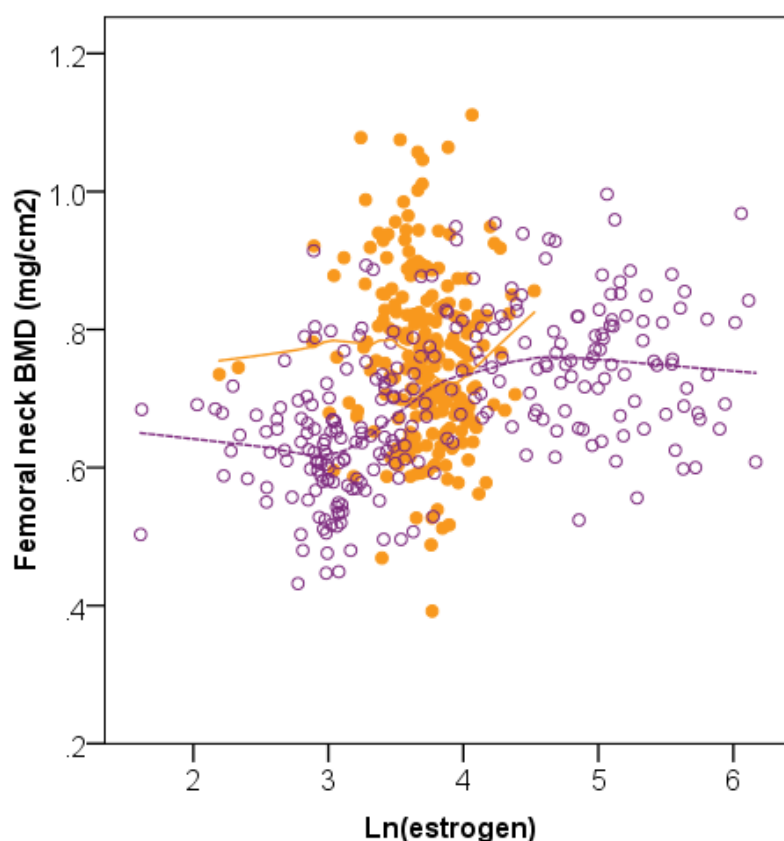


Figure 21. Bone mineral density (g/cm²) of the femoral neck as a function of serum estrogen levels (°log of ng/mL) in a sample of Vietnamese men (orange line, dot) and women (violet line, circle)

4.4.4 Low aglycone content in commercial soy drink products (IV)

4.4.4.1 Commercial soymilk products

The composition and total content of isoflavone for each product are shown in **Table 6**. Most of the soymilk samples had a similar total isoflavone content (glycosides and aglycones) ranging 60-80 mg/L except S1. In the S1 preparation, the concentration was as high as 122 mg/L ($p < 0.001$).

The predominant isoflavones in all products were glycosides, accounting for around 80% of the total isoflavone content. Glycitein and genistein were the two most abundant aglycones, and levels found in all products except S1 were 3-6 folds higher than for daidzein (**IV**). In the S1 sample, levels of daidzein ($p < 0.001$) and glycitein ($p < 0.01$) were markedly higher whereas the amounts of genistein were similar to that of the other preparations in paper **IV**.

Table 6. Isoflavone content (mg/L) of commercial soymilk products from Vietnam & Sweden

Product	Total		
	glycoside	aglycone	isoflavone
VN1	54.0	7.2	61.1
VN2	65.4	5.6	71.0
VN3	71.2	6.3	77.5
S1	107.7	14.0	121.8
S2	64.2	9.8	73.9
S3	65.6	7.0	72.5

4.4.4.2 “Home-made” soymilk

There was a large variation in total isoflavone and glycoside content in soymilk prepared from beans of different origins ($p<0.001$). The US bean based soymilk R2 had the highest total isoflavones, followed by the UK bean based preparation R3 ($p<0.001$). The Vietnam bean based preparation R4 had the lowest amount of total isoflavones, which was less than half of that of the R2 ($p<0.001$). The differences were mainly due to the variation in the amounts of glycoside content (**Table 7**).

Table 7. Isoflavone content (mg/L) of “home-made” soymilk prepared at the lab from beans of different origin. (Samples R2-4 were prepared with prolonged heating for 3 hours at 80°C).

Product	Total		
	glycoside	aglycone	isoflavone
R1-US	81.3	10.2	91.5
R2-US	128.9	10.1	139.0
R3-UK	86.5	11.7	98.1
R4-VN	57.6	5.8	63.4

The amounts of individual and total isoflavones were markedly increased after prolonged heating in the R2 preparation. The total isoflavone content was 38% higher in the R2 than in the R1 sample, which was prepared from the same US beans according to the standard procedure. The difference was mainly attributed to an increase in the glycoside content whereas levels of aglycones were quite similar (**Table 7**).

The theoretical maximum yield of aglycone after total transformation of its glycoside in all investigated 10 preparations was low. The two US bean (R1 and R2) and the UK bean based preparations had higher total isoflavone content than all commercial products, except for S1. The isoflavone distribution in the different preparations was quite similar. The relative amounts of daidzein, glycitein and genistein were around 20-30%, 35-45% and 30-40%, respectively. Among the three Vietnamese products, the total isoflavone content in VN1 was significantly lower than that in VN2 ($p<0.05$) and VN3 ($p<0.01$). Among the Swedish samples, the content in S1 was significantly higher than in S2 and S3 ($p<0.001$, respectively).

4.4.4.3 Total protein content and isoflavone levels

The amounts of total protein, total glycoside, total aglycone and total isoflavones for the ten different soymilk preparations are presented in **Fig 22**.

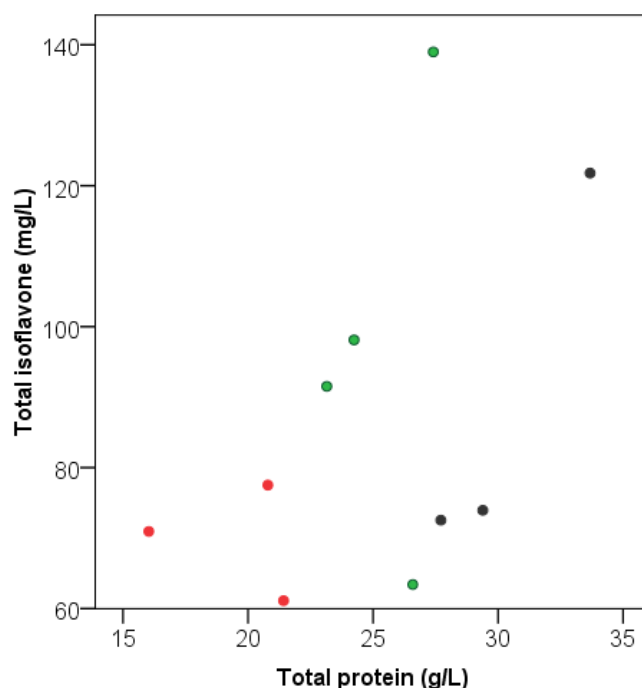


Figure 22. Total protein content and isoflavone levels in different soymilk preparations [samples from Vietnam (red), Sweden (black) and home made (green)]

Total protein content of the commercial soymilk preparations from Vietnam was generally lower than for the Swedish and home-made products. Also, the amounts of glycoside and aglycone and total isoflavone were lower. However, there was no apparent relationship between the amount of total protein and the isoflavone content in different soymilk preparations.

5 DISCUSSION

5.1 PEAK BONE MINERAL DENSITY IN VIETNAMESE WOMEN AND MEN (I & II)

Peak BMD values are important to calculate the T-score that is a basis for the classification of osteoporosis (WHO, 1994). Using different values of pBMD (mean and SD) will influence the number of individuals classified to have osteopenia/osteoporosis in a population. As illustrated in **Table 10 and study I**, values for pBMD of Vietnamese women and men (either measured with Hologic equipment in **study II&III** or with Lunar equipment in **study I**), were comparable with data from other Asian countries but somewhat lower than Caucasian data.

Table 10. Comparison of young adult bone mineral density (g/cm²) of the femoral neck in women and men from different Asian countries (all measurements were done with the Hologic equipment)

Population	Peak BMD at femoral neck (mean and SD)	
	Women	Men
Vietnamese ^{II}	0.80±0.10	0.85±0.11
Vietnamese in urban city ¹	0.80±0.11	0.85±0.13
Japanese ²	0.83±0.12	na
Chinese ²	0.81±0.12	0.94±0.15
White American ³	0.86±0.12	0.93±0.14

¹(Ho-Pham et al 2011), ²(Wu et al 2003), ³(Looker et al 1997)

Values for BMD measured with the Hologic densitometer (**II & III**) were lower at all three studied sites and also the peak was reached at different ages in comparison with data measured by GE Lunar (**I; Table 3**). Given the sampling variation and the lack of calibration between densitometers of the same brand, the differences among Asian populations seem insignificant (**I; Table 10**). Some of the differences in prevalence of osteoporosis could be partially due to the difference in bone densitometry technologies. However, the effect of measurement technology to diagnose osteoporosis

is likely to be small because the prevalence will be determined from BMD T-scores, and T-scores are standardized to the mean and standard deviation as obtained from each densitometer and each population. Still, potential differences between densitometry technologies could be important when repeated measurements are performed in the same individual. Therefore, longitudinal measurements to follow change in BMD should be carried out using the same equipment.

Peak BMD values in Vietnamese men were also higher than in women at the femoral neck and total hip in concordance with previous reports (Looker et al 1997; Wu et al 2003), but not for the lumbar spine. The **age at pBMD** was quite similar for both sexes. For the femoral neck the peak was reached about 3 years earlier than for the lumbar spine (Ho-Pham et al 2011; Looker et al 1997). Our data suggest that Vietnamese women may achieve their peak BMD at a later age (between 25 and 30 years) than Caucasian women among whom the peak BMD was reached between 20-25 years (Henry et al 2004; Nguyen et al 2001). The underlying factors to explain this apparent difference are not fully clear. However, it is well-known that Asian girls tend to have a later menarche (average age of 13 years) than Caucasian girls (12 years). It is also possible that nutritional factors and lower levels of estrogen and other sex steroids may contribute to the difference.

After definition of the specific Vietnamese pBMD, we could now obtain more appropriate T-score values to have more precise diagnosis of osteoporosis in both women and men. The prevalence of osteoporosis classified by using the specific Vietnamese T-score was 17.9% for women and 8.5% for men. In comparison the corresponding figures when using the reference data from the manufacturer of the densitometer were 11% & 14.5% respectively. These marked differences illustrate the importance of using population specific data for the diagnosis of osteoporosis.

5.2 PREVALENCE OF OSTEOPOROSIS IN VIETNAMESE WOMEN AND MEN (I & III)

BMD is an important predictor of future fracture (Cummings et al 2006; Marshall et al 1996; Nguyen et al 1993) and individuals with osteoporosis are at increased risk (Marshall et al 1996). It has been shown that 65% of individuals with osteoporosis will sustain a fracture within the next 10-15 years (Kung et al 2007a); (Nguyen et al 2007).

The data presented in **Table 4** suggest that approximately a quarter of Vietnamese women aged 50+ suffer from osteoporosis and are at high risk of hip fracture. Even more women may have a risk for lumbar compression and pain as a consequence of osteoporosis in this site. Also among men aged 50+, a significant number of individuals with osteoporosis were identified. Thus, it seems clear that osteoporosis represents an important burden of public health in Vietnam.

A comparison between the prevalence of osteoporosis among women and men aged 50+ years as reported from different countries is presented in **Table 10**.

Table 10. Prevalence of osteoporosis among women and men aged 50+ years reported from different countries

Population	Prevalence (%) of osteoporosis at femoral neck	
	Women postmenopausal / 50+	Men 50+
Vietnamese ^I	23.1	na
Vietnamese ^{III}	17.9	8.5
Vietnamese in urban city ¹	28.6	10.0
Vietnamese American ²	37.0	-
Thai ³	29.5	12.6
Indonesian ⁴	~15.0	-
Chinese ⁵	10.1	-
Chinese ⁶	31.2	10.4
Hong Kong ⁷	49.6 ⁶	-
Japanese ⁸	17.0	-
Korean ⁹	12.4	5.9
Australia ¹⁰	21.0	-
U.S White ¹¹	20.0	6.0

¹(Ho-Pham et al 2011); ² Marquez M, et al (Melton et al 2002); ³ Limpaphayom KK, et al (Limpaphayom et al 2001); ⁴Tirtarahardja G, et al (Tirtarahardja G et al 2006); ⁵ Wu XP, et al (McNutt et al 2003); ⁶ Lynn HS et al (Lynn et al 2005); ⁷Ho SC, et al. (Ho et al 1999); ⁸Iki M et al (Kaneki et al 2001); ⁹ Yang SO, et al (Yang SO et al 2006); ¹⁰ Henry MJ, et al (Pasco et al 2000); ¹¹ Looker A, et al (Looker et al 1997). ^INote that the estimate in this study was limited within the age range of 50 and 65 years.

Our material included only few women above 65 years of age, none in study **I**, and around 40 in study **III**. Therefore, it could be that the prevalence of osteoporosis has

been underestimated. Even so, the prevalence (between 17-23%) is similar with reports from Australia (Pasco et al 2000) and America (Looker et al 1997) but lower than in Thai (Limpaphayom et al 2001) and Hong Kong women (Cummings et al 2006). However, the prevalence of osteoporosis in the present study appears to be substantially higher than in Chinese (McNutt et al 2003), Japanese (Kaneki et al 2001), Korean (Yang SO et al 2006) and Indonesian women (Tirtarahardja G et al 2006).

While the peak bone mass of Vietnamese women was found comparable to that of other Asian girls, the prevalence of osteoporosis among women aged 50+ was still higher than in many other countries. One possible explanation could be that of a cohort effect. Women aged 50+ were born and grew up during many years of war, when the country's economy was in great hardship and many women suffered from malnutrition which may have resulted in a low BMD. In contrast, the younger cohort aged between 20 and 30 years was born and raised after the independence in 1975, during which economic and nutritional conditions have significantly improved.

In summary, this study has, for the first time, provided an estimation of peak BMD in Vietnamese women and men of urban and rural residence. Among women, the values for peak bone mass in this study were found comparable to Asian data but the prevalence of osteoporosis was higher than in some other Asian and comparable to Caucasian populations. In men, the peak bone mass was found somewhat lower than for other Asian and Caucasian populations, but the prevalence of osteoporosis was quite similar. These data in the future may be useful as a valuable reference base to diagnose osteoporosis and for the clinical management of its consequences within the Vietnamese society.

5.3 DETERMINANTS OF BONE MASS (II & III)

5.3.1 Urban and rural differences in bone mineral density and prevalence of osteoporosis (II & III)

A consistent trend in osteoporosis research has been that the incidence of fracture is higher in developed than in developing countries. Also within different countries, the incidence is higher in urban than in rural communities (Pongchaiyakul et al 2005). Although other factors may also be involved, BMD is generally regarded as the most important primary determinant of fracture risk (Pongchaiyakul et al 2005).

Here we found the overall mean BMD values of urban women to be lower as compared to women of rural residence. After menopause, this difference was

significant ($p<0.01$) for BMD values at the femoral neck and total hip (**Table 6**). Also the prevalence of osteoporosis for these sites (**III**) was found to be higher among menopausal women from urban areas.



Figure 22. Field work in rural Vietnam. Courtesy of Reuters



Figure 23. Modern Hanoi cityscape, courtesy of Wikimedia

Working and living indoors and also air pollution in cities and as a consequence reduced sun exposure to the skin could contribute to a lower average BMD in urban women (**Fig 22 & 23**). However, in the present study we also found them to have lower levels of circulating estradiol than their rural counterparts. The explanation for this difference is not clear but it could possibly reflect differences in physical activity and dietary habits. Like tobacco smoking, heavy air pollution in large cities might eventually

reduce aromatase activity and thus estrogen synthesis (Barbieri et al 1986). None of the postmenopausal women used hormone replacement therapy.

5.3.2 Vitamin D deficiency and association with bone mineral density in Vietnamese women and men (II)

Although there is a lack of data, it is commonly believed that as consequence of less sun exposure, vitamin D deficiency is more prevalent in Caucasian than in tropical populations (Mithal et al 2009). However, here we found that more than 30% of the women and 16% of the men in a Vietnamese population had serum levels of 25(OH)D below 20 ng/mL (50 nmol/L). In fact, the prevalence of vitamin D deficiency in our study was comparable to reports on Caucasian Americans in the USA (Looker et al 2008). The variations in the prevalence of vitamin D deficiency might be due to differences in ethnicity, pigmentation, nutrition and sun light exposure.

In our material, being a woman, of younger age, living in a city, and winter season were independent predictors of vitamin D deficiency. The difference was more apparent among the women than for the men. This finding could possibly reflect an effect of different clothing practices on vitamin D synthesis (Ho-Pham et al; Mithal et al 2009). Following the rapid economic growth and an increasing cultural influence from e.g. western countries, white skin may have become part of a modern “beauty concept”, especially attractive to the younger generation of women (**Fig 24**). The trend among younger people to try to avoid exposure to sunlight was also observed in a previous study on urban residents in Ho Chi Minh City (Ho-Pham et al).



Figure 24. Clothing habits and air pollution may contribute to vitamin D deficiency in urban citizens. Courtesy of VietnamNet.

Urbanization is known as a predictor of low levels of vitamin D and has been identified as a risk factor for deficiency in previous reports from Asia and the Middle East (Harinarayan et al 2007). Both men and women more often work indoors compared to those in rural areas. Also, air pollution in cities could contribute by acting as a barrier against UV light. However, in women the prevalence of vitamin D deficiency was also high in rural areas, and significantly higher than in men. This may indicate that women in general are more prone to cover their skin with clothing than men.

There is no general consensus on which circulating levels should be representative for sufficient amounts of vitamin D, but certainly all authorities agree that a cut off level below 20 ng/mL is a strong indicator of deficiency (Heaney 2004; Holick & Chen 2008; Lips 2001; Mithal et al 2009). For levels >20 ng/mL, a plateau effect on e.g. bone markers has been suggested (**Fig 25**). Such low levels as <20 ng/mL have been clearly linked not only to osteoporosis but also to a variety of severe conditions like hypertension, other cardiovascular diseases and cancer (Bischoff-Ferrari; Giovannucci et al 2008).

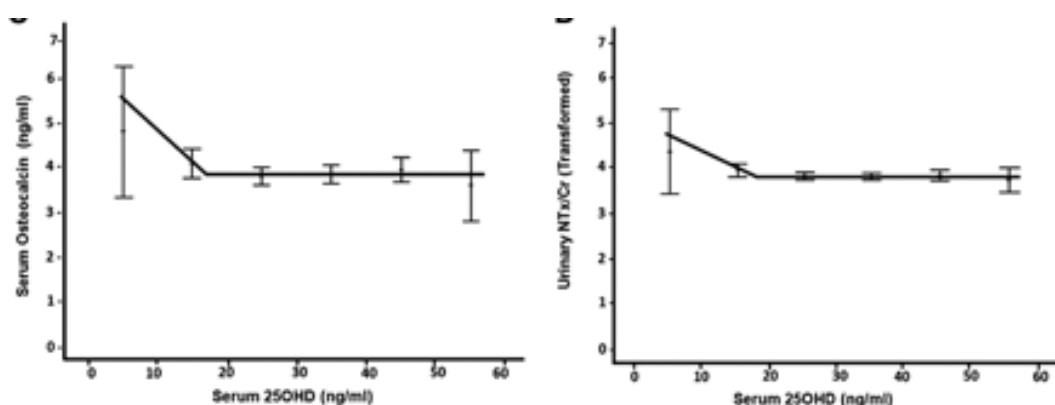


Figure 25. Relationship between serum 25(OH)D levels and the bone markers osteocalcin (left) and uNTx/Cr (right). A threshold level is indicated after values > 20 ng/mL adapted from (Sai et al 2011).

According to some authors, even levels below 30 ng/mL should be regarded as vitamin D insufficiency and still represent a significant risk factor. However, this notion is more controversial and uncertain (Mithal et al 2009; Prentice 2008). In the present material, more than 80% of the women and 60% of the men had 25(OH)D values below 30 ng/mL.

Synthesis of vitamin D is dependent on solar radiation of the skin. Latitude and seasonal variations influence the amount of possible solar radiation that a population

may receive. Here, the prevalence of vitamin D deficiency was much more pronounced in blood samples collected during the winter season (December and January) than in samples from autumn (October and November) and summer (May and June). This difference was apparent in both women and men and there was a significant trend for seasonal variations. This finding is in agreement with several previous reports, and seasonal variations in 25(OH)D concentration have been found even in subtropical locations (Mithal et al 2009). Adjustment for seasonal variation would be important for the definition of threshold values for vitamin D deficiency in different populations.

In women, the use of oral contraceptives was associated with a lower risk of vitamin D deficiency. Activated vitamin D is bound to a binding protein in the circulation and estrogen is known to stimulate the production of this protein in the liver (Riggs et al 2002). Furthermore, estrogen stimulates hydroxylation of vitamin D in the skin. By these two mechanisms oral contraceptives may enhance the levels of vitamin D.

Vitamin D deficiency will increase bone resorption, cortical bone loss and the risk of fracture (Bischoff-Ferrari; Bischoff-Ferrari et al; Lips 2006; Mason et al). Age, gender, body weight, smoking, alcohol and coffee have been implied as other risk factors for osteoporosis (Grossman). Also, smoking, alcohol, and coffee consumption were more frequent in men. In the present material, serum levels of 25(OH)D were positively associated with BMD at different sites in women. In men, vitamin D concentrations were also positively associated with BMD at the total hip and lumbar spine.

These results indicate the importance of sun exposure for vitamin D status. Serum levels of 25(OH)D and vitamin D deficiency were significantly associated with bone mass in both women and men. The high prevalence of vitamin D deficiency should raise the concern about bone health, including risk of osteoporosis and fracture within the Vietnamese society.

5.3.3 Sex hormone levels as determinants of bone mineral density in women and men (III)

Our study supports the notion that estrogen levels have a stronger association and are more important as predictors for BMD than testosterone in both men and women.

The critical role of estrogen in bone metabolism of both sexes is consistent with reported data. While BMD in men was previously considered to correlate mainly with testosterone (Faustini-Fustini et al 1999), recent findings rather indicate a direct

correlation between BMD and estrogen than with testosterone in healthy men regardless of their age (Hoppe et al). In men, estrogens are produced via conversion from androgens by aromatase activity in peripheral tissues, including bone (Ongphiphadhanakul et al 1998). Apart from age and BMI, we found estrogen levels to be significantly predictive for BMD of the femoral neck and total hip in men. This is in agreement with a case report of an adult man with estrogen resistance caused by a mutation in the estrogen receptor gene (Smith et al 1994). While blood levels of testosterone were within the normal range, this man suffered from increased bone resorption and osteopenia (Smith et al 1994).

Although estrogen was generally more important, we still found that testosterone remained a significant predictor for BMD at the lumbar spine. Men older than 50 years gained 8-14% of BMD at the lumbar spine but not at the femoral neck after receiving intramuscular testosterone therapy for moderate hypogonadism (Tracz et al 2006). Testosterone, like estrogen, appears to stimulate bone turnover, acting directly or indirectly via conversion of estrogen in human osteoblasts to increase androgen receptor expression and stimulate bone cell proliferation and mineralization (Tracz et al 2006).

In summary, the present data confirm the high prevalence of osteoporosis in the Vietnamese population also in men, and that estrogen levels are essential for bone mass in both men and women. The results should have clinical implications and increase awareness about an important health issue within the Vietnamese society.

5.4 CAN SOY INTAKE PREVENT AGAINST OSTEOPOROSIS? (IV)

Treatment for osteoporosis for a long time was based on HRT but its long-term safety is currently questioned and uncertain (Hickey et al 2005). Bisphosphonates and selective estrogen receptor modulators (SERMs) e.g. raloxifene or tamoxifen offer alternative treatments. However, these drugs are relatively expensive and their affordability in developing countries, where 1.5 billion elderly (60 years and more) are estimated to be living by 2050 (Mirkin 2001), is limited.

While many scientific panels have now reached a consensus on the effect of phyto-estrogen on cardiovascular disease prevention (Erdman 2000; J.H.C.I 2002; U.S 1999), the same conclusions have not been reached for prevention and treatment of osteoporosis (Branca 2003; Weaver & Cheong 2005). There are conflicting data on the effectiveness of soy isoflavones on bone metabolism in postmenopausal women. A daily

intake of 500 mL of soymilk containing 76 mg of aglycones showed favorable effects on BMD as compared to the same amount of soymilk containing less than 1mg in a two-year randomized trial (Lydeking-Olsen et al 2004). In contrast, soy protein intake 18 mg + 105 mg isoflavone aglycone equivalents showed no apparent effects on BMD (Kenny et al 2009). Contradictory findings have also been reported for bone turnover. Yamori et al showed that an isoflavone-rich test drink (40 mg of glycosides) reduced bone resorption (Yamori et al 2002); while others had found no such effect from intake of soy with a content of 135 mg of total isoflavones/day (Cheong et al 2007). The inconsistent findings may reflect insufficient amounts of bioactive phytoestrogen i.e. aglycones and individual variations in sensitivity and isoflavone metabolism (Cassidy et al 2006)

In our study, most of the soymilk products had a total isoflavone content around 60-80 mg/L (calculated maximum yield of bioactive aglycone as 40-50 mg/L). They contained only 10-20% of the bioactive aglycones, which can be absorbed directly through the intestine. Even an individual with a high capacity to metabolize would need to consume several liters of soymilk per day in order to achieve a threshold of around 90-120 mg/day of aglycones to be effective on bone (Ma et al 2008a; Wong et al 2009). Certainly, such consumption is not feasible in everyday life not even among high soy consumers as in Vietnam.

A large variety of factors may influence soy bean isoflavone content e.g. type of bean, crop year, growing temperature and location of agriculture. The variation in isoflavone content among different commercial products could also reflect their storage and processing (Eisen et al 2003; Lee et al 2003; Mathias et al 2006). The “home-made” soymilk (R1 and R2) from beans of US origin was found to provide a higher total isoflavone content than most of the commercial products. In this study, the commercial VN1 product was prepared from the same source of US beans, and therefore, the difference in isoflavone between the US bean and VN1 preparation was likely due to differences in processing. For the “home-made” preparation, the whole beans including the hull were used whereas in the industrial process VN1 was prepared after removing the hull (manufacturer personal communication). The hull of soy-bean itself has a very low isoflavone content but its removal may cause loss of the hypocotyl axis, which is a major source of soy aglycones (Wong et al 2009).

We found prolonged heating for 3 hours to markedly enhance the β -glucoside content of the “home-made” soymilk preparations. In R2 as compared to R1, the total isoflavone content increased by 38%. Heating soymilk at 80⁰C will convert the

conjugated malonyl-glucosides into the non-conjugated β -glucosides, which have the potential for further transformation into active isomers. Also, prolonged heating did not change the aglycone content, which is in agreement with previous data (Xu et al 2002).

The present study found considerable variation in total isoflavone content when soymilk was prepared from beans of different origins and the differences were mainly due to glycoside amounts. Previous studies have also shown significant variations in the content of active aglycones and their glycosides in different commercially available soy foods in the US, Australia and Korea (Lee et al 2003; Setchell & Cole 2003).

Long term storage of soy beans has also been suggested to change the isoflavone profile probably due to the reduction of the amount of malonyl-glycosides (Lee et al 2003). It is possible that differences in duration and conditions of storage could explain the overall low isoflavone content and the poor effect of long-term heating to increase the glycosides in the sample of Vietnamese beans (R4).

Given the increasing interest and consumption of soy products, there is an apparent need to establish universal guidelines and uniform industry standards for labeling of aglycone content. At present, consumers and health professionals are confused about what types and amounts of different soy consumables might confer any beneficial health effects. The food labeling claim for soy protein in prevention of coronary heart disease has been approved by many health authorities e.g. in the US, UK, France, and Asian countries but has also been seriously questioned (Sacks et al 2006; Xiao 2008). Variations in the content of active aglycones and their bioavailable glycoside isomers in different products could well account for many of the inconsistencies in reported outcomes from observational and clinical studies.

It is often suggested that the isoflavone content of a soy product can be deduced from information of total protein content. The present study showed that such assumptions may not be justified. We found no apparent association between total protein and its isoflavone content in the different commercial and “home-made” soymilk preparations.

Accurate labeling of soy food in general and soymilk in particular would be a major step forward to support consumers worldwide. Such a measure would also allow health professionals and researchers to better explore the health benefits and risks of soy in dietary intervention studies.

5.5 CRITICAL ASSESSMENT AND FUTURE PROSPECTS

The present results should be interpreted in relation to some strengths and weaknesses. The sample was randomly drawn from the general population after careful screening, which should support its representativeness and external validity. The sample size was statistically adequate to estimate a prevalence of osteoporosis between 10-20%. The use of DXA technology with reliable measurement of BMD is also a strength of this study. However, the design was cross-sectional and therefore the estimate of pBMD and age at pBMD could be subject to uncontrolled bias. Ideally, these values should be determined in a representative cohort which should be followed from 5 years to 30 years of age. However, it is unlikely that such a study is feasible and therefore, a cross-sectional study is an alternative design. The estimated osteoporosis prevalence in postmenopausal women in study I was based on a rather small sample size, particularly among those aged 60-65 ($n = 24$), which could result in an increased sampling error. Because the maximum age of participants in this study was 65, the osteoporosis prevalence could have been underestimated. Studies on larger numbers of both old women and old men are needed in the future.

This is one of the largest studies of vitamin D status in the Asian population. Therefore, the estimates of vitamin D deficiency in age- and sex-subgroups would seem quite reliable. The results could probably be generalized for both urban and rural settings at least for the northern part of the country. The study population was highly homogeneous, which reduces the effects of potential ethnic confounders that could compromise the estimates. Nevertheless, the study has some potential weaknesses. Levels of 25-hydroxyvitamin D₂ (ergocalciferol) and 1,25(OH)D were not measured in this study. However, the occurrence of these vitamin D metabolites in blood is very low and levels of 25(OH)D are considered to adequately reflect vitamin D status. This was a cross-sectional study, so no causal inferences could be made for the observed relationships between factors.

To the best of our knowledge, this is the first study to investigate the correlation between sex steroid hormones and BMD in a Vietnamese population. The clinical material comprised both men and women randomly recruited from both rural and urban areas according to a rigorous selection scheme. Furthermore, the present study population had a wide age range from 13-83, which allowed the comparison of the relation between sex hormones and BMD in different age groups. Nevertheless, the study had clear limitations. Serum levels of the important sex hormone-binding globulin

(SHBG) were not quantified; and thus, the concentrations of free sex steroid hormones which are bio-active at target tissues could not be evaluated (Elmlinger et al 2005). In addition, as the study design was cross-sectional, conclusions on the long-term influence of sex hormones on BMD could not be drawn. Ideally, this relationship should be determined in a representative cohort in which a large number of men and women are followed.

The aglycone content of commercial soy drinks was analyzed in only a small sample of products. This pilot study should be expanded to larger materials and also performed for other forms of soy food e.g. tofu and soy powder. Still, analyses were performed with HPLC, a high quality technique with high sensitivity. The conclusion that currently available commercial soymilk products contain low amounts of aglycone is unlikely to change. So, it seems that unless new products become available, a strategy to promote soy food intake would have only little influence to prevent osteoporosis.

5.6 PUBLIC HEALTH AND CLINICAL IMPLICATIONS

Osteoporosis is an important health problem in both women and men in the Vietnamese society. Accurate diagnosis is dependent on relevant data for BMD from the specific population that should be monitored and eventually be subject of intervention.

In the present work, such early reference data have been presented. However, in the future, even larger numbers of individuals should be examined by densitometry to further establish accurate reference values for the population.

Vitamin D deficiency has been linked to osteoporosis, and also to risk of cancer, autoimmune disorders and cardiovascular diseases (Bischoff-Ferrari; Cutolo & Otsa 2008; Holick 2004). The high prevalence of vitamin D deficiency in the present material should raise the awareness of potentially important health issues within the Vietnamese society. Young women in urban areas may be an especially important target group for information about possible risks associated with vitamin D deficiency. Efforts to change attitudes towards sun exposure and the perception that white skin is a mark of attractiveness and a measure of high social status should be carried out. Defining resident groups at risk for vitamin D deficiency should be important to stimulate prevention strategies employed in a clinical setting. Vitamin D supplementation, increased exposure to UV light, fortification of food products with vitamin D and recommendations for better dietary intake could all be important tools and help to reduce potential health problems on a national level.

Estrogen levels are essential for bone mass in both men and women. In the clinical setting, both sex steroid hormones, estrogen and testosterone should be investigated for both men and women with osteoporosis. To the general public, information on possible risks associated with reduced sex hormone levels e.g. from smoking should be given.

Rural citizens have better bone mass when compared with their urban counterparts. Thus, a healthy lifestyle as in some rural communities should be promoted e.g. the importance of healthy nutrition, moderate physical activity and a clean environment should be emphasized.

6 GENERAL CONCLUSIONS

This Thesis has, for the first time, presented

- Reference values for pBMD in Vietnamese women and men. These data allowed the calculation of T-scores and thus an accurate identification of osteoporosis. Among women, values for pBMD in this study were found comparable to other Asian data but the prevalence of osteoporosis was higher and comparable to Caucasian populations. In men, the pBMD was found somewhat lower than for other Asian and Caucasian populations, but the prevalence of osteoporosis was quite similar. As determined at the femoral neck, the prevalence of osteoporosis in women was 17-23% and for men 9%. Even more women (37-49%) may have a risk for lumbar compression and pain as a consequence of osteoporosis in the spine. These data clearly suggest that osteoporosis is an important public health problem. In the future, our results may be useful as a valuable reference base to diagnose osteoporosis and for the clinical management of its consequences within the Vietnamese society.
- Significant predictors of BMD in men were age, BMI, and serum levels of estrogen. In postmenopausal women, age, weight and residence (urban vs rural) were the most important predictors. Postmenopausal women living in urban areas experienced osteoporosis more than rural residents. For all women, also blood levels of estrogen and testosterone were significant determinants.
- Serum levels of 25(OH)D were significantly associated with bone mass in both women and men. The prevalence of vitamin D deficiency, even when using the low cut off value of < 20 ng/mL, was very high and estimated to 30% in women and 16% in men. Partly this finding might be explained by low exposure to sunlight (urban residency and winter season) especially in women younger than 30 years of age. The high prevalence of vitamin D deficiency should raise the awareness of potentially important health issues such as osteoporosis but also about other serious diseases within the Vietnamese society.
- The total isoflavone content of different soymilk preparations were low, around 60-80 mg/L and there were only 10-20% of bioactive aglycones. This is far below the reported threshold levels to exert significant effects on bone. Consumption of several liters of soymilk per day would be needed to achieve any protective effect.

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