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**NUTRITIONAL STATUS OF VITAMIN D AND
DIETARY INTAKE OF KEY BONE HEALTH
NUTRIENTS IN SAUDIA
ARABIAN WOMEN - IMPLICATIONS FOR
BONE HEALTH**

A thesis presented for the degree of Doctor of philosophy

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For
My parents
My husband
My children
with my love

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ABSTRACT

Osteoporosis is a common highly prevalence public health problem affecting both gender in all age stages in worldwide. Little information is known about the bone health and lifestyle characteristics of women living in Middle Eastern countries in general and Saudi Arabia in specific. The complete information and statistical figures of osteoporosis prevalent and vitamin D deficiency among population it has not yet been identified. The strong correlation between dietary intake and bone health has been explored in Western populations but no data are available in Middle Eastern countries.

The aims of this study were four fold: (i) to examine the extent of poor bone health in the Saudi population of postmenopausal and premenopausal women; (ii) to investigate the effect of lifestyle factors including physical activity levels and sun-time exposure on bone integrity; (iii) to determine the extent of vitamin D deficiency in the population and the effect of this status on bone mass and calcium/bone metabolism; (iv) to evaluate the dietary quality and quantity in Saudi women and investigate fully the effect of diet on bone health indices.

As part of our investigation, a total of 212 Saudi Arabian apparently healthy women were voluntarily participated in this study. A total of 112 postmenopausal and 100 premenopausal women. They were aged 45-60 years and 20-30 years respectively. Bone mineral density (BMD) was determined at the lumbar spine (L2-L4) and femoral neck using dual x-ray absorptiometry (DXA). Calcaneal bone mass was measured by broadband ultrasound attenuation (BUA). All subjects were interviewed concerning their habitual dietary intake, physical activity levels and general lifestyle. Information on dietary intake of each individual was obtained using 3-day estimated food diaries. The amount of food consumed (in grams) for the five

food groups was calculated for each subject. Intakes were converted to frequency of consumption (time/d) by dividing food groups by average portion sizes. Using the only existing Food Composition Table for the Middle East, the nutrient values for energy, protein, fat, fibre, calcium, phosphorus, iron, vitamin C, vitamin D and potassium were chosen for the five food groups identified and calculated per 100g. Bone resorption was assessed by measurement of pyridinium crosslinks (PYD) and (DPD) using a second morning urine sample. Bone formation was assessed by bone specific alkaline phosphatase (BSAP) and osteocalcin (OC). Serum 25(OH)D, 1,25(OH)D, PTH, calcium, and phosphorus were measured.

Bone health indices indicated a high prevalence of low bone mass in these groups. According to WHO criteria (WHO, 1994), a total of 52% of postmenopausal and 37% of premenopausal women were osteopenic at the lumbar spine. Osteoporotic prevalence was 13% and 2% respectively. Similar results were found for the femoral neck. Physical activity levels were low and exposure to sunlight was low. A significant correlation was found between period of sunlight exposure (min/d) and axial BMD and calcaneal bone mass. The 'quality' of food consumption by Saudi Arabian women does not follow the recommended food guidelines, and the intakes of energy, fibre and potassium in women are lower than those recommended in western population but intakes of phosphorus are somewhat higher. Calcium, vitamin C and iron are around recommended levels. Vitamin D deficiency is highly prevalent in Saudi women, with 78% of women being below the classical threshold of 12ng/ml. A low milk consumption was associated with higher bone resorption in both postmenopausal and premenopausal women which remained significant after adjustment for the key confounding factors of age, weight, height and menopausal status. A low intake of fruit and vegetables and nutrients associated with high fruit

and vegetable intake including vitamin C, potassium and estimates of net endogenous acid production were found to be related to poorer indices of bone health.

These results are a cause for concern. It indicates that bone health is poor and dietary and lifestyle factors are not favourable to skeletal integrity in Saudi Arabian women.

LIST OF ABBREVIATIONS

PBM	:	peak bone mass
GH	:	growth hormone
IGF	:	insulin-like growth factor-1
HYP	:	hydroxy proline
PYD	:	hydroxy proline
NTX-I	:	n-telopeptides
CTX-I	:	c- telopeptides
TRACP	:	tartarat-resistan acid phosphatase
BSP	:	bone sialoprotein
PINP-PICP	:	n-and c- propeptides of type-1 collagen
OC	:	osteocalcin
BSAP	:	bone specific alkaline phosphatase
ALP	:	alkaline phosphatase
GPL	:	glycin-phosphatidy linositol
TNS	:	tissue-non specific
DPD	:	deoxypyridinoline
ECF	:	extracellular fluid
PTH	:	parathyroid hormone
CT	:	calcitonin
TH	:	thyroid hormone
7-DHC	:	7-dehydrocholesterol
HPLC	:	high-performance liquid chromatography
CBPA	:	competitive enzyme binding protein assay
RIA	:	radioimmunoassay
TMP	:	tetra methyl benzidine
CTR	:	ct receptor
WHO	:	world health organization

BMC	:	bone mineral density
SD	:	standard deviation
BMD	:	bone mineral density
DEXA	:	dual energy x-ray absorptiometry
QUS	:	quantitative ultrasonometry
VOS	:	velocity of sound
DB	:	measure in decibels
BUA	:	broadband ultrasonic attenuation
SOS	:	speed of sound
QUI	:	quantitative ultrasound index
NSP	:	non starch polysaccharides
DBP	:	vitamin D binding protein
COMA	:	the committee on medical aspect of food policy
FFQ	:	food frequency questionnaires
BMI	:	body mass index
AP	:	anterior posterior
DXA	:	dual x-ray absorptiometry
PMT	:	photomultiplier tube
QA	:	quality assurance
ELISA	:	enzyme linked immuno assay
OD	:	optical density
PNPP	:	p-nitrophenyl phosphate
NIH	:	national institute of health
EIA	:	enzyme immuno assay
TMP	:	tetra methyl benzidine
ANOVA	:	analysis of variation
ANCOVA	:	analysis of covariance
EARs	:	estimated average requirements
RDA	:	recommended dietary allowance
PNI	:	reference nutrition intake
DRVs	:	dietary reference values
AI	:	adequate intake

P	:	phosphorus
IOM	:	institute of medicine
APOSS	:	aberdeen prospective osteoporosis screening study
NEAP	:	net endogenous acid production
NMG	:	non-milk group
LMG	:	low-milk group
HNG	:	high-milk group
PFF	:	Proximal femur fracture

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Chapter 1

General Introduction

1.1 Bone health

Bones are the organs of skeletal system, together with cartilage makes up the skeleton. Development and maintenance of bone health is essential to skeletal integrity. Adult bone health is governed by two factors: the maximum attainment of peak bone mass (PBM) which is achieved during growth and early adulthood, and the rate of bone loss that occurs with ageing, with the menopausal years being a time of concern for women. Both aspects are determined by a combination of genetic, endocrine, and mechanical and nutrition factor, and there is evidence of extensive interaction between them.

Healthy lifestyle maintains bone health; adequate diet and regular weight bearing physical activity appropriate for individual are beneficial to bone health. Adequate vitamin D status can be achieved from exposure of the skin to sunlight. Diet plays an important role in the skeletal growth and maintenance of bone health throughout life. There are many clinical disorders associated with faulty nutrition, ranging from the obvious effects of deficiency of key nutrients especially vitamin D and calcium, to the many other dietary components that affect bone health in obvious or more subtle way. Calcium is the major mineral component of bone, a substrate for bone formation and an antiresorptive agent. Vitamin D plays a role along with calcium in preserving bone.

Poor bone health will result in increased risk of osteoporotic fracture. Osteoporosis is a major public health problem through its association with fragility fracture. These fractures are associated with excess mortality (hip and vertebral fractures) and morbidity (all fractures) and hence represent a major public health burden.

1.2 Bone

Bone is a highly specialized form of connective tissue. It is a complex living tissue where the extracellular matrix is mineralized, giving rigidity and strength to the skeleton with some degree of elasticity. Bones serve three functions; (a) mechanical supports and protects of tissues to provide a framework that enable body movements; (b) protective of organs and bone marrow; and (c) metabolic as a storage site for calcium and phosphate (Baron, 1999). Thus the most important role of bone is the homeostatic regulation of blood calcium levels, which is crucial for life. There is increasing evidence that the central control of development and renewal of the skeleton is more sophisticated than previously known (Ducy *et al.*, 2000).

1.2.1 Anatomy of bone

The bones in the body are divided into basic but overlapping shapes. The limb bones and many of the hand and foot bones are long bones and tubular in shape with expanded ends. The bones of the cranial vault, shoulder, pelvis, and rib cage are flat and tabular. The bones of the ankle, wrist, and spine are blocky and irregular (Eriksen *et al.*, 1994). At the gross level, all bones in the skeleton have two basic structural components: compact and spongy bone. Compact (cortical), bone is the solid, dense bone found in the wall of bone shafts and external bone surfaces. Spongy, trabecular, cancellous bone is the porous, lightweight, honeycomb structure. This bone is found where tendons attach, in the vertebral bodies, in the end of long bones, in short bones, and sandwiched within flat bones. The molecular and cellular compositions of compact (cortical) and spongy (trabecular) bone tissue are identical; it is only the difference in porosity (Ross *et al.*, 1995).

1.2.2 Molecular structure of bone

At the molecular level a bone tissue is basically the same in all mammals. The first component is a large protein molecule known as collagen, which constitutes about 90% of bone organic content. Collagen molecules intertwine to form flexible, slightly elastic fibers in bone. A dense inorganic filling of hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, the second component, stiffens the collagen of mature bones. Bone is a highly connective tissue. There are two surfaces at which the bone is in contact with the soft tissues: an external surface (the periosteal surface) and an internal surface (the endosteal surface). These surfaces are lined with osteogenic cells organized in layers, the periosteum and the endosteum (Baron, 1999).

Three primary cell types are involved in forming and maintaining bone tissue. Osteoblasts are bone-forming cells responsible for synthesizing and depositing bone material. Osteoblasts are often concentrated beneath the periosteum. They make large quantities of a material known as osteoid (pre-bone tissue), an uncalcified organic matrix rich in collagen. Calcification of bone occurs when hydroxyapatite crystals deposited into the osteoid matrix and when surrounded by bony matrix, the osteoblasts are called osteocytes.

These osteocytes cells are reside in lacunae and responsible for maintaining bone tissue. Osteoclasts are responsible for the resorption (removal) of bone tissue. Remodeling of bone takes place at the cellular level as osteoclasts remove bone tissue and osteoblasts build bone tissue. These opposing processes of bone formation and resorption allow bones to maintain or change their shape and size during growth (White, 2000).

1.2.3 Bone mineral and mineralization

The composition of bone allows it to perform its mechanical, protective and homeostatic functions. This composition varies with age, anatomic location, diet and health status. Mineral accounts for 50% to 70% of adult mammalian bone, the organic matrix for 20% to 40%, water for 5% to 10%, and lipids for <3% (Lian *et al.*, 1999). The mineral, an analogue of the geologic mineral, hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], provides mechanical rigidity and load bearing strength to the bone composite. In contrast to large geologic hydroxyapatite crystals, bone mineral crystals are extremely small ($\sim 200 \text{ \AA}$ in their largest dimension). Bone mineral contains numerous impurities (carbonate, magnesium, acid phosphate). These small imperfect crystals are more soluble than geologic apatite, allowing bone to act as a reservoir for calcium, phosphate, and magnesium ions (Glimcher, 1998).

Calcium and phosphate ions are required for normal bone formation. Calcium and phosphate deficiency impair mineralization. Calcium deficiency results in increased resorption, as bone being used as a reservoir to maintain extracellular calcium concentration. Phosphate acts as a regulator of bone metabolism (Marcus *et al.*, 1996).

1.3 Bone metabolism

Osteologists distinguish between “modeling” as bone sculpting during growth, and “remodeling” as the process of continuous removal and replacement of bone during life. Proper Bone formation, growth, and repair are critically dependent on the accurate organization of all these processes. A variety of systemic hormones, such as the growth hormone (GH)- insulin-like growth factor-1 (IGF1) signaling system, thyroid hormone, estrogens, glucocorticoids, and vitamin D regulate these processes (Gorski, 1998).

1.3.1 Bone growth and modelling

The framework of the skeleton is apparent early in fetal developments, long before mineralization. The long bones attain their future shape and proportions and left-right symmetry by about 26 weeks of gestation. The skeleton of a newborn baby has about 25g of calcium, an acquired from the maternal placental circulation. From birth to the end of pubertal period of growth, the healthy child increases in length by about threefold, and accumulates large quantities of calcium and phosphate within the skeleton (Gertner, 1999).

The size and shape of each bone and of the whole skeleton are genetically predetermined but growth and the environment influence the expression of this potential. Endocrine factors, nutrition, physical forces and local growth factors all contribute.

In childhood, the skeleton is modelled to meet the needs for growth and strength. Growth in length of bones occurs in the layer of epiphyseal cartilage (the growth plate) by a process of chondrogenesis followed by ossification. This ceases at maturity. Growth in width of bones occurs by intramembranous ossification and

continues at a slow rate through out life while endosteal resorption also continues over the life span. These processes constitute modeling (Heaney, 1994).

Bone grows in size during the first two decades of life, with a spurt during adolescence. This is followed by a period of consolidation. Peak adult bone mass is reached at a bout the age of 28 years for cortical bone and a little earlier for trabecular bone (Recker *et al.*, 1992).

1.3.2 Bone remodelling

The adult skeleton is in a dynamic state, continually broken down and reformed by the action of osteoclast and osteoblast this occurs at sites that are mechanically weakened by stress and where stress micro fractures may develop. Bone remodeling operates to renew ageing bone, to remove fatigue fractures, to adept the skeleton to physical stress (related to physical activity and load bearing) and to release ionized calcium as needed. The actual stimulus for initiation of remodelling cycle is unknown. The remodeling cycle at both cortical and trabecular sites involves a similar sequence of cellular activity. It occurs throughout the skeleton but dominant in trabecular bone since this has ten times the surface area of cortical bone (Riggs and Melton, 1992).

An initial phase of osteoclastic resorption is followed by a prolonged phase of bone formation mediated by osteoblasts. Initially osteoclasts excavate a resorption pit. Then osteoblasts are migrating to line the pit (Figure 1.1). These cells create an osteoid matrix which fills the pit and which is subsequently mineralized. In young adults, the creation of resorption pits with release of calcium is matched by the calcification of newly formed osteoid repairs to earlier pits. Thus there is no net change in calcium balance (Kanis, 1994). In the menopause, an increase in the rate

of bone resorption leads to the creation of many more resorption pits but bone formation fails to increase to restore the lost bony tissue completely. This results in an overall loss of bone (osteoids, cells and mineral) from the skeleton and this is more rapid in trabecular bone because of its large surface area. The amount of bone made under normal condition equal to the amount removed. When this balance is upset, bone loss occurs. In most bone diseases (osteoporosis, hyperthyroidism, hyperparathyroidism), bone resorption is increased more than bone formation (Benker *et al.*, 1988.)

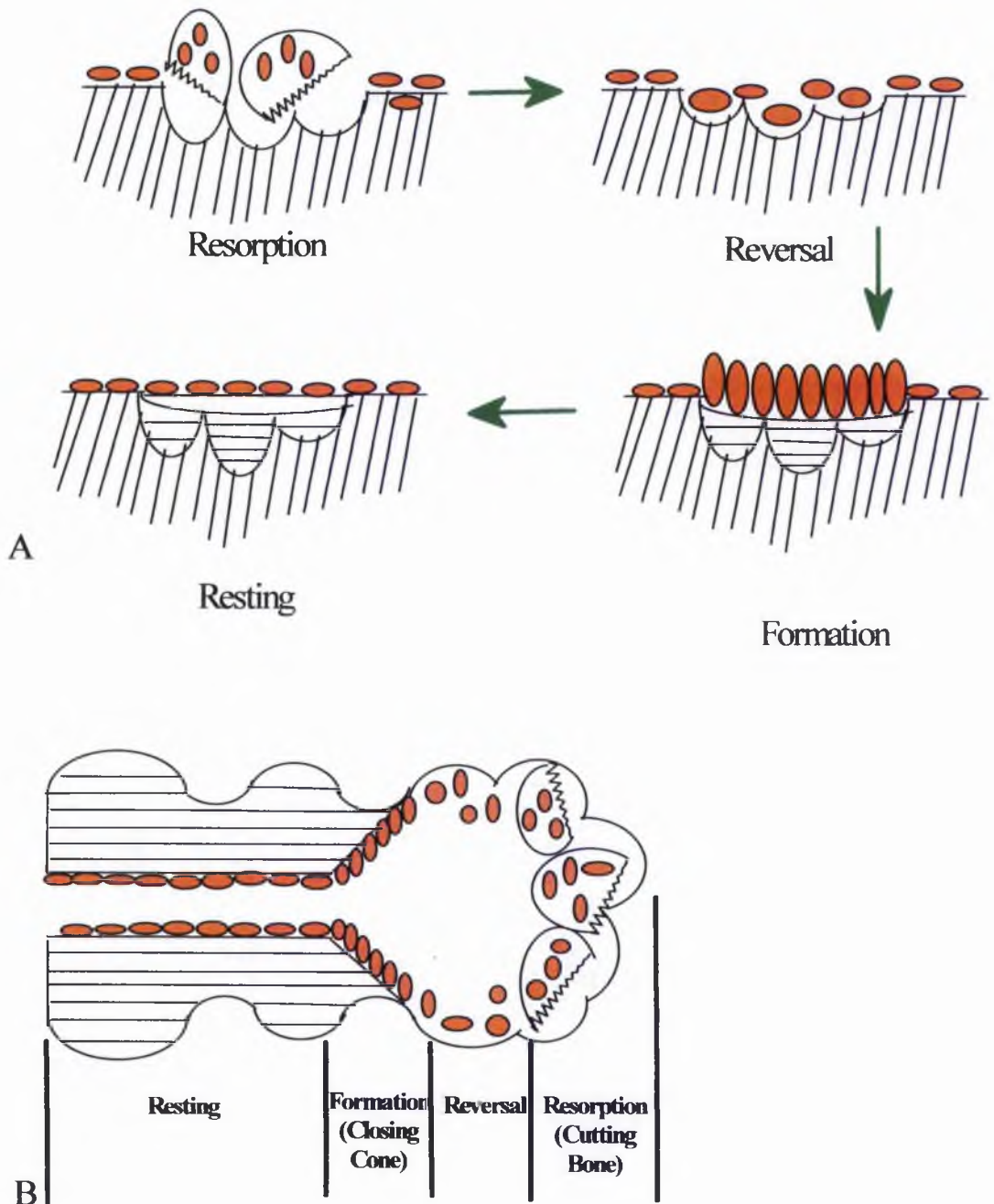


Figure 1.1: Bone remodelling in trabecular (A) and cortical (B) bones (From Riggs and Melton, 1992)

1.3.3 Biochemical markers of bone metabolism

The organic phase of bone is principally composed of collagen (90%) and other smaller matrix proteins, glycoproteins and proteoglycans. The cellular and extracellular components of the skeletal matrix form the basis for the development of biochemical markers that specifically reflect either bone formation or bone resorption. The skeleton throughout life undergoes continuous remodeling with removal of old bone and replacement with new bone. Bone turnover is always initiated by osteoclasts eroding a mineralized surface. When osteoclasts resorb bone, they secrete a mixture of acid and neutral proteases that act sequentially to degrade the collagen fibrils into molecular fragments.

The biochemical markers of bone resorption therefore include collagen breakdown that include hydroxyproline (HYP), hydroxylysine glycosides and the pyridinoline cross-links (PYD). These cross-links range in size from the free amino acids to segments of the N- telopeptides (NTX-I) and C- telopeptides domains (CTX-I) (CTX-MMP). Other markers of bone resorption include tartrate-resistant acid phosphatase (TRACP) and bone sialoprotein (BSP). The process of bone resorption is followed by the recruitment of osteoblasts to the outer edge of the erosion cavity.

The biochemical markers of bone formation are products of osteoblastic synthesis. These include N- and C- propeptides of type I collagen (PICP)(PINP), osteocalcin (OC) and bone-specific alkaline phosphatase (BSAP) (Garnero *et al.*, 1996; Tsukahara *et al.*, 1996). There is much interest in whether the predictive value in respect of future bone loss is improved if markers both of formation and

resorption are assessed simultaneously to reflect the dynamic process of bone turnover (Johnell *et al.*, 2001).

1.3.3.1 Markers of bone formation

Osteocalcin:

Osteocalcin is a small protein (49 amino acids) synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. It is characterized by the presence of three residues of the calcium-binding amino acid, γ -carboxyglutamic acid (Gla), these Gla residues are responsible for facilitating the binding of the protein to hydroxyapatite and maintaining its secondary structure (Gundberg, 2001). Osteocalcin is deposited in the extracellular matrix of bone, but a small amount enters the blood. Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level reflect the rate of bone formation. Serum levels of osteocalcin are elevated in patients with diseases characterized by high bone turnover rate and reflect the expected changes in bone formation. The chief route of circulating osteocalcin catabolism is renal glomerular filtration and degradation. The plasma half-life is about 20 min in humans. Osteocalcin levels follow a circadian rhythm characterized by a decline during the morning to a low around noon, followed by a gradual rise, which peaks after midnight (Gundberg *et al.*, 1985). The major advantages in using osteocalcin as a clinical index of bone turnover its tissue specificity, wide availability and relatively low variation within person (12% – 22%).

Alkaline phosphatase:

Alkaline phosphatase (ALP) (orthophosphoric- monoester phosphohydrolase) belongs to a large group of proteins that are attached to the extracellular surface of cell membranes via a carboxyl-terminal glycan-phosphatidylinositol (GPI) anchor (Moss, 1982; Low and Saltiel, 1988). Although the exact metabolic function of ALP is unknown, it is an enzyme that catalyses the alkaline hydrolysis of monophosphate ester groups and is present in high concentration in the intestinal epithelium, kidney tubules, bone, liver and placenta (Harris, 1989). Four gene loci code for ALP: the three tissue-specific genes encode the intestine, mature placenta and germ cell enzymes and the tissue-nonspecific (*tns*) gene is expressed in numerous tissues (including bone, liver, kidney and early placenta). Tissue nonspecific ALPs are the products of a single gene, but tissue specific differences are found in their electrophoretic mobility, stability to heat and sensitivity to a variety of chemical inhibitors. These differences are due to variations in their carbohydrate side chains (Weiss *et al.*, 1988).

The bone isoform of ALP is produced by osteoblasts as a tetramer and is initially anchored to the outer surface of the osteoblast cell membrane to inositol via a glycan ester (glycosylphosphatidylinositol) (Seargeant and Stinson, 1979). Bone ALP is released from the osteoblast cell surface via a glycosylphosphatidylinositol-specific phospholipase D enzyme. The phospholipase enzyme cleaves ALP from the outer surface of the membrane, releasing it into the circulation in two forms:

- (1) An anchorless, soluble dimeric form.
- (2) An anchor-intact, insoluble form (Anh *et al.*, 1998).

The anchorless, soluble form predominates and constitutes 35 – 40% of the total ALP found in the circulation in health. With either hepatobiliary or bone disease, these different isoforms differ in their concentration in blood. Its primary physiological role in bone is associated with calcification of the skeleton and bone formation. Bone ALP catalyses the hydrolysis of phosphate esters at the osteoblast cell surface to provide a high phosphate concentration for the bone mineralization process as part of the osteoblast cell role in bone remodelling (Fishman, 1990). As a result, bone ALP level is raised in the circulation during periods of active bone formation and bone growth. During life, there are two age-dependent physiological peaks of high bone ALP activity, during infancy and at the time of puberty when bone growth is accelerated by the effects of sex steroids (Behnke, 1998).

Earlier methods for bone-specific ALP measurements relied on the physicochemical properties of the enzyme and on the instability of the enzyme in the presence of elevated temperatures (Moss and Witby, 1975).

The heat-inactivation methods:

Take advantage of the fact that the bone isoform is more susceptible to higher temperatures than the liver isoform. Patient samples are incubated at 56°C for 10-min, and the difference in enzyme activity before and after incubation at 56°C typically reflects the bone isoform contribution to total enzyme activity.

Electrophoresis:

On agarose gels following precipitation with neuraminidase.

High-performance liquid chromatography:

Has also been used to separate the liver and bone isoforms. These methods are tedious, time-consuming and not practical in the setting of routine clinical laboratory testing (Moss and Edwards, 1984; Magnusson *et al.*, 1992).

Immunoassay methods:

The development of monoclonal antibodies, which react, with the bone-specific isoform of ALP in the late 1980 was considered a breakthrough for the routine assessment of this isoform of ALP enzyme by immunoassay (Hill and Wolfert, 1989).

(1) Tandem-R Ostase: The first commercial two-site immunoassay relies on the use of two monoclonal antibodies, both of which react preferentially with the bone isoform. This assay measures the mass amount of the enzyme in a two-site immunoradiometric assay (Garnero and Delmas, 1993). This assay required an overnight incubation and provide low degree of cross-reactivity with liver ALP.

(3) Tandem-MP Ostase: To improve on assay time for bone-specific ALP, Hybritech developed an activity-based assay. This assay uses a single biotinylated monoclonal antibody to bind the serum bone ALP, followed by the addition of the p-nitrophenyl phosphate substrate to produce an activity-based result (Broyles *et al.*, 1998). This activity assay uses a 60-min incubation time.

(4) Metra Biosystems, Alkphase-B: This immunoassay based on enzyme activity measurements for bone-specific ALP. This assay uses a single monoclonal antibody that is bound to a microtiter plate and captures bone ALP from the serum followed by reactivity with the substrate p-nitrophenyl phosphate to assess enzyme activity (Gomes *et al.*, 1995). This assay required a 3-hours incubation.

Measurement of bone ALP along with a specific bone resorption marker allows determining whether coupling of bone resorption to bone formation is normal.

1.3.3.2 Markers of bone resorption

Pyridinium cross-links:

Newly deposited collagen fibrils in the extracellular matrix are stabilized by intramolecular and intermolecular cross-links. The main cross-links in skeletal tissues are the trivalent structures, deoxypyridinoline (DPD) and pyridinoline (PYD). The pyridinoline cross-links occur at two sites placed symmetrically at about 90 residues from the ends of the 1000-residue helical domain in the collagen fibril (Figure 1.2). Pyridinolines act as mature cross-links in types I, II and III collagens of all major connective tissues other than skin (Eyre *et al.*, 1984, 1988). One of the first maturation products of the intermediated cross-links to be identified was pyridinoline (PYD) or hydroxylysyl pyridinoline (HP), a trifunctional 3-Hydroxy-Pyridinium compound (Fujimoto *et al.*, 1978). An analogue, deoxypyridinoline (DPD) or lysyl pyridinoline (LP) has identified in bone (Ogawa *et al.*, 1982) (Figure 1.3). Bone represents the major reservoir of total collagen in the body and turns over faster than most major connective tissues (Eastell *et al.*, 1997). Osteoclasts attach to the bone surface and secrete acid and hydrolytic enzymes that resorb bone, releasing bone minerals and collagen fragments. There are only 40-50% of the Pyridinium cross-link are free and 60% are peptide-bound, many investigators have measured total pyridinolines (peptide bound plus free). Their concentrations in urine reflect only the degradation of insoluble collagen fibers and not of any precursors. The ratio PYD: DPD in urine is similar to the ratio in bone suggesting that both are derived predominantly from bone. DPD is described as a more bone-specific marker since it is more restricted to mineralized tissues (Seibel *et al.*, 1992). The Pyridinium cross-link markers provide distinct advantages. First, they are not influenced by dietary intake (Colwell *et al.*, 1993). Second, they are formed only at the final stages of

fibril formation and therefore unaffected by degradation of newly synthesized collagen. Finally, they are not further metabolized nor are they reused in collagen biosynthesis.

Initially, the assays for Pyridinium cross-links were:

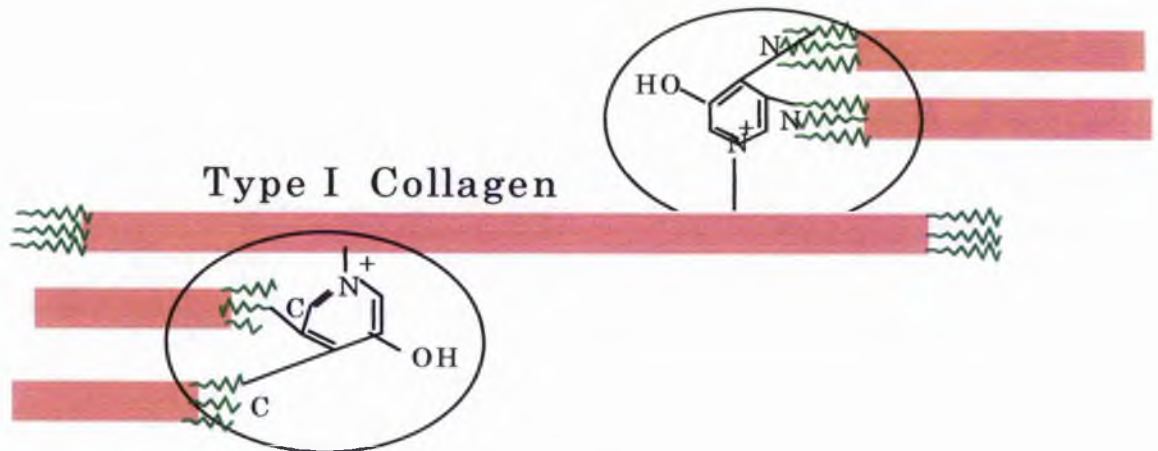
(1) HPLC methods:

With a hydrolysis by acid of the urine sample to liberate peptide bound and conjugated forms, followed by solid-phase extraction, separation by HPLC, and Quantitation by fluorescence (Eyre *et al.*, 1984; Black *et al.*, 1988); despite later automation of the procedure (Pratt *et al.*, 1992), these procedures considered time-consuming.

(2) Immunoassay method:

The observation that the ratio of free to peptide-bound cross-links was similar in urine from healthy individuals and patients with metabolic bone disorders (Robins *et al.*, 1990; Abbiati *et al.*, 1993) opened the way for direct analysis of urine samples without the need for the hydrolysis step. This in turn led to the development of specific immunoassays for DPD (Robins *et al.*, 1994) or for both Pyridinium cross-links (Gomez *et al.*, 1996). Commercial kits are available which measure both DPD and PYD in combination (Pyrilinks II, METRA Biosystems) or DPD alone (Pyrilinks D, METRA Biosystems). Urine for measurements of bone turnover markers should be collected as random, fasting, 2-hours post-voiding samples.

Cross-linked N-telopeptides



Cross-linked C-telopeptides

Figure 1.2: Cross-linked N-and C-telopeptides of type I collagen (From Seibel *et al.*, 1992)

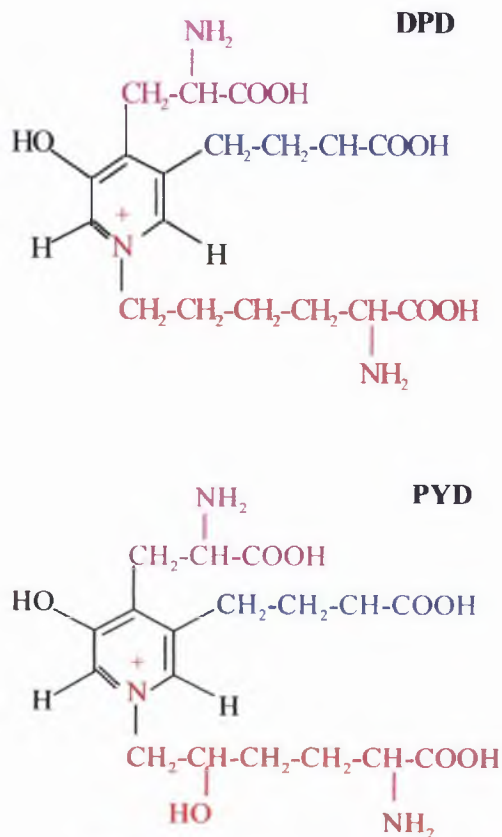


Figure 1.3: Structure of the pyridinium cross-links, pyridinoline (PYD) and deoxyypyridinoline (DPD) (From Seibel *et al.*, 1992)

1.4 Calcium metabolism

1.4.1 Calcium

Calcium is the most important mineral constituent of the skeleton. The adult skeleton contains about 1 kg of calcium and is in equilibrium with the plasma calcium at a concentration of about 2.25-2.60 mmol/l (9-10.4 mg per 100ml). Fifty percent of the extra skeletal calcium is found in the extracellular fluid (ECF). Changes in the concentration of plasma-ionized calcium are usually accompanied by changes in the total amount of calcium in the extracellular fluid since there is a passive distribution of ionized calcium throughout the ECF compartment. Within the plasma 50% of calcium is bound to proteins, mainly albumin (Smith, 2000).

Calcium balance is a function of the integrated fluxes across gut, kidney and bone. Calcium normally enters the body only by its intestinal absorption (Russell, 2001). The true absorption of calcium is greater than the net absorption because some calcium is returned to the gut lumen in biliary, pancreatic and intestinal secretion. Absorption occurs throughout the length of the small intestine and depends both on active transport and simple passive diffusion processes across the gut cell wall. The major sites for active transport are in the duodenum and upper part of the jejunum and predominate when the dietary intake of calcium is low and depend on vitamin D. A large amount of calcium is filtered and most of them are reabsorbed so that only 1-3% is excreted into the urine (Kanis, 1994). Studies with radioisotopes (calcium and strontium) have shown that in normal human adults there is a large exchangeable pool of calcium between bone and the ECF. This exchangeable pool of calcium is important in plasma calcium homeostasis (Staub *et al.*, 1989), but should be distinguished from the movements of calcium that occur in bone as a result of mineralization and bone resorption. Provided that serum calcium

is stable, the total excretion of calcium reflects the net input of calcium to the ECF, largely from gut and skeletal sources.

These fluxes are continually changed and are affected by a variety of factors including several hormones. These hormones can be subdivided into controlling and influencing hormones. The controlling hormones are the major regulating hormones such as parathyroid hormone (PTH) increases renal tubular reabsorption of calcium and bone resorption, calcitonin (CT) inhibits bone resorption and 1,25-dihydroxyvitamin D (calcitriol) increase intestinal absorption for calcium, the secretion of each of which is altered in response to changes in plasma ionized calcium concentrations. The influencing hormones are those other hormones such as thyroid hormones (TH), growth hormones (GH) and adrenal and gonadal steroids which have important effects on calcium metabolism but whose secretion is determined primarily by factors other than change in plasma calcium (Russell, 2001).

Calcium balance

Falling blood calcium signals the parathyroid glands to secrete PTH, then PTH stimulates the activation of vitamin D. Both PTH and vitamin D stimulate calcium reabsorption in the kidneys. Vitamin D enhances calcium absorption in the intestines. Vitamin D and PTH stimulate osteoclast cells to break down bone, releasing calcium into the blood. All these actions result in higher blood calcium levels, which inhibit PTH secretion.

Rising blood calcium signals the thyroid gland to secrete calcitonin, which inhibits the activation of vitamin D. Calcitonin prevents calcium reabsorption in the kidneys and limits calcium absorption in the intestines. It also inhibits osteoclast cells from breaking down bone, preventing the release of calcium. These

actions result in lower blood calcium levels, which inhibit calcitonin secretion (Parfitt, 1980).

1.4.2 Parathyroid hormone

Parathyroid hormone (PTH) is a hormone synthesized by the chief cells of the parathyroid gland. PTH consists of a single polypeptide chain containing 84 amino acids, but only the first 32-34 amino acids (reading from the N-terminal end) are necessary for biological activity. PTH cleavage occurs naturally, partly in the liver, to produce a short N-terminal biologically active fragment and a larger inactive C-terminal fragment. This cleavage may be necessary for PTH to act on bone (Kanis, 1994). In the circulation, PTH consists of several polypeptide fragments that are degraded in the liver and kidney (Juppner *et al.*, 1999).

The major stimulus to PTH secretion is a decrease in the plasma calcium. The target organs for PTH action are bone, kidney and indirectly on gut. PTH acts on the kidney to increase mainly the distal tubular reabsorption of calcium and to depress the proximal tubular reabsorption of phosphate. This action gives a rise in plasma calcium and a fall in plasma phosphate. On bone, PTH stimulates osteoclasts to resorb bone, with an increase in their numbers and activities. Osteoblasts mediate this action since isolated osteoclasts do not respond because they contain no PTH receptors. A direct effect of PTH on osteoblasts has been demonstrated in cell and organ culture. When isolated osteoblasts are treated with PTH they secrete factor(s) that stimulate osteoclasts to resorb bone (McCauley *et al.*, 2001).

Furthermore, PTH stimulates the activity of the renal 1α -hydroxylase, there by enhances the synthesis of $1,25(\text{OH})_2\text{D}_3$, which in turn increases the intestinal absorption of calcium and phosphate (Potts and Juppner, 1997).

1.4.3 Vitamin D

Vitamin D is derived from two sources, namely exogenous (diet) and endogenous (skin). Vitamin D is the generic term for two molecules. Ergocalciferol (Vitamin D₂) and Cholecalciferol (Vitamin D₃). The former is derived by UV irradiation of the ergo sterol that is widely distributed in plants and other fungi where as the later is formed from the action of UV irradiation on the skin. During exposure of skin to sunlight, 7-dehydrocholesterol (7-DHC) absorbs solar radiation with energies between 290 nm and 315 nm, which, causes the transformation of 7-dehydrocholesterol to previtamin D in the skin (DeLuca, 1988). Once formed, previtamin D undergoes a temperature-dependent isomerization over a period of a few hours, which is then metabolized to Vitamin D.

Vitamin D production by the skin is related to latitude, because clouds, ozone and other forms of atmospheric pollution absorb the short UV wavelengths of light necessary for the photo conversion of 7-dehydrocholesterol as they pass through the atmosphere. At higher latitudes the angle of the sun's rays is greater, so the path through the atmosphere is longer and less UV-B reaches the earth's surface. In Scandinavia and the UK (55° N), plasma 25(OH)D levels vary from winter to summer, conversion of 7-dehydrocholesterol occurs only between April and September (Stamp and Round, 1974). Similarly in Canada (52° N), conversion only occurs from April to September (Vieth, 2000). In Boston, USA, at latitude (42.2° N), the photo conversion of 7-dehydrocholesterol occurs only between March and October (Webb and Holick, 1988). By contrast, in countries nearer to the equator, such as Saudi Arabia at latitude (20° N) and Puerto Rico (18° N) there is no evidence of seasonal variation, 7-dehydrocholesterol conversions in winter is similar to the summer months (Ghannam *et al.*, 1999; Loveridge, 2000). The amount of

UV-B radiation penetrating the epidermis is influenced also the degree of melanin pigmentation of the skin, (if the skin is more pigmented it requires longer exposure to UV-B radiation to produce enough quantities of vitamin D), the amount of clothing covering the skin, window glass and sunscreens. The occurrence of several of these factors together may cause vitamin D deficiency (Pettifor *et al.*, 1996).

Despite the assumption that abundant sunshine ensures a decent vitamin D supply, people living in those sunny climes are not ensured of desirable vitamin D concentration. Culture, clothing, and shelter minimize the natural production of vitamin D by the skin (Vieth, 2000). In humans, synthesis in the skin provides the major contribution of vitamin D probably better than 80% (Heaney *et al.*, 2001), but the smaller dietary component is also of significance.

Vitamin D is stored in adipose tissue and muscle (Pettifor, 2003). Schroder and colleagues verifying that the active vitamin D metabolites are stored in adipose tissue that can in turn be released for target tissue uptake (Schroder *et al.*, 2000). It is well known that obesity is a protective factor for osteoporosis, which may be explained, in part, by the storage and release of active vitamin D metabolites in the adipose tissue (Ricci *et al.*, 1998). The lipid nature of vitamin D and its metabolites limit their concentration within the circulation, but a specific vitamin D transport protein (an α_2 globulin) binds a number of vitamin D metabolites (Loveridge, 2000). Either vitamin D₃ or D₂ have biological effect until they are converted to 25(OH)D₃ or D₂ in the liver by the enzyme 25-hydroxylase (Blunt *et al.*, 1968; DeLuca, 1979) and then to 1,25(OH)₂ D₃ or D₂ in the kidney by the enzyme 1 α hydroxylase (Fraser and Kodicek, 1970). The rate of conversion to 1,25(OH)₂D by the kidney is PTH dependent.

Many studies report a fall in the circulating concentration of 25(OH)D and 1,25 (OH)₂ D with advancing age (Fujisawa *et al.*, 1998; Blunosohn and Eastell, 1995). As the skin ages it is less efficient at synthesising vitamin D under the influence of sunlight (Jacobsen *et al.*, 1991) because of the thickness of the epidermis that declines with age. The amount of the 7-dehydrocholesterol is also reduced with age (Holick *et al.*, 1989). Gastrointestinal absorption of vitamin D is less efficient in older people (Barragry *et al.*, 1978) and there is age-associated reductions in 1 alphahydroxylase in the kidney may impair the conversion of 25(OH)D to 1,25(OH)₂D. There is also an age-associated decline in the trophic effect of PTH in enhancing the production of 1,25(OH)₂D in the kidney (Tsai *et al.*, 1984).

Plasma concentration of 1,25(OH)₂D is regulated too closely to be a sensitive measure of vitamin D status. The most commonly used index of status is plasma 25(OH)D that reflects both skin synthesis and dietary intake. The normal range is 25-75 nmol / l. At all ages, from neonates to older people, lower plasma levels of 25 (OH)D are associated with higher levels of PTH (Zeghoud *et al.*, 1997; Khaw *et al.*, 1994). Low plasma phosphate and raised chloride levels characterize the early stages of vitamin D inadequacy. If compensation fails, the level of alkaline phosphatase usually rises and eventually calcium may fall. Very low levels of plasma 25(OH)D (below 12 mmol/l) are associated with clinical disease such as osteomalacia and rickets.

1.4.3.1 Vitamin D metabolites

Many improvements been introduced within the last few years with respect to the methods of assessing circulating vitamin D metabolites. The current

assays are all stand-alone types as opposed to multiple-metabolite assays described in years past (Horst *et al.*, 1981; Lambert *et al.*, 1981); the stand-alone format was chosen because in a clinical situation, a battery of vitamin D metabolite values is seldom required. The assays for 25(OH)D and 1,25(OH)₂D have been optimized as single metabolite procedures (Hollis *et al.*, 1993). From a clinical standpoint only measurements of circulating 25 (OH) D and 1,25 (OH)₂ D supply useful information.

Measurement of 25-Hydroxyvitamin D

The most abundant metabolite of vitamin D in human plasma is 25 (OH) D. The high circulating concentrations of 25(OH)D make this metabolite a useful indicator of vitamin D status. Standard methods to measure 25(OH)D in biological specimen are high-performance liquid chromatography (HPLC), competitive enzyme binding protein assay (CBPA) and radioimmunoassay (RIA) (Bruce *et al.*, 1999).

1) *High-performance liquid chromatography (HPLC)*

The most successful approaches to measuring 25(OH)D have involved extraction of analyte from sample, followed by chromatographic purification, and measurement by absorptiometry of eluant from a chromatograph (HPLC) (Vieth *et al.*, 1995). 25(OH)D is mainly bound to a binding protein, therefore an initial extraction step using acetonitrile or methanol is required for release of the analyte from the binding protein. Further purification can be performed by solid phase (C₁₈-cartridges) or solvent (methylene chloride or chloroform) extraction. Subsequent evaporation to dryness is necessary for both methods to concentrate the sample.

Most of the HPLC- separations are based on regular phase columns (non-derivation silica) using in many cases hexane/isopropanol mixtures as mobile phase (Oberrauch *et al.*, 1999)

2) Competitive enzyme binding protein assay (CBPA)

The second approach was competitive protein-binding of the purified analyte with tritium-labeled 25(OH)D₃. These established methods all require the monitoring of, and correction for analyte recovery. To overcome the limitations of instrumentation respectively the use of radioactive isotopes, the competitive enzyme binding protein assay (CBPA) have been developed (Vieth *et al.*, 1995). This assay is performed in a 96-well microtiter plate and uses human Gc-globulin (vitamin D binding protein) a biotinylated 25(OH) D analogue as tracer, and peroxidase as reporter enzyme. There are two assay procedures for the competitive enzyme binding protein assay (CBPA) in measuring 25(OH)D in human plasma samples; Gc-globulin is directly labeled with horseradish peroxidase, or indirectly detected with a rabbit anti-Gc-globulin antibody. Gc-globulin is either directly labeled with peroxidase (periodate coupling according to the method of Nakane and Kawaoi, 1974, or indirectly using rabbit anti- Gc-globulin antibody. In the second case, Gc-globulin is first reacted with anti-Gc-globulin and then with a peroxidase labeled second antibody (Hollis, 1996).

Vitamin D is first extracted from serum or plasma by ethanol precipitation of the proteins. 25-50 µl samples are mixed with 4 volumes of ethanol, incubated for 30 at -20 °C and then centrifuged at 3000 X g for 5 minutes. The supernatants are used directly in the assay. The biotinylated 25(OH)D (tracer) is bound to the wells of streptavidin coated plates. After washing 20 µl of extracted standard or sample

and Gc-globulin are incubated for 2 to 4 hours or alternatively overnight at 4 °C. 25(OH)D in the extract competes with the 25(OH)D bound to the surface for the binding pocket of the Gc-globulin. Thus the amount of bound binding protein is inversely correlated to the concentration of 25(OH)D present in the sample. Finally, the peroxidase is quantitated by incubation with tetramethylbenzidine (TMP) and this reaction stopped after 20 minutes with 50 µl 0.1 NaH₂SO₄ (Hawa *et al.*, 1999).

3) *Radioimmunoassay (RIA)*

The first valid Radioimmunoassay for assessing circulating 25(OH)D was introduced in 1985 (Hollis and Napoli., 1985). This RIA eliminated the need for sample prepurification before assay. The method was still based on using [³H]25(OH)D as tracer. This shortcoming was resolved in 1993 when an ¹²⁵I-labeled tracer was developed and incorporated into the RIA for 25(OH)D (Hollis *et al.*, 1993). This ¹²⁵I-based RIA was designed around an antisera raised against the synthetic vitamin D analogue 23,24,25,26,27 pentanorvitamin D-C(22)-carboxylic acid. Coupling this compound to bovine serum albumin allowed the generation of antibodies that cross-reacted equally with most vitamin D metabolites.

1.4.4 Calcitonin

Calcitonin (CT) is a 32- amino-acid polypeptide hormone is produced by the C cells of the thyroid gland, which originate from the neural crest. It was originally identified as a hypocalcemic factor (Copp *et al.*, 1962). Its main biologic effect is to inhibit osteoclastic bone resorption. This property led to its use for disorders characterized by increased bone resorption as in osteoporosis, in which there is increase in bone resorption (Carstens & Feinblatt, 1991). Calcitonin also

was reported to inhibit osteocytes and stimulate osteoblasts. CT mediates its biological effects through the CT receptor (CTR). Ambient calcium concentration is the most important regulator of CT secretion. When blood calcium increases acutely, there is a proportional increase in CT secretion, and an acute decrease in blood calcium. Calcitonin degradation by the kidney, liver, bone and the thyroid gland. Most investigators find that women have lower CT levels than men and the serum concentration of CT decline with age (Deftos and Nolan, 1997).

1.5 Osteoporosis

The terminology associated with osteoporosis was developed in the nineteenth century by German pathologists to distinguish osteomalacia, osteoporosis and osteitis fibrosa cystica. Later, when X-rays were available, osteoporosis was not readily distinguished from osteomalacia and the two were often considered together. In 1941, Fuller Albright and colleagues defined osteoporosis pathologically as ‘a condition in which there is lack of bone tissue, but that tissue which remains is fully calcified’ (Albright *et al.*, 1941). This differentiated it from osteomalacia, a condition involving failure of bone matrix mineralization, usually caused by deficiency in vitamin D or disturbance in vitamin D metabolism.

Definition of osteoporosis and osteopenia that are now widely accepted are: Osteoporosis is a progressive systemic skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and risk of fracture, (Anon, 1993). There is proportional loss of all bone elements, cells, osteoid and mineral. The bone becomes porous, because of the imbalanced action of forming and resorbing cells (Dempster and Lindsay, 1993; Riggs and Melton, 1992). The definition proposed by a World Health Organization expert (WHO) group in 1994 was:

“A disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk” (WHO, 1994).

The categories of the disease are defined in terms of bone mineral mass density as follows

Normal - a value for bone mineral content (BMC) or bone mineral density (BMD) within one standard deviation (SD) of the young adult reference mean for that

gender. (There are no absolute standard values other than locally derived population means.)

Low Bone Mass (osteopenia) - a value for BMC or BMD more than one SD below the young adult mean but less than 2.5 SD below this value.

Osteoporosis - a value for BMC or BMD 2.5 SD or more below the young adult mean. Severe or established osteoporosis is the same but coupled with the presence of one or more fractures (Broll, 1996).

Osteoporosis is the most prevalent metabolic bone disease in the United States, Europe and other developed countries. The clinical significance of osteoporosis arises from the fractures; the common ones are vertebral, forearm, and femoral neck. Vertebral compression fractures occur at an earlier age than femoral neck fractures and occur in trabecular rather than cortical bone. There is an increased mortality rate following both hip and vertebral fractures. The most important determinants of osteoporosis are the peak bone mass attained in early adulthood and the amount of bone lost with aging (Smith, 2000). Peak bone mass has been defined as the highest level of bone mass achieved as a result of normal growth. The amount of bone present in the young adult (when bone mass is at peak) is determined by genetic factors (Matkovic *et al.*, 1999), gender, physical activity (Lanyon and Rubin, 1983), hormonal status and the nutritional intake, which includes calcium (Heaney, 1996). Most risk factors are in five major categories: age or age related; genetic; environmental; endogenous hormones and chronic diseases; and physical characteristics of bone (Wasnich, 1999)(Table 1.1). Osteoporotic fractures increase with age; wrist fractures show a rising incidence in the 50s age group, vertebral fractures in the 60s age group, and hip fractures in 70s age group (Figure 1.4). There is at least twofold higher incidence among women compared

with men for all age-related fracture sites. This can be explained by the fact that life expectancy is longer for women and due to the hormonal changes, which occur with menopause.

Annual incidence per
1000 women

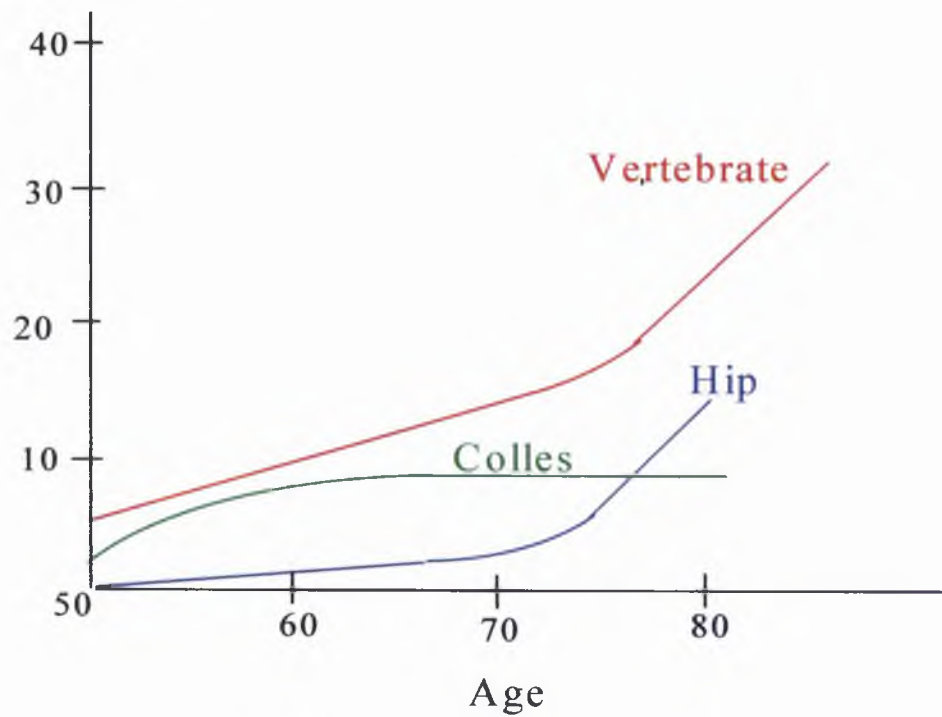


Figure 1.4: Representation of the incidence rates for vertebral, colles and hip fractures in women (From Wasnich, 1999)

Table 1.1: Risk factors for osteoporosis (From Wasnich, 1999)

Age

Each decade associated with 1.4 - 1.8- fold increase

Genetic

Ethnicity: Caucasians and Oriental > blacks and Polynesians

Gender: Female > male

Family history

Environmental

Nutrition; calcium deficiency

Physical activity and mechanical loading

Medications, e.g., corticosteroids

Smoking

Alcohol

Falls (trauma)

Endogenous Hormones and Chronic Diseases

Estrogen deficiency

Androgen deficiency

Chronic diseases, e.g., gastrectomy, cirrhosis, hyperthyroidism,

Hypercortisolism

Physical Characteristics of bone

Density (mass)

Size and geometry

Microarchitecture

Composition

1.6 Assessment of bone health

1.6.1 Fracture rate

An important effect of osteoporosis is fracture following minimal trauma. However, fracture is not always clinically apparent, even to the affected individual. The commonest fractures early after the menopause is of the vertebrae, but a substantial proportion pass unrecognized and only obvious at a later date from radiological examination or as a presumed cause of loss of height. Vertebral fracture cannot therefore be used as a reliable outcome marker for bone health. The other common sites for fracture in older people are the hip and the wrist, where few pass undiagnosed.

Recent trials of supplementation with calcium and/or vitamin D confirm that hip fracture rate can be used as an outcome (Chapuy *et al.*, 1994; Dawson-Hughes *et al.*, 1997). Studies that examine the effect of intervention have only been done in groups of elderly participants aged at least 75 years and over by which age hip fracture incidence is high enough to make studies feasible. This means that there are no data from intervention trials that use hip fracture as the outcome marker in younger postmenopausal women. Worldwide there were an estimated 1.66 million hip fractures in 1990 and it is estimated that this will increase to 6.26 million by 2050 (Cooper *et al.*, 1992).

1.6.2 Bone mineral density

Bone mineral density, as measured by DXA and related techniques Represents the amount of mineral within the bone envelope per unit area scanned (Prentice, 1995). Similarly, the measured of bone mineral content (BMC) is the mass of mineral within the scanned bone envelope. As a consequence, both BMC

and BMD are influenced by the size, shape and orientation of the bone, and can provide no information about internal structure. Measurement of bone mineral density has become an essential element in the evaluation of patients at risk for osteoporosis (Kains *et al.*, 1997).

1.6.2.1 Dual x-ray absorptiometry

Over the past decade, dual x-ray absorptiometry (DXA) has established itself as the most widely used method of measuring BMD because of its advantages of high precision, short scan times, low radiation dose and stable calibration. DXA equipments allows scanning of the spine and hip usually regarded as the most important measurement sites because they are frequent sites of fractures that cause impairment of quality of life, morbidity, and mortality. A measurement of hip BMD has been shown to be the most reliable way of evaluating the risk of hip fracture (Marshall *et al.*, 1996; Black *et al.*, 2000).

The fundamental principle behind DXA is the measurement of the transmission through the body of x-rays of two different photon energies. Because of the dependence of the attenuation coefficient on atomic number and photon energy, measurement of the transmission factors at two energies enables the “areal” densities (i.e., the mass per unit projected area) of two different types of tissue to be inferred (Blake and Fogelman, 1997b). In DXA scans these are taken to be bone mineral (hydroxyapatite) and soft tissue, respectively. Radiation dose to the patient is very low (1 to 10 μSv) (Njeh *et al.*, 1999a) and is comparable to the average daily dose from natural background radiation of 7 μSv . Three of the methods currently in common uses are offered by Hologic, Lunar and Norland. Lunar Radiation Corporation (Living stone, West Lothian) use a cerium-filtered spectrum that

contains two peaks at 50 and 70 keV. Hologic Inc. (Waltham, Massachusetts) produce the two energies by rapidly alternating source voltages and passing the beam through a rotating calibration wheel. The beam then passes through two filters to provide the energies required. The Norland (Norland Medical Systems Inc., Fort Atkinson, Wisconsin) system is based on samarium filter. DXA equipment is expensive and bulky but technical development of the machines has meant that standard scans of the spine and hips can be completed faster and the latest fan beam machines can perform a scan in less than one minute allowing greater throughput. This has been made possible by substituting a fan beam for the previous pencil beam, and by using a high-density array of detectors instead of a single detector. The new systems produce better quality images.

Today, there is general agreement that BMD measurements are the most effective way of identifying patients at risk for osteoporosis (Kains *et al.*, 1997). In particular, in 1994 a WHO study group recommended a clinical definition of osteoporosis based on expressing BMD measurements in standard deviation (SD) units called t- scores (WHO, 1994; Kains *et al.*, 1994). Taking the difference between a patient's measured BMD and the mean BMD of healthy young adults matched for gender and ethnic group, and expressing the difference relative to the young adult population SD calculate the t- score:

$$\text{T- score} = \frac{\text{measured BMD} - \text{young adult mean BMD}}{\text{Young adult standard deviation}}$$

A t- score result therefore indicates the difference between the patient's BMD and the ideal peak bone mass achieved by a young adult. According to WHO a woman should be classified as having osteoporosis if she has a t-score $\leq - 2.5$. An intermediate state of low bone mass (osteopenia) defined by a t-score between $- 2.5$ and $- 1.0$. A t-score $\geq - 1.0$ was taken to be normal. Finally, a fourth state,

established osteoporosis, denoted osteoporosis as defined above, but in the presence of one or more fragility fractures. Alongside the t- score, another useful way of expressing BMD measurements is in z- score units (Blake and Fogelman, 1997b). Like the t- score, the z- score is expressed in units of the population SD. But instead of comparing the patient's BMD with the young adult mean, it is compared with the mean BMD expected for a healthy normal subject matched for age, gender and ethnic origin:

$$\text{Z- score} = \frac{\text{measured BMD} - \text{age-matched mean BMD}}{\text{age-matched standard deviation}}$$

Although it is not widely used as t- scores, but remains a useful concept because it expresses the patient's risk of sustaining an osteoporotic fracture relative to their peers. Typically, every reduction of 1 SD in BMD equates to an approximately twofold increase in the likelihood of fracture (Marshall *et al.*, 1996). It follows that patients with a z- score < - 1.0 are at a significantly increased risk of fracture compared with individuals with a normal bone density for their age.

1.6.2.2 Quantitative Ultrasonometry

Quantitative ultrasonometry (QUS) for measuring the peripheral skeleton has raised considerable interest in recent years (Njeh *et al.*, 1997,1999a). BUA measures the attenuation of the signal as it passes through the patient's heel providing information on the density and structure of the bone. VOS measures the velocity of sound when passing through bone, measured either in transmission (speed of sound, SOS) or reflection (Gluer *et al.*, 1993).

There is a wide variety of equipment available, with most devices using the heel as the measurement site. The calcaneus is chosen because it encompasses a large volume of trabecular bone between relatively flat faces and is readily accessible for transmission measurements. The physical principles of QUS measurements are outlined in (Figure 1.5). An ultrasound pulse passing through Calcaneus bone is strongly attenuated as the signal is scattered and absorbed by trabecular bone. The power spectrum of the pulse transmitted through the patient's heel is compared to a reference trace measured through water alone. The additional attenuation measured through the patient's heel (measured in decibels (dB) increases linearly with frequency, and the slope of the relationship is referred to as the broadband ultrasonic attenuation (BUA: units dB/MHz).

BUA is reduced in patients with osteoporosis, because there are fewer trabecular bones in the calcaneus to attenuate the signal. As well as BUA, most QUS systems also measure the speed of sound (SOS) in the heel by dividing the distance between the ultrasound transducers by the propagation time (units: m/s). SOS values are reduced in patients with osteoporosis because, with the loss of mineralized bone, the elastic modulus of the bone is decreased. Some manufactures combine the BUA and SOS values into a single parameter referred to as "stiffness"

or the "quantitative ultrasound index " (QUI). These combinations have no particular physical meaning, but may improve precision and discrimination by averaging out errors such as those caused by temperature variation (Nicholson and Bouxsein, 1999).

With most early-generation QUS devices, the patient's foot was placed in a water bath to couple the ultrasound signal to the heel. However, later devices are dry contact systems in which rubber pads covered with ultrasound gel are pressed against the patient's heel. A major attraction of bone ultrasound devices is that they do not use ionizing radiation, is relatively inexpensive and portable.

Therefore ultrasound systems could be made much more widely available than conventional DXA scanners, which are largely restricted to hospitals. Moreover, recent evidence from several studies confirms that QUS measurements are predictive of hip fracture risk (Hans *et al.*, 1996; Bauer *et al.*, 1997; Pluijm *et al.*, 1999).

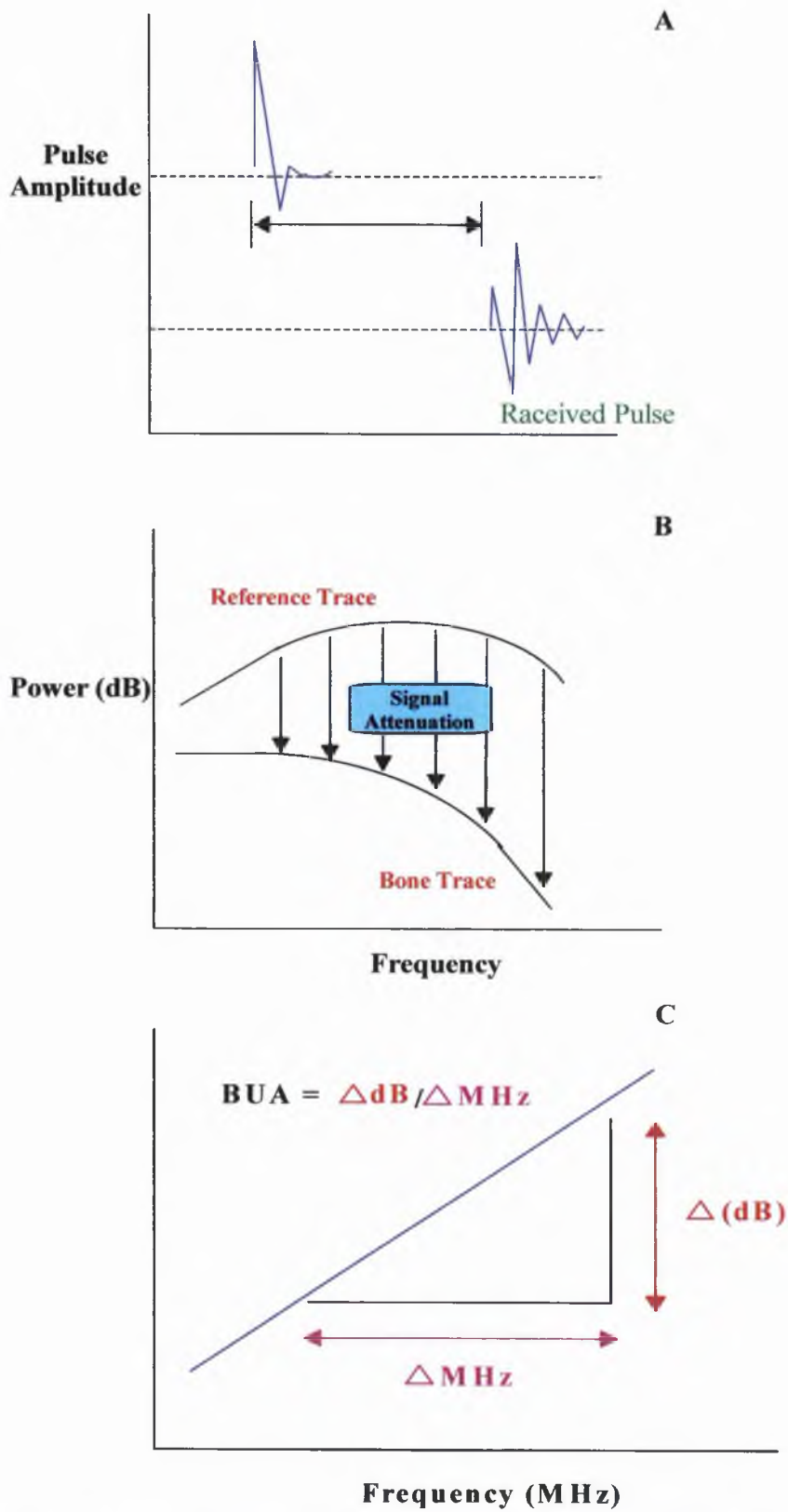


Figure 1.5: The physical principles behind the measurement of BUA and SOS
 (From Blake & Fogelman, 1997)

1.7 Nutrition and bone health

Sufficient nutrition considered to influences all aspects of bone health from the development of peak bone mass in childhood and adolescence through to the maintenance of bone mass in adulthood and the bone loss and fracture in the elderly. Nutrition is a modifiable pathogenic factor of osteoporosis that play a significant role in prevention, and treatment of osteoporosis (Heaney, 1996). The nutrients known to be very important are calcium, vitamin D, protein, and energy. Phosphorus, magnesium, trace minerals (manganese, copper, and zinc), and vitamins C and K are also considered to be important to bone health.

1.7.1 Dietary calcium

The chief sources of calcium in the diet are milk and dairy products (e.g. cheese, yogurt and ice cream). In addition, cereals and cereal products may supply with calcium, although this may be less well absorbed from wholegrain cereals due to the presence of NSP (non-starch polysaccharides) and phytate. Green leafy vegetables such as spinach, broccoli and kale contain good amount of calcium, but its absorption may be inhibited due to the presence of oxalates.

Other sources of calcium may include tofu (soybean curd), oysters, small fish, (such as sardines and whose bones (when eaten) supply calcium) dried figs, nuts (e.g. almonds and brazil nuts), parsley, watercress, sweet potatoes, black treacle and some seeds (such as sesame seeds) (Barasi, 1997). Hard water (contains many dissolved salts) can supply significant amount of calcium (Lentner, 1981) (Table 1.2).

Table 1.2: Sources of dietary calcium, mg /100 g (From Woolf and Dixon, 1998)

		Ca
Fruit		7 – 20
Vegetables	Leaf	50 – 150
	Root	25 – 50
Nuts		50 – 200
Bread	Fortified white	84
	Unfortified white	43
	Wholemeal	84
Milk	Whole	133
	Dried skimmed	1300
Cheese	Cheddar	750
Soya flour medium fat		244
Meats and poultry		5 – 10
Fish		5 – 60

Absorption of calcium

Calcium salts are not highly soluble, which makes their absorption from the diet a problem. Many factors can enhance or inhibit calcium absorption. The most important of enhancing factors is vitamin D, which helps to make the calcium-binding protein in the intestinal cells that needed for absorption and transporting calcium into the plasma. The ability to synthesize this protein is regulated by homeostatic mechanisms involving parathyroid hormone and $1,25(\text{OH})_2\text{D}$, in response to changes in circulating levels of plasma calcium. The calcium in milk is better absorbed than that from most other sources due to lactose (milk sugar) that enhances calcium absorption by keeping it in a soluble form. Other sugars and protein also enhance calcium absorption (Whitney *et al.*, 2002). Gastric acid secretion of the upper digestive tract facilitates the solubility of calcium. In old people acid secretion Reduced, reduces the absorption of calcium (Smith, 2000). Whenever calcium is needed, the body increases its production of the calcium-binding protein to improve calcium absorption. The result is obvious in the case of

growing children that absorb up to 60 percent of the calcium they consume. Then, when bone growth slows or stops, absorption falls to the adult level of about 30 percent. In addition, absorption becomes more sufficient during times of inadequate intakes. Many of the conditions that enhance calcium absorption inhibit its absorption when they are absent, sufficient vitamin D supports absorption, while its deficiency impairs absorption. Calcium absorption is reduced by phytic acid due to the formation of insoluble calcium phytate. Oxalates may also inhibit calcium absorption due to the insoluble nature of the calcium oxalate salt. Non-starch polysaccharides may trap some calcium making it unabsorbable in the small intestine.

Excretion of calcium

Calcium is lost from the body via the faeces and urine, with very small amounts lost in sweat. Loss in the faeces represents the calcium unabsorbed from the diet, together with endogenous calcium from digestive secretions, especially bile and cells shed into the digestive tract, approximately 100 mg/day. Urinary calcium represents the final adjustment of plasma calcium levels, with the majority of the calcium filtered being reabsorbed by the renal tubules up to 97 percent (Barasi, 1997).

Calcium deficiency

Calcium may be lacking in the body if absorption is impaired. A diet low in available calcium may also be a contributing factor. Deficiency of calcium in the body gives rise to the development of rickets in childhood, osteomalacia in adult life and osteoporosis in the elderly people (Barker, 2002). The National Osteoporosis Society recommends an increased calcium intake for those at risk of osteoporosis

(NOS 1999). Their recommendations are 1000 mg calcium per day for women aged over 45 years, 1500 mg calcium per day for women aged over 45 years and men aged over 60 years. These figures are based on the NIH Consensus Statement (NHI 1994).

1.7.1.1 Role of calcium in bone development

There is considerable interest regarding the role that calcium plays in both peak bone mass attainment and postmenopausal bone loss (Heaney, 2000; Speaker, 2000). During adolescence bone growth is considerable, and greater for boys. This requires a daily-absorbed amount of calcium of at least 250mg for girls and 300mg for boys, and it can be higher. It is agreed that it is important to meet these requirements in order to achieve a peak bone mass which reflects the genetic potential of each individual (Prentice, 1995). Several recent supplementation studies of children and adolescents 7-15 year, have observed increases in BMC and BMD of 2 - 6 % in response to supplementation with calcium salts (Johnston *et al.*, 1992; Lloyd, *et al.*, 1993; Andon, *et al.*, 1994; Lee, *et al.*, 1995; Lloyd, *et al.*, 1996; Bonjour, *et al.*, 1997; Stear, *et al.*, 2000a,b). In general, this has not been associated with alterations in skeletal size although increases at the lumbar spine have been reported (Lee, *et al.*, 1995; Bonjour, *et al.*, 1997). There is evidence that the increase in bone mineral appears early in the supplementation period with little additional effect thereafter (Johnston, *et al.*, 1992; Nowson, *et al.*, 1997), that it is associated with a decrease in bone formation and that it disappears on withdrawal of the supplement (Slemenda, *et al.*, 1993; Lee, *et al.*, 1994; Lee, *et al.*, 1995). The long-term benefit of Ca supplementation on bone mass remains to be determined (Ghatge, *et al.*, 2001).

Recent evidence suggests that milk may exert an anabolic effect on the growing skeleton that is different from that of calcium salts alone, possibly as a result of the associated increase in protein intake (Cadogan, *et al.*, 1997). Increase in bone mineral has also been observed after supplementation with milk and dairy products (Cadogan, *et al.*, 1997; Chan, *et al.*, 1991), some data suggest that milk basic protein (casein) suppresses osteoclastic-mediated bone resorption, which result in the prevention of bone loss in the animal model (Toba *et al.*, 2000).

1.7.1.2 Role of calcium in postmenopausal bone loss

The menopause is defined as the cessation of menses, which marks failure of the ovarian function to secrete the sex hormones, estrogen and progesterone. In the peri- and early postmenopausal period there is sparse evidence to link bone loss with customary calcium intake. In studies that have included both early postmenopausal and older women, calcium supplementation appears to have had little effect on BMD in women who are within the first five years after the menopause (Dawson-Hughes *et al.*, 1990). However, in general, women receiving calcium supplements had BMDs that were 1-3 per cent higher than those who did not receive supplements, particularly in region of the skeleton rich in cortical bone. Long-term studies suggest that any effects of calcium supplementation largely occur in the first 1-2 years (Elders *et al.*, 1994; Reid *et al.*, 1995) and that they are mediated by a reduction in bone turnover (Reid *et al.*, 1993; Prince *et al.*, 1995). There is now a general consensus of agreement that Ca is effective in reducing bone loss in late post-menopausal women particularly in those women with low habitual Ca intake (<400mg/d).

1.7.2 Dietary vitamin D

There are few sources of dietary Vitamin D present. It found mainly in foods of animal origin in which it has been already formed; egg yolk, butter and oily fish such as herrings, sardines, mackerel and salmon. Cod and halibut liver oils are very rich sources. Most homogenized milk, and spreading fats (including margarine, low- fat spreads) are artificially fortified with synthetic vitamin D. It might expect that milk and cheese are good sources of vitamin D, but in fact they contain only small amount of this vitamin (Table 1.3). Vitamin D, either natural or added, is stable in foods and storage; processing or cooking does not affect its activity (Loveridge, 2000).

Vitamin D activity was expressed in international units (IU), but is now expressed in micrograms: 1 IU= 0.25 μg . In the UK, the average dietary intake of vitamin D is 3 μg and the range is 0.5-8 μg / day. With the major source of vitamin D being derived from the skin, establishing any requirement is hard. There is no dietary reference value for adults living a normal lifestyle because they manufacture an adequate supply from the sun. For people who do not go out in the sun particular old people, an intake of 10 μg / day has been set (DoH 1991a). An intake of 10 μg / day is also recommended for pregnant and lactating women. The reference nutrient intake (RNI) for the housebound is 10 μg / day (Barker, 2002).

Absorption of vitamin D

This fat-soluble vitamin, when ingested, is absorbed through the intestinal walls with other fats with the aid of bile. About 50 percent of dietary vitamin D is found in the chylomicrons leaving the digestive tract in the lymph; most of the vitamin finds its way to the liver with the remnants of the chylomicrons. Vitamin D

synthesized in the skin diffuses into the blood, and then is picked up by a specific vitamin-D-binding protein (DBP), which transports it to the liver, some may remain free and deposited in fat and muscle (Barasi, 1997).

Vitamin D deficiency

In vitamin D deficiency, production of the protein that binds calcium in the intestinal cells slows. So even when calcium in the diet is adequate, it passes through the gastrointestinal tract unabsorbed, leaving the bones unsupplied. Thus vitamin D deficiency results in a poorly mineralized skeleton, causing rickets in children and osteomalacia and osteoporosis in the adults, which can cause fractures in the elderly (Whitney, 2002).

Vitamin D toxicity

There are some toxicity problems related to hypervitaminosis D. These occur with high doses of more than 1,000-1,500 IUs daily for a month or longer in adults, more than 400 IUs in infants, or more than 600 IUs daily in children (Hass, 1992)

Table 1.3: Vitamin D content of food, ug/100g (From Loveridge, 2000)

Cereals	
Grain, flours, starches	0
Milk and milk products	
Cow s milk	0.01 - 0.03
Human milk	0.04
Dried milk	0.21
Cream	0.1 - 0.28
Cheese	0.03 - 0.05
Yoghurt	Trace - 0.04
Eggs	
Whole	1.75
Yolk	4.94
Fats and oils	
Butter	0.76
Cod liver oil	210
Margarines and spreads*	5.8 – 8.0
Meat and meat products	
Beef, lamb, pork, veal	Trace
Poultry, game	Trace
Liver	0.2 – 1.1
Fish and fish products	
White fish	Trace
Fatty fish	Trace – 25
Crustacea and mollusks	Trace
Vegetables	0

1.7.2.1 Role of vitamin D in bone health

The effect of vitamin D and its metabolites on bone is very complex. It is known to stimulate matrix formation and bone maturation, enhance osteoclastic activity and may influence differentiation of bone cell precursors (Henry and Norman, 1984). Together with PTH and Ca, it regulates Ca and P metabolism and promotes Ca absorption from the gut and kidney tubules. A deficiency of vitamin D has been shown to reduce Ca absorption, increase PTH excretion thereby stimulating osteoclastic activity and thus increasing bone loss (Parfitt *et al.*, 1982). Vitamin D is vital for optimum growth and development in the younger population.

Vitamin D deficiency and insufficiency are important nutritional factors that require careful study in all population groups. Evidence is emerging that vitamin D "insufficiency" is more prevalent in children and adolescents than previously thought (El-Hajj Fuleihan *et al.*, 2000). Furthermore, there is growing concern that atmospheric pollution, particularly in developing countries, may have important implications for the vitamin D status of population groups, both 25-(OH) D and PTH were significantly different in children from high-pollution areas compared with those from low-pollution areas (Pulliyel *et al.*, 2000). In more recent study, on young and old men who were on daily oral supplementation with 800IU of cholecalciferol during eight weeks, both young and old had similar increases in plasma 25(OH) D during this period (Harris and Dawson-Hughes, 2001).

Of considerable interest in the area of vitamin D research were the finding of a global study of vitamin D status and parathyroid function in postmenopausal women with osteoporosis, bone mineral density (BMD) was found to 4% lower in women with serum 25(OH) D below 25 nmol / l. The results also suggested that women with low vitamin D levels (<25 nmol / l) had 30% higher serum PTH values

and higher levels of alkaline phosphatase, a marker of bone formation (Lips *et al.*, 2000).

1.7.2.2 Role of calcium and vitamin D in preventing fracture

Supplementation trials have shown that vitamin D improves Ca absorption, lowers PTH levels, and reduced bone loss in postmenopausal women (New, 1999). Vitamin D and Ca supplementation have been shown to significantly reduce fracture rates in both institutionalized (Chapuy *et al.*, 1992) and free-living elderly populations (Dawson-Hughes *et al.*, 1997). In an interesting paper presented by (Grados *et al.*, 2000), supplementation with 500 mg of calcium and 400 IU of vitamin D₃ twice a day for 12 months in a group of elderly women with vitamin D insufficiency resulted in significant increases in BMD at the femoral neck, femoral trochanter, and whole body sites and significant alteration in bone remodeling. Recent study reported on the effect of 800 IU vitamin D together with 1200mg Ca supplementation on the risk of falling and on muscle strength in an elderly population, a 49% reduction of falls was observed and of interest, markers of bone turnover were significantly reduced (Bischoff *et al.*, 2001).

1.7.3 Dietary protein

Early studies have shown that high dietary protein intake is a powerful determinant of urinary calcium loss (Margen *et al.*, 1974). Protein is metabolized to organic acids and these may be buffered at the expense of bone (Bushinsky, 1989). Total nutrition, and specifically adequacy of protein and energy intake, is important. First, malnutrition predisposes to falls. Second, soft tissue mass over bony prominences (e.g., lateral hip) distributes the energy sustained in falls and thereby

reduces point loads on bone. Finally, adequacy of protein intake is a major factor in determining outcome after hip fracture (Delmi *et al.*, 1990). An adequate amount of dietary protein is essential to maintain production of hormones and growth factors that modulate bone synthesis (Heaney, 2001). At any age, the balance of the total dietary acid and alkaline load is critical to the excretion of calcium. This is achieved with a diet balanced in protein foods that generate acid and fruits and vegetable that provide the alkali to neutralized the acid (Tucker *et al.*, 1999). The Committee on Medical Aspects of Food Policy (COMA) recommended average daily intakes of protein for adults the RNI is 55.5 g / day for men and 45 g / day for women (DOH 1991).

1.7.4 Dietary phosphorus

Phosphorus is often considered along with calcium major mineral constituent of bone. It contributes 50% of the weight of bone mineral. It is widely available in both animal and plant foods including meat, poultry, fish, eggs and dairy products and in cereals, nuts and legumes. Small amounts occur in coffee and tea. It is widely present as food additive in processed meats, soft drinks and bakery goods. Total phosphorus is similar in animal and plant foods, typically 8-20 mg per gram of protein. Phosphorus in protein from meat, poultry and fish is found as phosphate bound to amino acid side chain, which is released during digestion. While phosphorus in plant is found as phytate are digested poorly (Massey and whiting, 2003). Dietary phosphorus deficiency is unlikely to occur in population eating normal diet. In fact, there is a concern that eating modern diet might result in excessive phosphate intake.

Phosphorus level in the body is regulated mainly by renal excretion under the influence of parathyroid hormone, which causes increased urinary loss. This will allow plasma calcium level to rise (Barasi, 1997).

1.8 Nutritional epidemiology

Nutritional Epidemiology objective is to provide the scientific evidence to support the understanding of the role of nutrition in the causes and preventing the illness. Epidemiology defined as having three aims: 1) to describe the distribution and size of disease, 2) to elucidate the etiology of diseases, 3) to provide the information necessary to manage the prevention and control of disease.

1.8.1 Dietary assessment

The goal of assessing diet in population groups is to enable estimation of food consumption patterns, to determine nutrient intake and to explore the effects of diet on health. There are two main approaches to individual dietary assessments, prospective and retrospective. Prospective methods involve recording current diet, while retrospective methods require recording either recent or past diet. There are many methods in use for the measurement of diet in cohort, cross-sectional and intervention studies. These methods generally consist either of the collection of observations from separate days investigation, as in (records, checklists and 24-hour recalls) or attempt to obtain average intake by asking about the usual frequency of food consumption, as in the (diet history and food frequency questionnaires FFQ) (Margetts and Nelson, 2001).

1.8.1.1 Food frequency questionnaires

Food frequency questionnaires (FFQ) are the most frequently used in cohort studies in epidemiology. They provide a means of studying intake retrospectively. They designed to assess usual eating habits, over recent months or years. To assess the frequency of food consumption, accompanying the food list is a

multiple response grid in which subjects are asked to say how often they usually consume an item of food or drink and how much they usually have on the days they consume it. These questionnaires vary from very short with only nine food items to assess a single nutrient (calcium) in a study of osteoporosis (Nelson *et al.*, 1988; Cooper *et al.*, 1988) to food lists numbering 190 items or more for assign a wide variety of nutrients.

1.8.1.2 Food diaries:

In this technique the food eaten is simply recoded in a notebook, without being weighed and the portion eaten is being quantified with tables of average portion sizes that based on measurements of typical portions (MAFF, 1993). This method requires that the subject is literate and physically able to write. The method remains subject to possible changes in the diet by the respondent and failure to record all food eaten. However, if subjects are adequately instructed, reasonably comprehensive records can be obtained. Generally, women produce more reliable records by this method than men (Barasi, 1997).

1.8.2 Food guides:

Food guides provide a framework to show how food can be combined together in a days' eating to provide an overall intake, which contain the appropriate range of nutrients. They grouped together foods that provide similar nutrients that could be interchangeable in the diet and made a number of 'serving' of foods from each group to be taken daily. Food guides were first appeared and used in the United States in 1916. They consisted of five food groups – milk and meat, cereals, vegetables and fruits, fats and fat foods, sugars and sugary foods. Further

developments occurred between the 1940s and 1970s, to the number and components of the groups used. These changes increased the understanding of the role of diet in health and disease prevention, in particular the consumption of fat, sugar and alcohol.

The most recent version of the USA Food Guide is a pyramid (Figure 1.6). This indicates that the food in the groups at the base of the pyramid should be present in the greatest amounts and 'support' the diet. Upper layers should be consumed in smaller amounts. Those at the top (fats, oils and sweets) should be used sparingly and are the least important components of the diet. The guide indicates the numbers of servings as a range; individuals with lower nutritional needs (e.g. children) should take the lower number of servings. The guide does not apply to infant feeding. Also the guide does not imply that any single food is essential in the diet; no food alone provides all the necessary nutrients. The important is to include variety in the diet, so shortcoming in one food is likely to be compensated by an adequate intake in another food (Barasi, 1997).

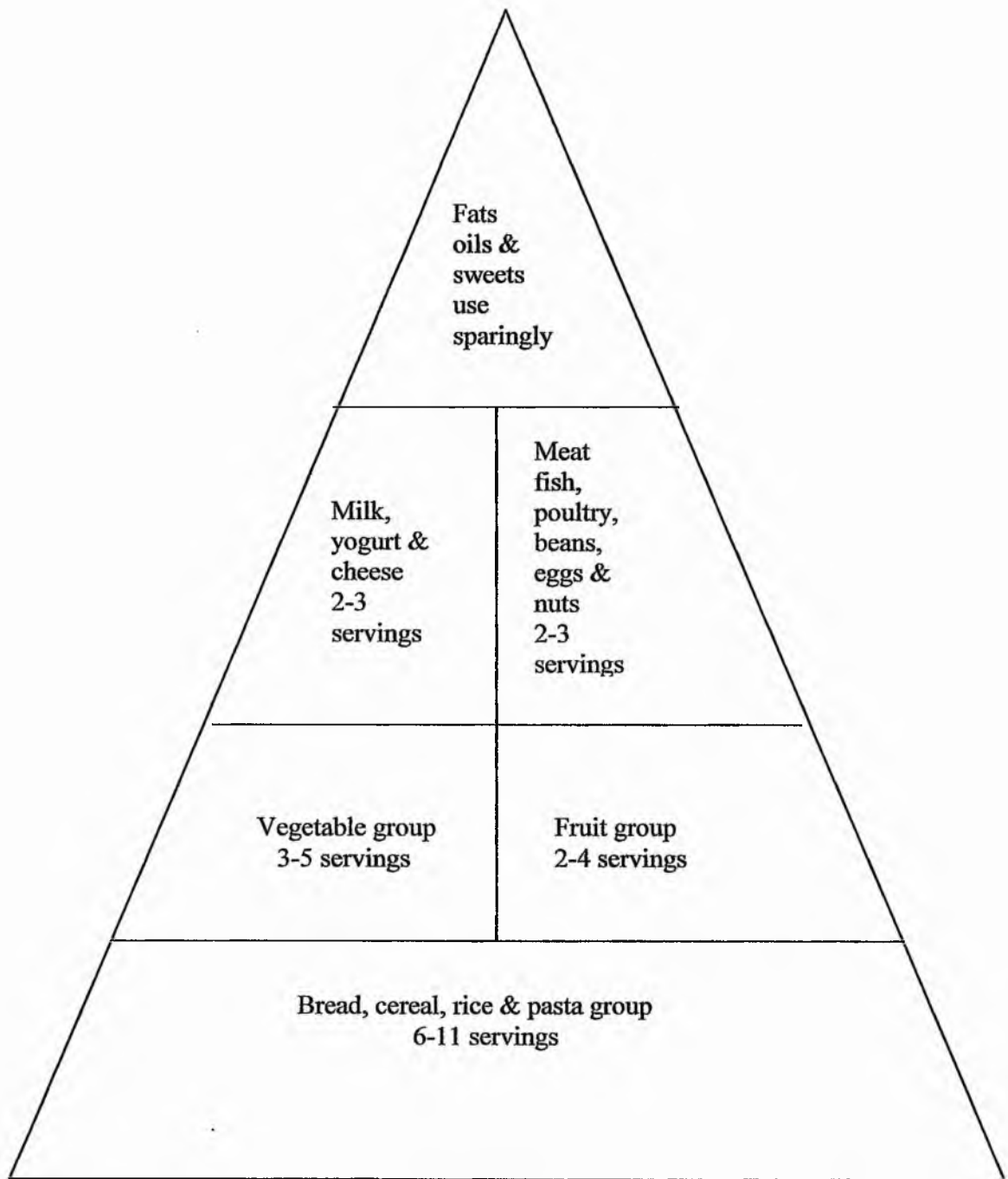


Figure 1.6: The USA Food Guide pyramid: a guide to daily food choices (from US Department of Agriculture/US Department of Health and Human Services).

In the UK, a National Food Guide was launched for the first time in 1994. The design is a tilted plate incorporating five food groups in the following proportion:

Food group	Segment size as % of Whole plate
Bread, other cereals and potatoes	33
Fruit and vegetables	33
Meat, fish and alternatives	12
Milk and dairy foods	15
Fatty and sugary foods	8

The emphasis in the UK Guide is on foods rather than nutrients and shows the importance of considering the diet as a whole rather than concentrating on specific foods that may be 'good' or 'bad'. The purpose of this guide is to provide a message of balance and proportion of the food group in a practical way and to allow choices based on personal preferences and dietary habits and take into account availability, costs, cultural norms and ethnic diets. The best way to use the food guide is to calculate the proportion of the total daily diet provided by foods from the different food groups in terms of numbers of servings eaten during the day and the contribution of each of these servings to the whole diet, which is important. Adding together the numbers of servings gives the following percentages of the total:

Bread / cereal group	6 servings = 28%
Fruit and vegetables	3 servings = 14%
Meat and alternatives	4 servings = 19%
Dairy foods	4 servings = 19%
Fats and sugars	4 servings = 19%

In this way it is possible to focus on which parts of the diet need attention (Barasi, 1997).

1.9 Physical activity

More than one century ago, the German scientist Julius Wolff proposed the theory that is now known as 'Wolff's Law; i.e. 'bone accommodates the forces applied to it by altering its amount and distribution of mass'. More recently, this concept has been refined to a general theory of bone mass regulation, known as the mechanostat model (Frost, 1987, 1992). There is long standing recognition that physical activity protects against risk of fracture in postmenopausal women (Cooper *et al.*, 1990; Jaglal *et al.*, 1993). Since physical activity is a lifestyle factor, which can be modified, it is important to identify the nature and level of those activities. The loading on the bone either from gravitational force, or from muscular tension influences its functional strength (Frost, 1993). Both arise through weight-training (lifting weights) involves muscular tension alone. Most activities have both weight bearing and non-weight bearing components.

In general, weight-bearing activities have a positive effect in increasing BMD in young women (Taaffe *et al.*, 1997) and premenopausal women (Bassy and Ramsdale, 1994) and in helping to maintain BMD after the menopause (Chow *et al.*, 1987). Dynamic exercises, which increase the loading on the weight-bearing skeleton, show a maximum effect from high impact activities such as jogging and jumping (Ayalon *et al.*, 1987; Dalsky, 1988; Kohrt 1995). Walking, since it is a weight-bearing activity needs to be brisk to have an effect on BMD (Krall and Dawson-Hughes, 1994). Muscular activities without weight bearing or impact, such as swimming (Grimston *et al.*, 1993) and cycling (Rico *et al.*, 1993) do not influence BMD.

An active lifestyle at all ages promotes good general health, and diverse physical activities bring benefit to the health not only of bones, but also of other

body systems. All physical activity contributes to energy expenditure (which is desirable). Physical activity such as walking may stimulate appetite. This would be valuable for those elderly people whose low food intake makes nutrient inadequacies more likely. Walking at a normal pace appears not to confer benefit for BMD except perhaps in those who are extremely sedentary (i.e. walking less than 10 minutes per day in total), but it does contribute to improved balance and muscle coordination, which in turn might help to prevent the falls which precipitate fractures. Participation for about 30 minutes in varied physical activities with a weight-bearing component on five days a week would be expected to promote stronger bones.

Examples include:

- For children and young adults: high impact activities such as jogging, jumping, or skipping and games which require these such as basketball, also energetic dancing;
- For middle-aged people: stair climbing, jogging, or walking briskly (a pace of at least four miles per hour, ideally on a gradient, step exercises, racket sports and hill walking);
- For older people: stair climbing and walking as briskly as realistic and safe, also dancing.

1.9.1 Role of physical activity on development and maintenance of the skeleton

It is well known that in the absence of weight-bearing exercise bone loss will occur at both axial and appendicular skeletal sites (New, 2001). Numerous studies in athletes show positive effects of training on different bone sites. Similar findings resulted from studies involving the effect of moderate and everyday exercise on bones of ordinary people and even children. In a study on monozygotic twin pairs aged 5 to 14 years suggest that different weight-bearing activities are positively associated with the development of bone mass in several skeletal regions including the hip (Slemenda *et al.*, 1991). In another study on a population of females and males from 13 to 28 years of age, only weight-bearing activity and weight contributed to the lumbar spine bone mineral density during puberty and young adulthood (Welten *et al.*, 1994). The optimal type and duration of weight-bearing exercise, however, remain to be determined (Buell *et al.*, 1999). Evidence suggests that the effect of exercise on bone mass is primarily due to its influence on bone volume rather than to the distribution of bone tissue within the bone as an organ (Haapasalo *et al.*, 2000). There is a positive effect of high intensity sport on bone mass in boys aged 10-16 years (Afghani *et al.*, 2001).

Recent studies showed the positive effects of high-impact exercise on bone health of early postmenopausal women (Engelke *et al.*, 2002; Mologhianu *et al.*, 2002; Brankovic and Pilipovic, 2002). A recent study indicated an improvement in both bone mass and quality of life in postmenopausal women who participated in a number of daily exercise routines (Eskiyurt *et al.*, 2002). These data were confirmed by a prospective study examining the relationship between daily physical activity (including walking, cycling, household activities, and sport) and risk of

falling in elderly men and women aged 65-88 years. A high level of total physical activity and walking was found to protect against falls (Pluijm *et al.*, 2002).

Furthermore, exercise may be of benefit in the prevention of osteoporosis, not necessarily via the mechanism of increasing bone mass but instead by increasing muscle strength, coordination, flexibility and balance, and thus reducing the tendency to fall (Eastell, 1999).

1.10 Bone health in Saudi Arabian women

Saudi Arabia is located in the Arabian Peninsula south west of Asia. The population is estimated to be approximately 14.87 million, with 38% of population above the age of 40 years. It is a sunny country with abundant sunlight all year round. There was not been much attention given to on bone health in Saudi Arabia But due to significant socioeconomic progress and with the improvement of living condition and health care system, there is expectancy to prolong life leading to an increase in the number of elderly in the population.

Very few studies have been published examining BMD in healthy Saudi females (Ghannam *et al.*, 1999; Desouki, 2003). The study published by Ghannam *et al* measured the bone mineral density (BMD) at the lumbar spine and the proximal femur in 321 Saudi females aged 10-50 years to establish normative data in Saudi females and to compare these data with their USA counterparts. This study concludes that BMD in healthy Saudi females was significantly lower than those USA females. The authors attributed this finding to increased number of pregnancies and longer duration of lactation together with prevalent vitamin D deficiency. The other pilot study presented by El-Desouki was to estimate the prevalence of osteopenia and osteoporosis in postmenopausal Saudi women aged 50-80 years. The results showed only (29%) of subjects (802 postmenopausal) are normal, whereas (31%) are osteopenic and (40%) were osteoporotic at the lumber spine. The conclusion of both studies indicated that the osteopenia and osteoporosis are common among postmenopausal Saudi women in the capitals Riyadh and their BMD are lower than that in their USA counterparts (Table 1.4). Ghannam *et al* have studied the correlation between vitamin D and bone health.

There is data to suggest that vitamin D deficiency is common in Saudi Arabia, although it remains identified in different age groups (Sedrani *et al.*, 1983; Al Arabi *et al.*, 1984; Fonseca *et al.*, 1984). The implications of this on indices of bone health remain undefined. However, there is evidence of a high level of fracture in urbanized communities (Al Nuaim *et al.*, 1995). Al-Nuaim *et al* reviewed case records of Saudi residents of Riyadh city who were 40 years or older and who were admitted to local hospitals over a period of 12 months (July 1990-1991) with diagnosed with proximal femur fracture (PFF). The incidence of PFF for male and female patients over the age of 50 years was 71 and 100 respectively with a female: male ratio 1: 4.

Recently, Ardawi *et al* (2004) determined the reference values of bone mineral density in healthy Saudis of both sexes in Jeddah area (Western region) and compared with US / Northern European / Lebanese reference data. They found that the prevalence of osteoporosis in the Saudi population (50-79 years) at the lumbar spine and total femur using the manufacturers vs. Saudi reference data was 38.3 %-47.7 % vs. 30.5 % - 49.6 % and 6.3 -7.8 % vs. 1.2 - 4.7 % respectively.

In summary, further research into the extent of vitamin D deficiency and prevalence of poor bone health is urgently required in Saudi postmenopausal and premenopausal women. No data exist on the effects of dietary intakes and lifestyle factors on bone mass and bone metabolism in Middle Eastern women. Given the public health implications of osteoporosis, further work is urgently required.

Table 1.4: Studies on prevalence (%) of osteopenia and osteoporosis and biochemical parameters in Saudi Arabian women

Reference	Subject No:	Age	Study population	Prevalence of osteopenia	Prevalence of osteoporosis	Site of measurement	25(OH) D mean ng/ml	PTH mean pg/ml	Hypovitaminosis 25(OH)D≤8ng/ml
Ghannam <i>et al</i> , 1999 Riyadh	Female 321	31-40 Years	Pre	19 %	1 %	LS	9.64 ± 6.24	55.9 ± 32	52 % of all population
				27 %	2 %	FN			
				45 %	17 %	LS			
EldeSouk 2003 Riyadh	Female 24	50-59 Years	Post	35 %	3.7 %	FN	10.66 ± 4.9	22.42 ± 13.12	—
				34 %	24 %	LS			
Ardawi <i>et al</i> , 2005 Jeddah	Female 320	≥50 Years	Post	42.2 %	30.5 %	LS	—	—	—
				58.6 %	4.7 %	FN			
				37 %	2 %	LS			
Khoja <i>et al</i> , 2005 Jeddah	Female 212	20-30 Years	Pre	23 %	4 %	FN	8.86 ± 3.6	39.35 ± 21.4	64.3 %
				52 %	13 %	LS			
				32 %	2 %	FN			
		45-60 Years	Post	32 %	2 %	FN	9.7 ± 4.4	61.9 ± 34.3	52.7 %

1.11 Aims of the study

The principal objectives of the study were as follows:

- 1. To determine indices of bone health (namely lumbar spine and femoral neck bone mineral density as measured by DXA and calcaneal bone mass as measured by (BUA) in Saudi Arabian women**
 - 1.1. To examine the extent of osteopenia and osteoporosis in young and old age groups**
- 2. To assess the impact of non-dietary life style risk factors on indices of bone health including physical activity levels, sun-exposure time and anthropometric measurements**
- 3. To assess dietary intake in terms of both “ quality ” and “ quantity” in Saudi Arabian women**
 - 3.1. To examine whether the composition of the Saudi Arabian diet follows the food Pyramid / plate model guideline**
 - 3.2. To determine the extent of nutrient insufficiency/deficiency of key bone health nutrients including calcium, vitamin D, protein, phosphorus, potassium, vitamin D and fibre as well as iron intake and estimates of dietary acidity in younger and older groups**
- 4. To examine the effect of dietary intake on indices of bone health**
 - 4.1. Specially to determine whether low consumption of milk, dairy products, vegetables, fruits, fish and meat has any effect on bone indices**
 - 4.2. To establish whether low dietary intakes of Ca, vitamin D, proteins and minerals have an effect on these indices**
- 5. To determine the status of vitamin D and its metabolites**
 - 5.1. To specifically examine the extent of vitamin D deficiency / insufficiency in**

Saudi young and old women

6. To assess the effect of vitamin D status on indices of bone health, namely bone mass and bone metabolism in both populations group

Chapter 2

Materials and Methods

2.1 Introduction

The study protocol was approved by King Abdul Aziz University Hospital in Jeddah, Saudi Arabia. The recommendations in the declaration of Helsinki were followed strictly in conducting the study. Informed consent was obtained for all the women.

2.1.1 Subject selection

A total of 100 premenopausal women aged 20-30 years and 112 postmenopausal women aged 45-60 years were followed. All volunteers were recruited from the city of Jeddah by local advertisements concerning the study and by distributing forms after lecturing about the aim of this search, which benefits women in the community all over the city. Informed consent was obtained from all subjects (Appendix I). An appointment letter was sent to all subjects. The exclusion criteria used for the study are shown in Table 2.1 below. It was made sure that no women selected suffered from any condition that would interfere with their bone metabolism.

2.1.2 Collection of anthropometric data

Subjects were weighed (while wearing minimal clothing) on an electric scale (Health O meter, Bridgeview, Illinois, USA) calibrated to 0.05 kg. Height (without shoe) was measured to the nearest 0.10 centimeter using a stadiometer (Health O meter, Bridgeview, Illinois, USA). Body Mass Index (BMI) was calculated using weight in kilograms divided by height in meters squared.

Table 2.1: Exclusion criteria

- ❖ If the subjects suffer from the following diseases they should be excluded
 - Inflammatory bowel disease
 - Insufficiency of the exocrine pancreas
 - Biliary cirrhosis, liver dysfunction
 - Diabetes
 - Renal osteodystrophy
 - Nephritic syndrome
 - Celiac disease
 - Rheumatoid arthritis, osteoarthritis, bursitis.
- ❖ If the subjects have the following, they should be excluded
 - Pregnancy
 - Lactating
 - Irregular menstrual cycle (amenorrhea)
 - Hysterectomy
 - Oophorectomy
- ❖ If the subjects are taking the following medications, they should be excluded
 - Steroids
 - HRT
 - Diuretics
 - Anticonvulsant drugs
 - Vitamin D supplement
 - Glucocorticoids drugs

Table 2.2: Levels of living and education of volunteer women

		Level of living			Level of education				
	no	High	Middle	Low	None	Primary	Middle	University	Higher
Post	112	55 61%	54 60%	3 3.36%	11 12.3%	33 36.9%	16 17.9%	31 34.7%	21 23.5%
Pre	100	58 59.7%	42 43.2%	1 1.03%	Nil	2 2.06%	64 65.9%	35 36.05%	Nil

2.2 Determination of bone health indices

2.2.1 Bone mineral density measurements

BMD measurements were determined at the lumbar spine (L2-L4) and at the left and right total hip (femoral neck, ward's triangle, and trochanter) using dual X-ray absorptiometry (DXA) with a Lunar DPX-IQ Version 4.7 scanner (Lunar Corp., Madison, WI, USA), according to the manufacturer's operator manual. Quality control procedures were carried out in accordance with the manufacture recommendations. Instrument variation was determined regularly by a daily calibration procedure using a phantom supplied by the manufacturer. Precision error of the phantom was 0.3% and for in vivo measurements was less than 1.2% for the spine and less than 2% for femoral regions. The calibration of the absorptiometry was checked daily. In addition, there was no significant drift over the period of the study using the DXA system.

All measurements were performed at the Radiology Department, King Abdul Aziz University Hospital by experienced technologists and reviewed by the Consultant Radiologist (Dr Abdul-Raouf A. Maimani, Head of the Radiology Department). The results of the measurements were expressed in g / cm^2 . Lunar USA normal database supplied by the manufacturer was used to derive Z scores (matched for age and weight) and t-scores (reference age 20-45 years). Since at the start of this study, no Saudi Arabian reference range existed. Subjects with osteopenia were defined by a t-score between -1.0 and -2.5 SD at a given skeletal site, and those with osteoporosis were defined by a t-score less than -2.5 SD, as recommended by the WHO report (WHO 1994). The precision of DXA machine in the King Abdul Aziz University Hospital was 1%.

2.2.1.1 Procedures of measurements

Spine Acquisition

The system performs an anteroposterior (AP) spine scan of the lumbar region. A scan begins in the lower vertebrae, adjacent to the pelvis, and moves upward toward L1.

Patient Positioning

The patient has to remove all attenuating materials from the scan region before a scan acquisition. Patient is positioned in the middle of the scan table: the centerline on the table pad run lengthwise down the center of the patient's body. The spine is centered and straight, both iliac crests are visible, and the scan starts in the middle of L5 and ends in the middle of T12 (Fig.2.1). The patient's head is directly below the horizontal line at the top of the table pad. The patient's arms are against

their sides with palms flat against the table pad. The patient's legs are position on the support block, such that the patient's thighs are at a 60°- 90° angles. The support block helps separate vertebrae and straightens the lower back. The laser light should be 2 inches below the patient's navel.

Vertebral Labels

The system can label all five lumbar vertebrae, as well as T11, T12, and the pelvis. Up to four labels can appear on the screen at one time. The program only calculates results for vertebrae labeled L1, L2, L3, or L4. Results are not calculated for vertebrae labeled T12 because the ribs interfere with the baseline calculations. Results are not calculated for vertebrae labeled L5 because the iliac crests interfere with the baseline calculations.

Femur Acquisition

A right or left femur scan begins in the soft tissue surrounding the femoral shaft and moves upward toward the femoral neck and greater trochanter. The scan provides BMD values for the femoral neck, ward's triangle, greater trochanter, shaft, and total femur regions.

Patient Positioning

The patient has to remove all attenuating materials from the scan region before a scan acquisition. Patient is positioned in the middle of the scan table: the centerline on the table pad run lengthwise down the center of the patient's body. The patient's head is directly below the horizontal line at the top of the table pad. The patient's arms are against their sides with palms flat against the table pad. Near the ankle of the leg that intends to scan, grasp and gently rotate the leg inward and

outward. While rotating the leg, press firmly on the outside of the patient's upper thigh to feel the greater trochanter roll under your fingers. The laser light should be 3 inches below the greater trochanter. Fasten the foot of the leg to be scanned to the angled side of the brace. Rotate the entire leg to the angle of the brace. Fasten the other foot to the straight vertical side of the brace. Ensure the patient toes point upward and the feet are perpendicular to the tabletop. Lift the foot brace and move it 3-4 inches in the direction of the left to be scanned: the femoral shaft must be parallel to the center longitudinal line on the scan table pad. Align the laser light with the longitudinal center of the thigh.

2.2.1.2 Interpreting DXA scans

Dual energy x-ray absorptiometry (DXA) enables quantitative assessment of bone mineral in specific regions of the body. The scanner continuously samples the bone mineral content (BMC) as it moves in a rectilinear pattern over the lumbar spine or other areas of interest. The quantities estimated by DXA are:

- Area, expressed in cm^2 , which is the two-dimensional projection of the bone;
- Bone Mineral Content (BMC) is the amount of bone at the site that is being measured), expressed in grams;
- Bone Mineral Density (BMD) is the bone mineral content divided by the area that is being scanned. It is an areal density, not a true density, expressed in grams per centimeter squared (g / cm^2).

(AP) spine

In the lumbar spine region, the BMD is usually calculated from L2 through L4. Results calculated from other than L2 through L4 are useful in

situations where L2, L3, or L4 cannot be analyzed due to artifacts or fractures in those vertebral regions.

Femur

In the proximal femur, the region of interest is usually the area 1.5 cm wide across the total femoral neck. Regions are defined by the software in the lower density Ward's triangle region, the neck, the greater trochanter, the shaft, and total femur regions. Bone loss in the proximal femur begins in the Ward's triangle region and proceeds outward from them. This makes the region an early indicator of bone loss, but the higher variance in measuring it, compared with the neck region, makes the latter zone a better discriminator.

The numeric data from the DXA printout should be reviewed. In the spine, bone mineral density values for each vertebra should be noted. Moving from L1 to L3, the bone mineral density should increase. Whereas from L3 to L4, it should be constant or should decrease slightly. The most common reasons for discrepant bone mineral density values among vertebrae are fractures and degenerative disease. In the hip, bone mineral density values of each region of interest should be noted, because fractures can occur at any position in the proximal femur (subcapital, cervical, Basicervical, intertrochanteric, subtrochanteric).

To compare individuals, results are corrected for body weight and height and expressed as (T) or (Z) score (Kanis *et al.*, 1996) (Figure 2.1-2.2). The T score compares the findings with a young adult reference range and the Z score with an age-matched reference range. For older subjects, the T score will fall with age as bone loss is universal with ageing and it will be less than the Z score. The T score is used in the WHO definition of osteoporosis and osteopenia, if the subject has a T

score in excess of 2.5 standard deviations Below the mean are regarded as osteoporosis, while values between 1 and 2.5 standard deviations below the mean are regarded as osteopenia or 'low bone mass'. So, osteoporosis is being defined as an absolute reduction in bone mass whether related to age or not. Osteoporosis is a systemic disease, it does not have the same effect on the entire skeleton, and T-scores at different skeletal sites are commonly quite different (Abrahamsen *et al.*, 1997). The principal uses of bone densitometry are to diagnose osteoporosis or assess risk of future fracture, and in these cases the results are being compared against a normal database.

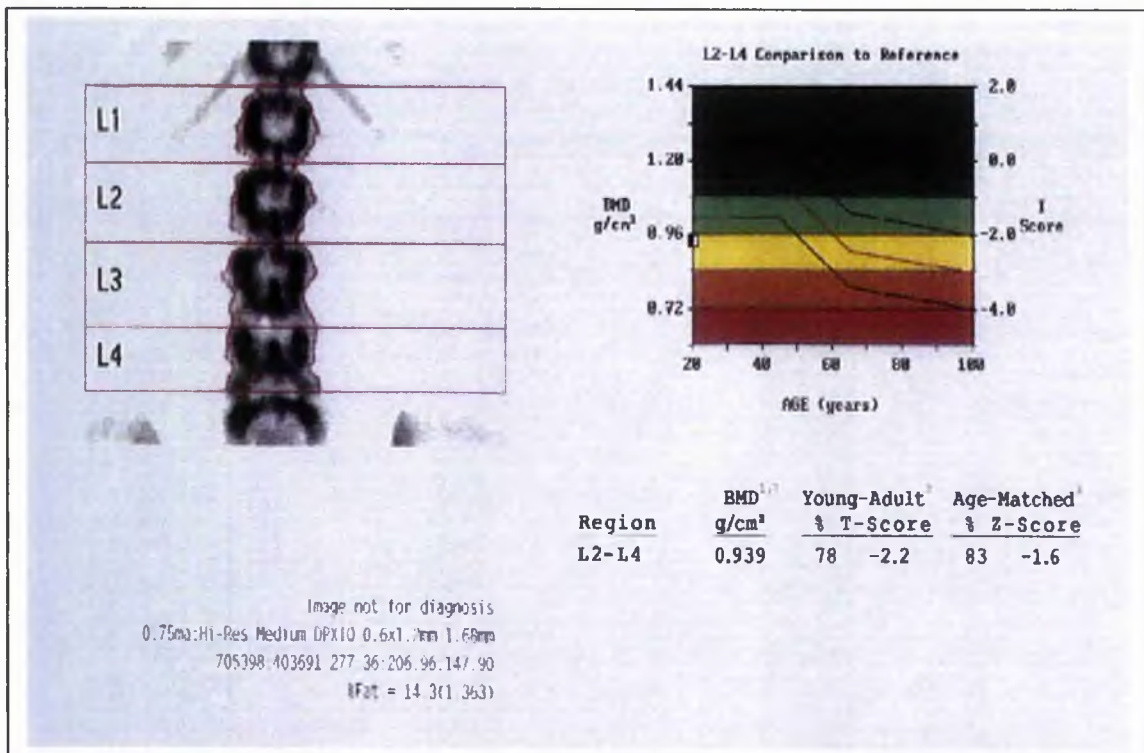


Figure 2.1: A dual energy x-ray absorptiometry measurement of bone density at the spine

score in excess of 2.5 standard deviations Below the mean are regarded as osteoporosis, while values between 1 and 2.5 standard deviations below the mean are regarded as osteopenia or 'low bone mass'. So, osteoporosis is being defined as an absolute reduction in bone mass whether related to age or not. Osteoporosis is a systemic disease, it does not have the same effect on the entire skeleton, and T-scores at different skeletal sites are commonly quite different (Abrahamsen *et al.*, 1997). The principal uses of bone densitometry are to diagnose osteoporosis or assess risk of future fracture, and in these cases the results are being compared against a normal database.

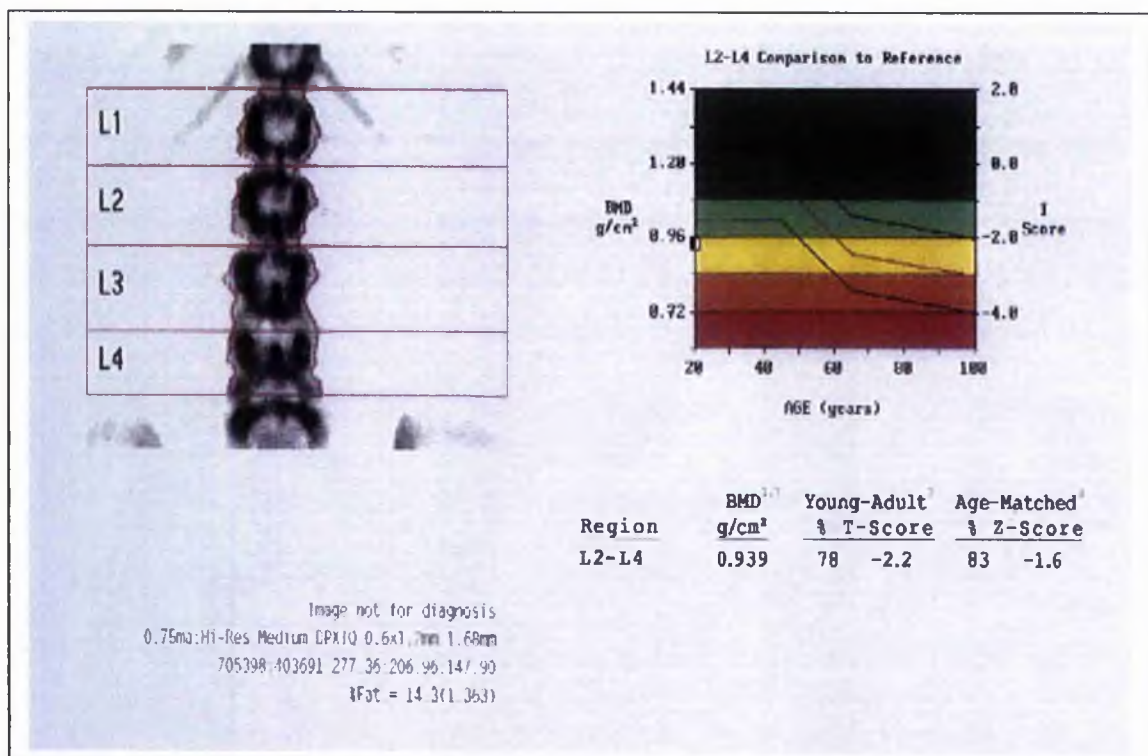


Figure 2.1: A dual energy x-ray absorptiometry measurement of bone density at the spine

Result Graph

After each sampling, the amplifier setting and the number of counts detected in the 38-keV and 70-keV channels (Low keV and High keV) appear on the screen. After 11 samples, the computer plots the results on a graph. The 70-keV peak should appear as a slope moving upward from left to right. The 38-keV peak should slope downward from left to right. The detector peak test then determines the optimal voltage setting for the PMT amplifier based on these curves.

Passing Value

To receive a passing value at the end of the detector peak test:

- The new peak value must be within 50 units of the previous QA peak setting. The actual peak setting is taken from the 70keV curve and not the 38-keV slope.
- The peak setting must be more than 250 units but less than 750 units.

Functional Measurements Test

The functional measurements tests begin after the detector peak test finishes. The system checks the internal mechanics and electronics of the scan table, and displays the final test values with a pass/fail evaluation.

Standard Values Test

After finishing the functional measurements test, the program advances to the standard values test. Standard values are used to convert uncalibrated scan data (in arbitrary computer units) to calibrated results (in grams of bone mineral). The Lunar system automatically prints results as part of the quality assurance tests.

Passing Evaluations Summary

Check the QA results printout to make sure that each test received a passing evaluation:

- The detector peak must be within 50 units of the previous peak setting, and the 70-keV and 38-keV peaks must be within 50 units of each other.
- Each functional measurement must receive a passing evaluation.
- The standard value must have %CV values of 2.00 or less. Mean values must not deviate from the expected value by more than 5.0%.

2.2.2 Quantitative ultrasound measurements

Quantitative ultrasound QUS measurements were obtained using a CUBA ultrasound densitometer (Mc Cue Ultrasonic, Winchester, UK). Participation occurred for three days at the manufacturers site (Parsonage Barn, Compton Winchester, Hampshire SO21 2AS England) in order for me to trained to be able to operate the instrument.

The ultrasound system consists of two transducers (emitting and receiving) faced with silicone rubber coupling pads. These are placed in direct contact on either side of the heel using a coupled gel. Broadband ultrasound attenuation BUA (decibels/megahertz) and velocity of sound VOS (meters/second) were measured in the left calcaneus (heel bone) in all subjects. Mean BUA and VOS were calculated from four measurements. We used the scanning protocol produced by the manufacturer. The precision of this instrument was 2.4% and 1% for BUA and VOS respectively.

2.2.2.1 Procedures of measurements

The software defaults to measuring the left foot, which is generally the non-dominant foot. (The normative data was collected on left feet). Patient contact surfaces (foot well, calf support, ultrasonic transducers and foot inserts) disinfected before each patient. Patient shoes and foot clothing removed in order to take a measurement. Alcohol impregnated wipes are used to clean the patient skin around the heel prior to the measurement. Locating the correct site on the calcaneus is important for accurate measurements, anatomical foot inserts is used according to foot length. Foot length being the distance from the back of the heel to the tip of the big toe. The foot is placed into the foot well, to identify where the big toe falls, to read the insert size needed. Insert A used on foot size below 230 mm in length, insert B for foot size from 230mm to 250mm in length and for feet over 250mm no insert is required.

To ensure that an unambiguous contact is made to each side of the heel, a coating of ultrasound gel applied to the face of each transducer pad and the skin on either side of the heel. The foot and especially the heel should be central with in the footwell of the CUBAClinical. The heel is pushed fully back into the footwell- this is especially important for accurate measurements. Fit the patient's calf in the calf support. The patient's calf should be positioned fully back in the support and secure this position with the straps. Then close the transducers to start the measurement. The system will take the measurement. After measurement, the result will displayed both in table format and graphically on the main screen.

2.2.2.2. Interpreting QUS scans

The CUBAClinical measures BUA (Broadband Ultrasound Attenuation). The more complex the structure of the bone, the more the sound wave will be blocked. Therefore normal bone has a higher attenuation (BUA) than osteoporotic bone (Fig.2.3). Likewise the greater the connectivity of tissue, the faster the sound wave will pass through it. As bone becomes osteoporotic the architecture diminishes and the speed of the sound wave slows down (Fig.2.4).

Normative data

The normative data was collected from a Caucasian population (n = ~ 3000). The left calcaneus was measured in accordance with the documented CUBAClinical measured protocol. Regression analysis of the results established the “ young normal ” (age 20) reference value for BUA. The mean and the 95th percentile then express the normative data with the normative range depicting +/- 2 standard deviation about the mean, measured at the 95th percentile. The BUA result can be compared to a mean normal population result and expressed either by Z score T score or % expected.

T and Z scores

These scores allow for the comparison of a patient's BUA value with normative population.

BUA Z Score

The number of population standard deviations that a BUA value is away from the population mean value for a particular age.

$$\text{Z score} = \frac{\text{Patient BUA} - \text{age-matched mean BUA}}{\text{age-matched standard deviation}}$$

BUA T Score

Similar to Z score but not age matched. Instead the patient's BUA is expressed in terms of the population mean and standard deviation at peak bone mass (young normal) say age 20.

$$T \text{ score} = \frac{\text{Patient BUA} - \text{young adult mean BUA}}{\text{Young adult standard deviation}}$$

<u>Status</u>	<u>T- score (SD)</u>
Average	Greater than -1.0 SD
Below Average	Less than -1.0 SD but Greater than -2.0 SD
Risk of osteoporotic fracture	Less than -2.0 SD

% Expected

A percentage analysis of the ultrasound result compared to an age matched normal mean.

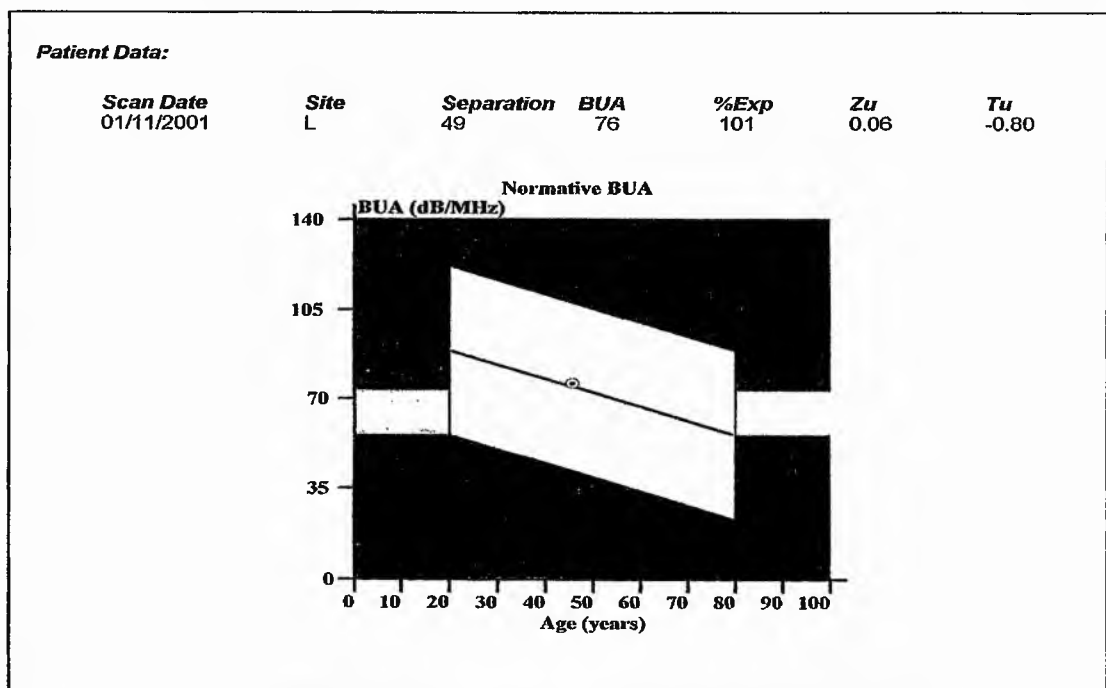


Figure 2.3: Broadband ultrasound attenuation BUA measurement at the heel

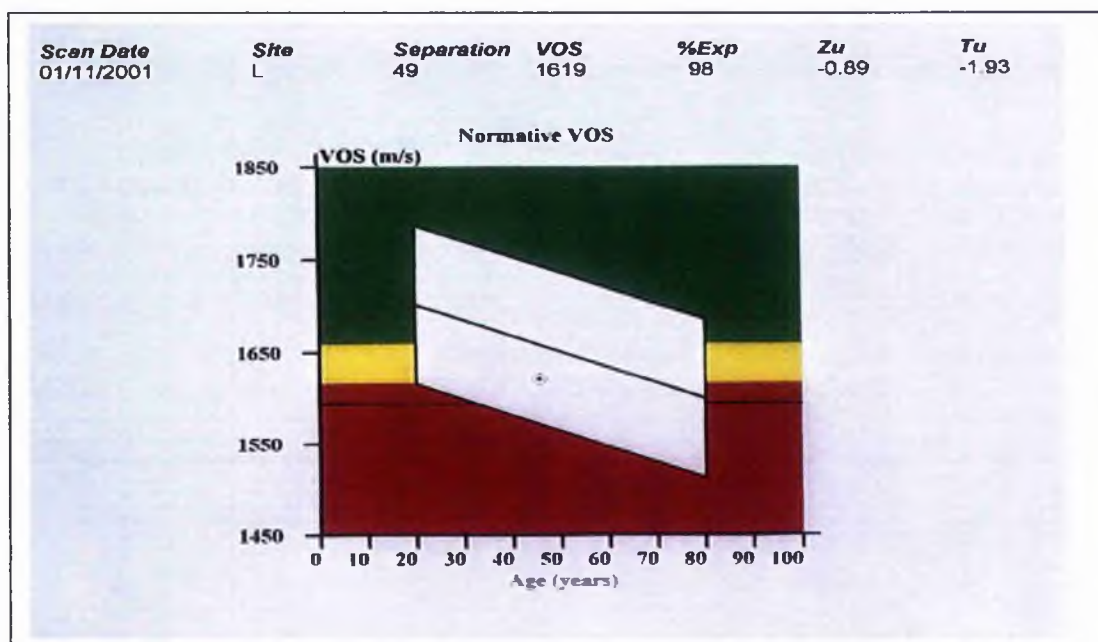


Figure 2.4: Velocity of sound measurement at the heel

2.2.2.3. Quality assurance tests

The CUBAClinical is a precision instrument; therefore it is essential at the beginning of each session a QA (Quality Assurance) test should perform to ensure that the machine is operating correctly. It is recommend checking the calibration of the instrument using the QA phantom supplied with the system.

Recommended limits

The certificate of calibration supplied with the CUBAClinical states the BUA and VOS results obtained by McCue PLC during factory calibration of this CUBAClinical with its designated phantom. Normally, BUA and VOS values with in tolerance of ± 2 BUA and ± 20 VOS m/s of the values stated on the certificate should be obtained.

Performing a OA Test

- Ensure that the faces of the transducers and the phantom are clean
- Place a 25mm diameter circle full of gel in the center of each face of the phantom using the tip of the gel bottle.
- Place the phantom in the CUBAClinical
- Close the transducer, by pressing the close button on the CUBAClinical
- Check that the phantom is pushed into the bottom of the footwell
- The system will wait for a setting period to allow distribution of the gel to stabilize and any trapped air bubbles to escape.
- After the settling period, the system will take the measurement.

2.3 Determination of lifestyle and dietary intake

2.3.1 Collection of lifestyle data

A brief lifestyle questionnaire through a personal interview was used to collect information on the level of physical activity such as walking, swimming, cycling and aerobics as hours per week. Information on the time spent in the sunlight as a period of exposure to sun minutes per day was also collected. The questions were also asked about an age, marital status and educational level. Frequency of caffeine consumption as cup per day, smoking habits and general lifestyle of the subjects were also included. The inquiry included questions about gynecological and reproductive history (Appendix II).

2.3.2 Methods of dietary assessment

There are four approaches to measuring food intake: diet records, diet recall, diet history, and food frequency questionnaire (FFQ). The method chosen in this research was the food frequency questionnaire and diet records.

2.3.2.1 Food frequency questionnaire

Dietary information was collected with a food frequency questionnaire (FFQ) through a personal interview (over the last year) (Appendix II). The questions were divided into two different parts: how many times a day usually a food has been eaten, which ranged from one to three times per day, and how many days a week this food was eaten, which ranged from a day to seven days. There was also "twice a month" and "never" categories. The women were asked how often, on average, per day, per week they had consumed these foods dairy products, eggs, fish, cereals and

fats, using predefined frequency categories. Additional open questions about consumption of dairy products were included (e.g. type of milk, number of glasses of milk per day and type of dairy products).

2.3.2.2 Food diary

Food diaries were given to the participants to write down everything they ate or drank in three days, including any snacks, sweets and drinks between meals and any tablets or supplements. They were asked to record all the ingredients in foods like casseroles, salads or fruit salads and describe any sauces or dressings. They have been asked to give as much detail as possible about the type and amount of food and drink they have had, such as (glass full, tablespoons and teaspoon). They were asked to indicate if foods or meals had been prepared at home, ready-made and reheated or bought as a take away. The method of cooking was to determine whether the food was grilled or boiled and the type of fat or oil used for frying. They were asked to note if the skin on items such as baked potatoes, chicken and fruit were eaten.

2.3.3 Coding and analyzing of dietary information

Results concerning the coding and subsequent analysis of the dietary data can be found in chapter 5.

2.4 Collection and treatments of blood and urine samples

Blood and urine specimens were obtained after an overnight fast. Blood was drawn in the fasting state and collected in two kinds of evacuated tubes: 1) Plain evacuated tubes 5ml 13×75 MM. 2) Lithium Heparin (LH 851. IU) tubes 5ml 13×75 MM. After collection, the sample was then allowed to clot for 70min at room temperature, and centrifuged at 3000× g for 10min to separate the serum and plasma. The extracted serum and plasma was stored at – 85 C° until assayed for calcium, calciotropic hormones and bone turnover marker levels. Second morning urine sample before 11.00 AM was obtained in to sterile tubes and stored at - 85 deg C° until analyzed for pyridinoline and deoxypyridinoline.

2.5 Analysis of bone turnover markers

Monitoring of bone metabolism by biochemical means depends upon measurement of enzymes and proteins released during bone formation (such as alkaline phosphatase, osteocalcin and collagen propeptides), and of degradation products produced during bone resorption.

2.5.1. Measurement of bone formation markers

For assessing the rate of bone formation, circulating levels of serum osteocalcin and serum bone alkaline phosphatase can be employed

2.5.1.1. Analysis of osteocalcin (OC)

Several commercial assay kits are available for serum osteocalcin measurements. These include bovine or human, monoclonal or polyclonal antibody

based immunoassays. These are in radioimmunoassay or Enzyme linked immunoassay (ELISA) format.

Principle of the Osteocalcin assay

In this study we used the Metra™ Osteocalcin immunoassay quantitatively to measure intact (de novo) osteocalcin in serum. This assay is a competitive immunoassay; it uses osteocalcin-coated strips, a mouse anti-osteocalcin antibody. This anti-mouse IgG-alkaline phosphatase conjugate and a p-Nitrophenyl phosphate substrate to quantify osteocalcin in serum.

Reagents and Materials

The Metra™ Osteocalcin EIA (activity-based assay) Kit, contains the following:

Reagents	Materials
Substrate tablet	p-Nitrophenyl phosphate
Anti-Osteocalcin	Purified murine monoclonal anti-osteocalcin antibody in a buffered solution containing nonionic detergent, stabilizers, and sodium azide (0.05%) as preservative
Osteocalcin Standards	(0,2,4,8,16,32 ng/ml) Lyophilized osteocalcin, purified from human bone, containing buffer salts and stabilizers
Low/High Controls	Lyophilized osteocalcin, purified from human bone, containing buffer salts and stabilizers
Enzyme Conjugate	Lyophilized goat anti-mouse IgG antibody conjugated to alkaline phosphatase containing buffer salts and stabilizers
Coated Strips	Osteocalcin purified from human bone adsorbed on to strip wells

Stop Solution	1N NaOH
Wash Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative
Substrate Buffer	A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as preservative

Reagents preparation

All reagents should be equilibrated to room temperature (20-25) prior to use.

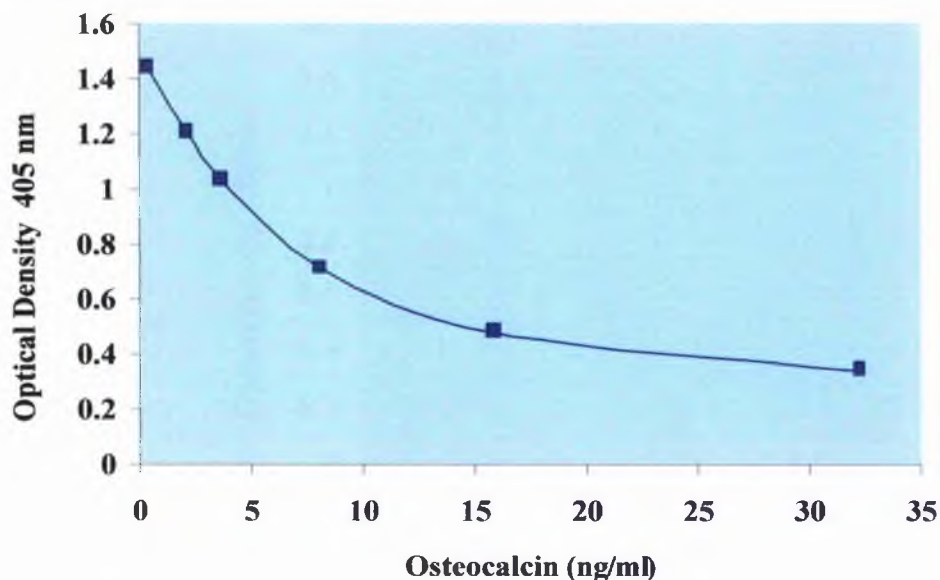
Coated Strips	Remove Strip well Frame and the required number of coated Strip from the pouch
Wash Buffer	Prepare required amount of 1X wash buffer by diluting 10X Wash Buffer 1:10 with deionized water. Store at room temperature (20-25°C). Use 1X Wash Buffer with in 24 hours of preparation.
Enzyme Conjugate	Prepare Enzyme Conjugate with in 2 hours of use. Reconstitute each required vial of Enzyme Conjugate with 10ml of 1X Wash Buffer. Allow the pellet to completely dissolve.
Osteocalcin Standards and controls	With in 1 hour of use, reconstitute Standards and controls with 0.5ml of 1X Wash Buffer. Allow at least 15 minutes for the pellet to completely dissolve. Reconstituted Standards and Controls should not remain at room temperature for more than 2 hours.
Working Substrate Solution	Prepare Working Substrate Solution with in 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer. Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

Assay procedure

1. Desired number of Coated Strips is placed in the strip well Frame prior to use.
2. 25 μ L of Standard, Control, or Sample is added to each well of the Coated Strip. This step completed with in 30 minutes.
3. 125 μ L of Anti-Osteocalcin is added to each well and incubated for 2 hours at room temperature (20-25°C).
4. Invert/empty strips. 300 μ L of 1X Wash Buffer is added to each well and invert/empty strips. Repeating two more times for total of three washes. Vigorously blot the strips dry on paper towels after the last wash.
5. 150 μ L of the reconstituted Enzyme Conjugate is added to each well.
6. Incubation for 60 minutes at room temperature (20-25°C).
7. Wash 3 times with 1X Wash Buffer.
8. 150 μ L of reconstitute Working Substrate Solution is added to each well
9. Incubation for 35-40 minutes at room temperature (20-25°C).
10. 50 μ L of stop Solution is added to each well to stop the reaction.
11. Optical Density (OD) is read at 405nm.
12. For MetraTM Osteocalcin assay result Quantitation software with 4-parameter calibration curve fitting equation is used for analysis.

Representative Standard Curve

Standard Osteocalcin levels: 0, 2, 4, 8, 16, 32 ng/ml



4 Parameters: Concentration $^B = (OD \cdot A) / (C + D \cdot OD)$ A=1.4E+0; C=1.7E-2;
D=7.7E-2; Rms Error=1.5E-1

Interpretation of results

Sample results are expressed as ng/ml.

Performance characteristics

Antibody Specifications

The monoclonal anti-osteocalcin antibody was raised against bovine osteocalcin, which exhibits significant homology with human osteocalcin. This antibody is believed to recognize only intact (de novo) osteocalcin and not fragments from resorbed bone tissue.

Sensitivity

The minimum detection limit of this assay is 0.45 ng/ml, determined by the upper 3 SD limit in a Zero standard study.

Precision

Within-run and between-run precision was determined by assaying three samples.

Results are provided below.

CV% = Percent coefficient of variance

$$CV\% = \frac{SD \times 100}{Mean}$$

Osteocalcin ng/ml	Within run¹ CV%	Between run² CV%
5.50	11.8	10.8
10.72	9.0	7.8
21.86	7.9	8.0

¹ n=10 ²n=3 in 3 runs

Osteocalcin Expected Values

Females 3.7 – 10.0 ng/mL

2.5.1.2. Analysis of bone specific alkaline phosphatase (BSAP)

Over the years, a variety of methods have been developed that specifically isolate and measure the circulating bone-specific isoform of ALP distinct from the liver and kidney isoforms (see section 1.3.3.1).

For the Metra™ Bone-specific Alkaline Phosphatase assay, antibody technology was employed to produce a monoclonal antibody demonstrates specificity for (BSAP). The specificity of the monoclonal antibody used in the assay allowed for simple, convenient, reproducible and direct Quantitation of BSAP activity in serum.

Principle of the Bone-specific Alkaline phosphatase assay

In this study we used a commercial immunoassay based on enzyme activity measurements for bone-specific (AP) Metra™ Bone-specific Alkaline Phosphatase (BSAP) which utilizing a single monoclonal antibody coated on the microtiter strip to capture BSAP in the sample. The enzyme activity of the captured BSAP is detected with a p-Nitrophenyl phosphate (pNPP) substrate.

Reagents and Materials

The Metra™ Bone-specific Alkaline phosphatase (activity-based assay) Kit, contain the following:

Reagents	Materials
Substrate tablet	p-Nitrophenyl phosphate
BSAP Standards A - F	(0, 2, 20, 50, 80, 140 U/L BAP) BAP purified from osteosarcoma SAOS-2 Cells in a buffered solution containing magnesium chloride, Zinc sulfate, surfactant, carrier protein, blue dye, and sodium azide (0.05%) as a preservative.
Low/High Controls	BAP purified from osteosarcoma SAOS-2 Cells in a buffered solution containing magnesium chloride, Zinc sulfate, surfactant, carrier protein, blue dye, and sodium azide (0.05%) as a preservative.
Assay Buffer	A buffered solution containing magnesium chloride, Zinc sulfate, surfactant, and sodium azide (0.05%) as a preservative.
Substrate Buffer	A 2-amino-2-methyl-1-propanol solution containing HEDTA, magnesium chloride, Zinc sulfate, and sodium azide (0.05%) as a preservative.
Coated Strips	Purified murine monoclonal Anti-BAP IgG antibody adsorbed onto strip wells
Stop Solution	1N NaOH
Wash Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative

Reagents preparation

All reagents are equilibrated to room temperature (20-25) before use.

1. Coated Strips

Remove Strip well Frame and the required number of coated Strip from the Pouch.

2. Wash Buffer

Prepare required amount of 1X wash buffer by diluting 10X wash Buffer 1:10

with deionized water. Store at room temperature (20-25°C). Use 1X Wash Buffer

with in 24 hours of preparation.

3. Working Substrate Solution

Prepare Working Substrate Solution with in 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer.

Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

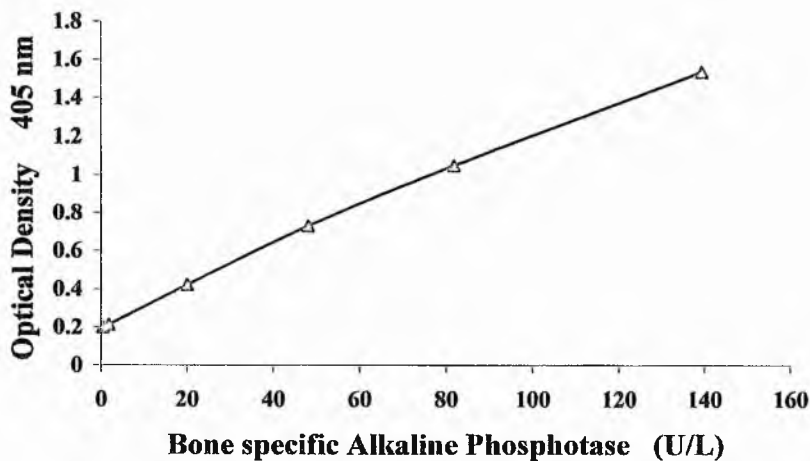
Procedure

1. Required number of Coated Strips is placed in the strip well Frame prior to use.
2. 125 μ L of Assay Buffer has been added to each well.
3. 20 μ L of Standard, control and Sample has been added to each well. Step is completed within 30 minutes, with gentle swirl of the strips to ensure mixing of sample and buffer.
4. 3 hours incubation at room temperature (20-28°C).

5. Invert/empty strips. 250µL of 1X Wash Buffer is added to each well and invert/empty strips. Repeating three more times for total of four washes. Vigorously blot the strips dry on paper towels after the last wash.
6. 150µL of reconstitute Working Substrate Solution is added to each well.
7. Incubation for 30 minutes at room temperature (20-28°C).
8. 100µL of stop Solution is added to each well to stop the reaction.
9. Optical Density (OD) has been read at 405nm.
10. To analyze the assay results, Quantitation software with a quadratic calibration curve fitting equation has been used.

Representative Standard Curve

Standard BSAP levels: 0, 2, 20, 50, 80, 140 U/L.



4 Parameters: Concentration^B=(OD-A)/(C+D*OD)
A=1.4E+0; B1.5E+0; C=2.1E-2; Rms Error=1.1E+0

Interpretation of results

1. Sample results are expressed as U/L.
2. 1 Unit represents 1 μmol of pNPP hydrolyzed per minute at 25°C in 2-amino-2-methyl-1-propanol buffer.

Performance characteristics

Antibody Specifications

The bone-specific alkaline phosphatase antibody has selective, high affinity for the bone-specific alkaline phosphatase isoform, low cross-reactivity to the liver form of alkaline phosphatase, and negligible binding of intestinal and placental isoenzymes.

Sensitivity

The minimum detection limit of this assay is 0.7 U/L, determined by the upper 3 SD limit in a Zero standard study.

Precision

Within-run and between-run precision were determined by assaying 3 serum samples in 6 runs. Results are provided below.

BSAP (U/L)	Within-run ¹ CV%	Between-run ² CV%
6	6.7	5.3
18	3.9	4.7
49	15	8.9

¹n=6 runs

²n=5 runs

CV% = Percent coefficient of variance

$$= \frac{SD}{Mean} \times 100$$

BSAP Expected Values

	Age (yr)		Range (U/L)	Median
Females	25-44	Premenopausal	11.6 – 29.6	18.3
Females	≥ 45	Postmenopausal	14.2 – 42.7	25.0

2.5.2. Measurement of bone resorption markers

The collagen molecules form fibrils, are mineralized and remain part of bone for many years. During osteoclastic bone resorption these cross-links are released into the circulation and excreted in the urine as free pyridinoline (free PYD) or free deoxypyridinoline (free D-PD). Collagen cross-links show a circadian rhythm and collection should be standardized: the first or second morning void of urine should be used.

2.5.2.1. Analysis of deoxypyridinoline (DPD) crosslinks

There are many different ways to measure these metabolites of collagen. The original assays for the hydroxyproline crosslinks required high-performance liquid chromatography (HPLC) (Nishi *et al.*, 1999). More recently, antibodies have been raised to various regions of the hydroxyproline cross-links, and they can now be measured in urine by a number of commercial assays.

Principle of deoxypyridinoline crosslinks (DPD) assay

The Metra DPD assay is a competitive enzyme immunoassay in a microtiter strip well format utilizing a monoclonal anti-DPD antibody coated on the strip to capture DPD. DPD in the urine sample competes with conjugated DPD-alkaline phosphatase for the antibody and the reaction is detected with a pNPP substrate. Metra DPD results are corrected for urinary concentration by creatinine.

Reagents and Materials

The Metra™ Deoxypyridinoline crosslinks (activity-based assay) Kit, contain the following:

Reagents	Materials
Substrate tablet	p-Nitrophenyl phosphate
Enzyme Conjugate	Lyophilized goat anti-mouse IgG antibody conjugated to alkaline phosphatase containing buffer salts and stabilizers.
DPD Standards A-F	(0, 3, 10, 30, 100, 300 nmol/L) DPD purified from bovine bone in 10mmol/L phosphoric acid containing sodium azide (0.05%) as preservative.
Low/High Controls	DPD purified from bovine bone in 10mmol/L phosphoric acid containing sodium azide (0.05%) as preservative.
Coated Strips	DPD purified murine monoclonal Anti-DPD antibody adsorbed on to strip wells
Stop Solution	1N NaOH
10X Wash Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative
Assay Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative
Substrate Buffer	A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as preservative

Reagents preparation

All reagents are equilibrated to room temperature (20-25) before use.

1. Wash Buffer

Prepare required amount of 1X wash buffer by diluting 10X wash Buffer 1:10 with deionized water. Store at room temperature (20-25°C). Use 1X Wash Buffer within 24 hours of preparation.

2. Enzyme Conjugate

Prepare Enzyme Conjugate within 2 hours of use. Reconstitute each required vial of Enzyme Conjugate with 7ml of Assay Buffer.

3. Working Substrate Solution

Prepare Working Substrate Solution within 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer. Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to be completely mixed.

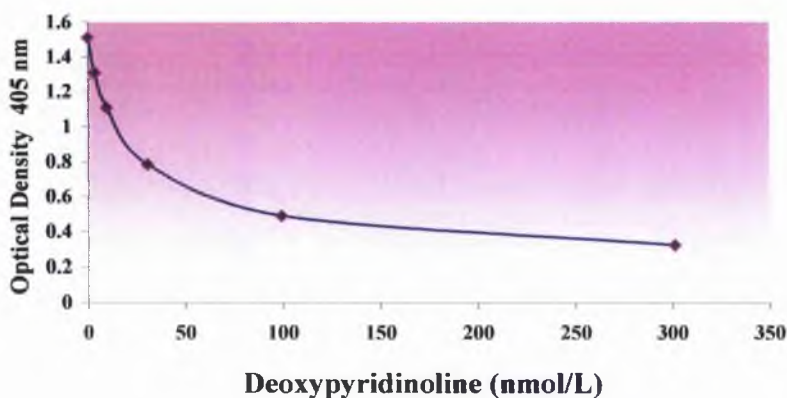
Procedure

1. Sample, Standards and Controls has been diluted 1:10 with Assay Buffer (e.g. 50µl sample + 450 Assay Buffer).
2. Required number of Coated Strips is placed in the strip well Frame prior to use.
3. 50µl of diluted Standard, Control or Sample is added to each well of the coated Strips. This step completed within 30 minutes.
4. 100µl of reconstituted Enzyme Conjugate is added to each well.
5. Incubation for 2 hours at 2-8 °C in the dark.

6. Invert/empty strips. 250µL of 1X Wash Buffer is added to each well and invert/empty strips. Repeating two more times for total of three washes. Vigorously blot the strips dry on paper towels after the last wash.
7. 150µl of Working Substrate Solution is added to each well.
8. 60 minutes incubation at room temperature (20-28°C).
9. 100µl pf Stop Solution is been added to each well.
10. Optical Density (OD) is read at 405nm with in 15 minutes.
11. Quantitation software with a 4-parameter calibration curve fitting equation was used to analyze the assay results.
12. Concentration of samples and controls are determined from the Standard curve.

Representative Standard Curve

Standard DPD levels: 0, 3, 10, 30, 100, 300 nmol/L



4 Parameters Concentration^B= $(OD-A)/(C+D*OD)$ A=1.5E+0; B=9.0E-1;
C=1.1E-2; D=5.6E-2; Rms Error=6.1E-1

Interpretation of results

Results obtained were corrected for variations in urine concentration by dividing the DPD value (nmol/L) by the creatinine value (mmol/L) of each sample (creatinine mg/dL x 0.088 = mmol/L). The final DPD results will be expressed as nmol DPD /mmol creatinine.

DPD Expected Values

	Age (yr)	Mean (nmol/mmol)	SD	Range
Females	25-44	5.0	1.4	3.0-7.4

Performance characteristics

Antibody Specifications

The monoclonal anti-DPD antibody has selective, high affinity for free DPD and negligible binding to DPD peptides and free or peptide bound pyridinoline (PYD)

Sensitivity

The minimum detection limit of this assay is 1.1 nmol/L, determined by the upper 3 SD limit in a Zero standard study.

Precision

Within-run and between-run precision were determined by assaying 3 urine samples in 6 runs. Results are provided below.

Sample	DPD (nmol/mmol)	Within-run ¹ CV%	Between-run ² CV%
1	32	7.0	12
2	94	3.8	9.5
3	237	9.0	8.4

¹n=6 runs

²n=6 runs

CV% = Percent coefficient of variance

$$= \frac{SD}{\text{Mean}} \times 100$$

2.5.2.2. Analysis of pyridinium crosslinks (PYD)

Preliminary immunodetection methods for pyridinium crosslinks (PYD) indicated that no cross-links were present in free form in urine due to the presence of specific diastereoisomerers. Direct immunoassays for the cross-links and a number of ELISA systems for measuring free PYD are available. Antibody technology was employed to produce a monoclonal antibody that demonstrates specificity for pyridinium crosslinks. The specificity of the monoclonal antibody used in the assay allowed for simple, convenient, reproducible and direct Quantitation of PYD and DPD in urine.

Principle of pyridinium crosslinks (PYD) assay

The Metra DPD assay is a competitive enzyme immunoassay in a microtiter strip format utilizing a monoclonal anti-pyridinium crosslinks antibody to measure PYD and DPD in urine. The PYD and DPD in the sample compete for the antibody with PYD coated on the strip. The reaction is detected with a pNPP substrate. Metra PYD results are corrected for urinary concentration by creatinine.

Reagents and Materials

The Metra™ Pyridinium crosslinks (activity-based assay) Kit, contain the following:

Reagents	Materials
Substrate tablet	p-Nitrophenyl phosphate
Enzyme Conjugate	Lyophilized goat anti-mouse IgG antibody conjugated to alkaline phosphatase containing buffer salts and stabilizers.
PYD Standards A-F	(0, 15, 40, 100, 250, 750 nmol/L) PYD purified from human urine in 10mmol/L phosphoric acid containing sodium azide (0.05%) as preservative.
Low/High Controls	PYD purified from human urine in 10mmol/L phosphoric acid containing sodium azide (0.05%) as preservative.
Coated Strips	PYD purified from bovine bone adsorbed on to strip wells
Stop Solution	1N NaOH
10X Wash Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative
Assay Buffer	Nonionic detergent in a buffered solution containing sodium azide (0.05%) as preservative
Substrate Buffer	A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as preservative

Reagents preparation

All reagents are equilibrated to room temperature (20-25) before use.

1. Wash Buffer

Prepare required amount of 1X wash buffer by diluting 10X wash Buffer

1:10

with deionized water. Store at room temperature (20-25°C). Use 1X Wash Buffer with in 24 hours of preparation.

2. Enzyme Conjugate

Prepare Enzyme Conjugate with in 2 hours of use. Reconstitute each required vial of Enzyme Conjugate with 7ml of Assay Buffer.

3. Working Substrate Solution

Prepare Working Substrate Solution with in 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer. Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

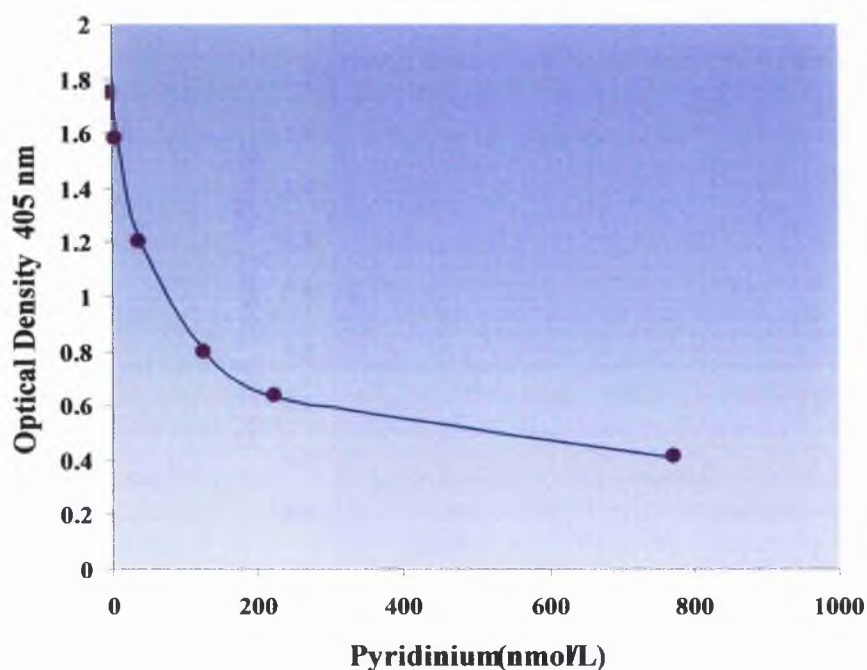
Procedure

13. Sample, Standards and Controls has been diluted 1:10 with Assay Buffer (e.g. 50µl sample + 450 Assay Buffer).
14. Required number of Coated Strips is placed in the strip well Frame prior to use.
15. 50µl of diluted Standard, Control or Sample is added to each well of the coated Strips. This step completed with in 30 minutes.
16. 100µl of reconstituted Enzyme Conjugate is added to each well.
17. Incubation for 3 hours at 2-8 °C in the dark.
18. Invert/empty strips. 250µL of 1X Wash Buffer is added to each well and invert/empty strips. Repeating two more times for total of three washes. Vigorously blot the strips dry on paper towels after the last wash.
19. 150µl of Working Substrate Solution is added to each well.
20. 60 minutes incubation at room temperature (20-28°C).
21. 100µl pf Stop Solution is been added to each well.

22. Optical Density (OD) is read at 405nm with in 15 minutes.
23. Quantitation software with a 4-parameter calibration curve fitting equation was used to analyze the assay results.
24. Concentration of samples and controls are determined from the Standard curve.

Representative Standard Curve

Standard PYD levels: 0, 10, 40, 100, 250, 750 nmol/L.



Interpretation of results

Results obtained were corrected for variations in urine concentration by dividing the Pyridinium crosslinks value (nmol/L) by the creatinine value (mmol/L) of each sample ($\text{creatinine mg/dL} \times 0.088 = \text{mmol/L}$). The final PYD results will be expressed as nmol PYD /mmol creatinine.

PYD Expected Values

Sex	Age (nmol/mmol)	Mean (nmol/mmol)	SD (nmol/mmol)
Females	16.0 – 37.0	25.5	7.5

Performance characteristics

Antibody Specifications

The monoclonal anti-Pyridinium crosslinks antibody has selective, high affinity for free PYD and negligible binding to PYD peptides

Sensitivity

The minimum detection limit of this assay is 7.5 nmol/L, determined by the upper 3 SD limit in a Zero standard study.

Precision

Within-run and between-run precision were determined by assaying 3 urine samples in 6 runs. Results are provided below. For samples 1 through 3 represent low normal, high normal, and elevated resorption.

Sample	PYD (nmol/mmol)	Within-run ¹ CV%	Between-run ² CV%
1	89	7.3	10.1
2	315	2.5	3.1
3	609	4.7	10.7

¹n=6 runs

²n=6 runs

CV% = Percent coefficient of variance

$$= \frac{SD}{\text{Mean}} \times 100$$

2.6 Biochemical analysis of blood samples

2.6.1 Measurement of 25-Hydroxy Vitamin D (CPBA method)

Principle

Serum 25-OH vitamin D was measured by a competitive protein-binding assay (CPBA). This is based on the competition of 25-OH Vit D present in the sample with biotinylated 25-OH Vit D (tracer) for the binding pocket of vitamin D binding protein (VDBP, Gc-globulin). Since all circulating 25-OH Vit D is bound to VDBP in vivo, samples have to be precipitated with organic solvents to extract the analyte. The supernatant is used without further treatment in the test.

Procedure

In the first step, vitamin D binding protein, anti vitamin D protein antibody, and samples / standards / controls are added. The 25-OH Vit D present in the sample then competes with the 25-OH- Vit D-Biotin, bound to the well, for the specific binding sites of the binding protein. Hence, with increasing concentrations of 25-OH Vit D in the sample the amount of binding protein immobilized to the well via the tracer is reduced. Simultaneous addition of an antibody specific for this protein yields a complex, which is finally quantitated by incubation with a host specific peroxidase labeled antibody using TMP as enzyme substrate. The amount of color developed is inversely proportional to the amount of 25-OH Vit D present in the standards / samples/ controls (i.e. the less vitamin D that is present in the sample, the higher the intensity of the color developed) (Hawa *et al.*, 1999). A standard curve is plotted and the concentrations of 25-OH Vit D in the samples are calculated from this curve.

Precision

Within-run and between-run precision was determined by assaying 3 samples in 9 runs. Results are provided below. For samples 1 through 3 represent low normal, high normal, and elevated levels.

Sample	25(OH) D Mean (nmol / l)	Within-run ¹ CV%	Between-run ² CV%
1	34.0	11.0	14
2	50.83	13.8	12.9
3	79.31	18.0	10.4

¹n=9 runs

²n=6 runs

CV% = Percent coefficient of variance

$$= \frac{SD}{\text{Mean}} \times 100$$

2.6.2 Measurement of 25-Hydroxy Vitamin D(HPLC method)

Principle

This analysis has been done in the laboratory of Dr. Jacqueline Berry in the Department of Medicine, University of Manchester (Supra-Regional Assay Center). Serum 25-OH vitamin D was also measured by another technique that is high-pressure liquid chromatography (HPLC) with ultraviolet quantification. This assay involves the use of a tritiated recovery standard, which means that a recovery correction has to be applied to the HPLC results. Anovel method developed in Dr. Mawer and Berry laboratory, where this measurement had been done, overcomes this problem by the use of an ultraviolet absorbing internal standard, which is

estimated on the same HPLC run as the samples, thus enabling an automatic correction to be made.

1. **Sample extraction.** Serum (0.5 ml) is placed into a 12 x 75- mm borosilicate glass culture tube containing 1000 cpm of [³H]25(OH)D₃ in 25µl of ethanol to monitor recovery of endogenous compound through the extraction and chromatographic procedures. Following a 15-min incubation with the tracer, 1 plasma volume of HPLC-grade acetonitrile is added to each sample. The sample is then vortex-mixed for 1 min, followed by centrifugation at 1000g for 10 min. The supernatant is removed into another 12 x 75-mm culture tube, and 1 plasma volume of distilled water is added.
2. **Solid-phase extraction chromatography.** C18 silica Sep-Pak cartridges (500 mg) and Sep-Pak racks were obtained. The C18 cartridges are washed in order with 5 ml of HPLC-grade methanol. The sample is then applied to the cartridge and eluted through the cartridge under vacuum into waste. This initial step is followed by 5 ml of 30%water in methanol (discard); then 3ml of acetonitrile is added and collected as 25(OH)D. this acetonitrile fraction is dried in a heated water bath at 55°C, under N₂. The lipid residue is then resuspended in 1 ml of 1.5% isopropanol in hexane and capped. The C18 cartridges can be cleaned and regenerated by washing with 2 ml of methanol and reused many times.
3. **Silica cartridge chromatography.** Silica Bond-Elut cartridges (500 mg) and Vac-Elut cartridge racks were obtained. The silica cartridges are washed in order with 5 ml of HPLC-grade methanol, 5 ml of

HPLC- grade isopropanol, and 5 ml of HPLC-grade hexane. The sample, in 1 ml of 1.5% isopropanol in hexane, is then applied to the cartridge and eluted through the cartridge under vacuum into waste. This initial elution is followed by 4 ml of 1.5% isopropanol in hexane (discard) and 6 ml of 5% isopropanol in hexane. This 6-ml fraction contains 25(OH)D₂ and 25(OH)D₃ and is subsequently dried in a heated water bath at 55°C, under N₂.

4. **Quantitative normal-phase HPLC.** The final quantitative step is performed with normal-phase HPLC, using a 0.4 x 25-cm Zorbax-Sil column packed with 5-µm spherical silica. The mobile phase is comprised of Hexane/dichloromethane/isopropanol (50:50:2.5, v/v) at a flow rate of 2ml/min. The sample residue from the silica cartridge is dissolved in 150µl of mobile phase and injected onto the HPLC column previously calibrated with varying amounts of 25(OH)D₂ and 25(OH)D₃ (1-100 ng). This HPLC system provides clear resolution of 25(OH)D₂ and 25(OH)D₃. Elution and final quantitation of 25(OH)D₂ and 25(OH)D₃ is by direct UV monitoring at 265 nm. The 25(OH)D₃ portion is collected, dried under N₂, and subjected to liquid scintillation counting to determine the final endogenous recovery of 25(OH)D₂ and 25(OH)D₃ from the sample. Calculations are then performed and the results reported in ng/ml 25(OH)D₂ and/or 25(OH)D₃.

2.6.3 Measurement of 1,25-Dihydroxy Vitamin D (EIA method)

Principle

Serum 1,25-dihydroxyvitamin D was measured by a competitive enzyme immuno assay (EIA). This is based on the competition of 1,25-dihydroxyvitamin D present in the sample with labeled 1,25-dihydroxyvitamin D tracer for the binding site of the vitamin D specific antibody.

Procedure

In this assay, the biological active vitamin D metabolite 1,25-(OH)₂ D₃ has to be extracted with two separate extraction columns. This procedure is necessary to separate the 1,25-(OH)₂ Vit D from other vitamin D metabolites especially from the 25-OH Vit D and the 24,25-(OH)₂ Vit D. After evaporation, samples, calibrators and control are dissolved in ethanol and antibody (mouse- anti 1,25-(OH)₂ vitamin D) is added. The samples then have to be transferred to the microtiterplate. 1,25(OH)₂ Vit D present in the sample competes with the tracer for the specific binding site of the specific antibody. So, with the increasing concentrations of 1,25 (OH)₂ Vit D in the sample, the amount of antibody immobilized to the well via the tracer is decreased. 1,25 Vit D is indirectly measured with a host specific peroxidase labeled antibody using TMB (tetramethylbenzidine) as enzyme substrate. The intensity of the color is indirectly proportional to the concentration of 1,25 (OH) Vit D in the sample (Armbruster *et al.*, 2000). A dose response curve of the absorbance unit vs. concentration is generated using the results obtained from the calibrators. Concentrations of 1,25 (OH)₂ Vit D present in the patient samples are determined directly from this curve.

Precision

Within-run (intra-assay) and between-run (inter-assay) precision was determined by assaying 3 samples in 8 runs. Results are provided below. For samples 1 through 3 represent low normal, high normal, and elevated levels.

Sample	1,25(OH)D Mean (pg / ml)	Within-run ¹ CV%	Between-run ² CV%
1	18.46	9.2	7.3
2	40.93	6.9	6.6
3	51.65	10.0	9.0

¹n=8 runs

²n=5 runs

CV% = Percent coefficient of variance

$$= \frac{SD}{\text{Mean}} \times 100$$

2.6.4. Measurement of intact-PTH [Parathyroid hormone]

Principle

Intact parathyroid hormone (1-84) was analyzed by a two-site enzyme-linked immunosorbent assay [ELISA] with two different goat polyclonal antibodies to human PTH have been purified by affinity chromatography to be specific for well defined region on the PTH molecule. One antibody is prepared to bind only the mid-region and C-terminal PTH 39-84 and this antibody is biotinylated. The other antibody is prepared to bind only the N-terminal PTH 1-34 and this antibody is labeled with horseradish peroxidase [HRP] for detection. Although mid-region and C-terminal fragments are bound by the biotinylated anti-PTH (39-84), only the intact PTH 1-84 forms the sandwich complex necessary for detection. The capacity of the biotinylated antibody and the streptavidin-coated microwell both has been adjusted to exhibit negligible interference by inactive fragments, even at very elevated levels.

Procedure

In this assay, standards, controls, and samples are incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stopping solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of intact PTH in the sample. A dose response curve of absorbance unit vs. concentration is generated using results obtained from the calibrators. Concentrations of intact PTH present in the controls and patient samples are determined directly from this curve.

Precision

Within-run (intra-assay) and between-run (inter-assay) precision was determined by assaying 3 samples in 10 runs. Results are provided below. For samples 1 through 3 represent low normal, high normal, and elevated levels.

Sample	PTH Mean (pg/ml)	Within-run ¹ CV%	Between-run ¹ CV%
1	34.83	7.8	9.2
2	63.29	6.9	11.3
3	101.59	9.0	14.1

¹n=10 runs

²n=8 runs

CV% = Percent coefficient of variance

$$= \frac{SD \times 100}{Mean}$$

2.6.5. Measurement of calcium (Ca)

The quantitative determination of serum calcium was carried out by a fully automated clinical chemistry analyzer that uses colorimetric methods with endpoint determination as described by Gindler and King (1972). Determining calcium is based on the reaction of calcium with o-cresolphthalein complexone in alkaline solution. Magnesium is masked with 8-hydroxyquinoline. The color intensity of the purple complex formed is directly proportional to the calcium concentration and is measured photometrically. The within run (intra assay) and between run (inter assay) coefficients of variation (CVs) were 1.0% and 1.7%, respectively.

2.6.6. Measurement of inorganic phosphorus (PHOS)

Phosphorus in serum was analyzed by a fully automated method based on the reaction of phosphate with ammonium molybdate to form ammonium phosphomolybdate without reduction to molybdenum blue as described by Henry (1974). The addition of an accelerator gives rise to a more rapid rate of reaction and the application of sample blanking yields more precise results. Inorganic phosphate forms an ammonium phosphomolybdate complex having the formula $(\text{NH}_4)_3 [\text{PO}_4 (\text{MoO}_3)_2]$ with ammonium molybdate in the presence of sulfuric acid. The complex is determined photometrically in the ultraviolet region (340 nm). The within run (intra assay) and between run (inter assay) coefficients of variation (CVs) were 0.9% and 1.8%, respectively.

CHAPTER 3

Extent of osteoporosis and osteopenia in Saudi Arabian postmenopausal and premenopausal women: effects of non-dietary lifestyle factors on indices of bone health

3.1 Introduction

There are few data available concerning the bone health and lifestyle characteristics of women living in Middle Eastern countries. In Saudi Arabia there are two studies have been published examined BMD in healthy Saudi females in Capital Riyadh city (Ghannam *et al.*, 1999; Desouki, 2000). The study achieved by Ghannam *et al* to measure the bone mineral density (BMD) at the lumber spine and the proximal femur in 321 Saudi females aged 10-50 years to establish normative data in Saudi females and to compare these data with their USA counterparts. The other study present by El-Desouki was to estimate the prevalence of osteopenia and osteoporosis in postmenopausal Saudi women. Recently, (Ardawi *et al.*, 2004) measured bone mineral density BMD at the lumber spine and femur regions in 915 males and 1.065 females aged 20-79 years living in Jeddah area, to determine reference values for Saudis of both sexes and to compare these data with US / Northern European and Lebanese reference data. None of the three studies have examined the influence of diet, physical activity and lifestyle risk factors on skeletal health.

Risk factors for bone health can be divided into non-modifiable and modifiable risk factors. Non-modifiable risk factors such as age, sex, ethnicity, etc. are important when identifying high-risk individuals. Age, is the important risk factor for both osteoporosis and fractures. With advanced age bone is lost in both men and women, leading to increased risk of fractures (Johnell, 2003). BMD fell with age in elderly men and women (Hannan *et al.*, 2000). The modifiable risk factors such as low bone mass, physical activity, nutrition, body weight etc. can be modified so that will have less impact. Body weight is a significant predictor of bone density and fracture risk. There is direct relationship between body weight and

bone mass. According to National Osteoporosis Foundation, low body weight is one of four major risk factors for osteoporosis (Felson *et al.*, 1993). The finding that bone is lost in many individuals during weight loss has major health implications. Recent studies indicate that major risk factor for fracture is a low body weight and that maintenance of body weight can prevent bone loss (Hannan *et al.*, 2000; Uusi *et al.*, 2001). A few studies have shown that increased body height is related to an increased risk of hip fractures as in the MEDOS, where the tallest quintile in women had an increased risk (Johnell *et al.*, 1995; Kanis *et al.*, 1999). While many studies indicate that excessive height loss reflect low bone mass and may predict osteoporosis related fractures (Hunt, 1996; Gunnes *et al.*, 1996).

An epidemiological study suggests that physical activity can favorably influence the development and maintenance of bone mass and delay the progression of osteoporosis. Short period of immobilization results in rapid bone loss; with trabecular bone loss exceed that of the cortex (Mazess and Wheadon, 1983). Several studies have reported a high bone density in physically active subjects as compared with that of sedentary subjects in both cross-sectional (Heinonen *et al.*, 1995; Etherington *et al.*, 1996; Slemenda and Johnston, 1994) and prospective studies on postmenopausal (Nelson *et al.*, 1994) and premenopausal women (Cooper *et al.*, 1995; Heinonen *et al.*, 1996). There are many studies on the relationship between physical activity and fracture rate, which indicate that individuals with increased physical activity have a lower fracture rate when compared to those with low physical activity. In this risk factor there seems to be an interaction with nutrition, those who are physically active also have a better food intake (Johnell *et al.*, 1995; Kanis *et al.*, 1999).

3.1.1 Study aim

The aim of this analysis therefore was to identify the extent of osteopenia and examine the effect of lifestyle and non-dietary risk factors on indices of bone health in Saudi Arabian women. The effects of body weight, height, and age on bone mineral density at five skeletal sites were analyzed. Impact of duration of physical activity per week on bone density was examined.

3.2 Study design

3.2.1 Subject selection

In brief details of subject recruitment can be found in (Section 2.1.1). A total of 100 premenopausal and 112 postmenopausal women they were aged 20-30 years and 45-60 years respectively and had not suffered from any known condition and were not taking any medication likely to affect bone metabolism. The exclusion criteria used for the study are shown in Table 2.1 (Page 61).

3.2.2. Anthropometrics and bone mass measurements

Details of the anthropometrics and bone mass measurement undertaken can be found in (Section 2.1.2). In brief weight and height were recorded and body mass index (BMI) was calculated. Bone mineral density (BMD) was determined at the lumbar spine (L2-L4), femoral neck, femoral ward, and femoral trochanter using dual x-ray absorptiometry (DXA) (Lunar Corp., DPX version 4.7) (Section 2.2.1). Calcaneal bone mass was measured by broadband ultrasound attenuation (BUA) (CUBA^{plus+} softwareV4) (Section 2.2.2).

3.2.3. Collection of lifestyle data

In brief, a questionnaire was used to collect information on current activity levels. Subjects were asked to record the amount of time they spent walking, swimming, etc per week. The questionnaire was adapted for the physical activity section of the Scottish Heart Health Study and had been previously used in the physical activity and bone health studies (New *et al.*, 1997, 2000). A copy of the questionnaire can be found in Appendix II. Specifically, the following questionnaire were asked concerning physical activity levels of:

- Walking
- Jogging
- Cycling
- Swimming
- Aerobic.

Information on details of the lifestyle data, caffeine consumption, smoking and general lifestyle of the subjects can be found in (Section 2.3.1).

3.3. Statistical analysis

Anthropometric data are shown in Tables 3.1 and 3.2 for postmenopausal and premenopausal respectively. The results are presented as mean values with their standard deviation (SD), median, minimum and maximum. Bone data are expressed in the form of bone mineral density (BMD), broadband ultrasound attenuation (BUA), velocity of sound (VOS), t- score and z- score. Values in the tables are given as mean values with their SD, median and range (min-max). Lifestyle factors data are expressed as physical activity hour per week. Values are presented in tables as mean values with their SD, median and range (min-max). The data were checked for normality and the variables were found to be not-normally distributed, non-

parametric tests were applied. Multiple linear regression analysis were conducted to examine the relation of BMD percent changes with risk factors.

3.4. Results

3.4.1 Anthropometrics data

The subjects' anthropometric data are shown in Table 3.1 for postmenopausal and in Table 3.2 for premenopausal. Women were average height and weight for the local population. The average body mass index BMI for the group of postmenopausal was 30.9 ± 5.4765 (kg/m^2) that is higher than normal BMI and according to (WHO, 1998). These women were considered overweight (obese class I). Grading of obesity according to body mass index (BMI) were: 30.0-34.9 (kg/m^2) Obese class I; 35.0-39.9 (kg/m^2) Obese class II; >40.0 (kg/m^2) Obese class III. On the other hand, the average of body mass index BMI for the group of premenopausal women was 24.0 ± 5.688 (kg/m^2), the normal range is from 18.5-24.9 (kg/m^2).

Table 3.1: Anthropometric data for 112 postmenopausal Saudi women

	Mean \pm SD	Median	Range
N=112			
Age (Y)	49.5 \pm 5.0	48.0	43.0 – 60.0
Weight (kg)	75.7 \pm 14.5	73.0	51.50 – 115.0
Height (cm)	156.4 \pm 6.1	156.0	142.00 – 174.00
BMI (kg/m^2)	30.9 \pm 5.4	30.20	21.1 – 47.2

Table 3.2: Anthropometric data for 100 premenopausal Saudi women

	Mean \pm SD	Median	Range
N=100			
Age (Y)	23.07 \pm 3.5	22.0	20.0 – 33.0
Weight (kg)	60.9 \pm 15.0	59.0	38.70 – 112.50
Height (cm)	159.1 \pm 5.8	158.0	145.0 – 173.0
BMI (kg/m²)	24.0 \pm 5.6	22.4	16.0 – 50.0

3.4.2. Bone mineral density and calcaneal bone mass

Table 3.3 and Table 3.4 summarizes bone mineral density BMD at the anterior-posterior lumbar spine and three sites of the left femur, broadband ultrasound attenuation BUA at the calcaneal site, velocity of sound VOS, t-score and z-score in both groups. As shown in Table 3.3, BMD values of lumbar spine had normal distribution with minimum, maximum and median values of (0.09 - 1.57) g/cm² and 1.07 g/cm². BMD values for femoral neck, ward, trochanter and total hip were also normally distributed with minimum, maximum and median values. For BUA values was also distributed normally with minimum, maximum and median values of (41.0-120.0) and 66.0. Table 3.4 shows that all the BMD and QUS measurement variables were significantly higher in premenopausal women compared with the postmenopausal women.

As shown in the tables bone health indices indicated a high prevalence of low bone mass in both groups. The percentage of women classified as osteopenic and osteoporotic was calculated for postmenopausal women aged 45-60 years and premenopausal women aged 20-30 years. According to WHO criteria (WHO, 1994) a total of 52% of postmenopausal and 37% of premenopausal women were osteopenic at the lumbar spine. Similar results were found for the femoral neck

32% of postmenopausal and 23% of premenopausal was osteopenic. For calcaneus they were 62% postmenopausal, 36% premenopausal was osteopenic. The percentages of women in the two groups at the lumbar spine defined, as osteoporotic were 13% and 2% respectively.

Table 3.3: Bone indices data for 112 postmenopausal Saudi women

	Mean \pm SD n=112	Median	Range
Lumbar spine BMD (g/cm²)	1.07 \pm 0.18	1.07	0.09 – 1.57
Femoral neck BMD (g/cm²)	0.91 \pm 1.6	0.89	0.61 – 1.44
Femoral ward BMD (g/cm²)	0.78 \pm 0.18	0.75	0.45 – 1.40
Femoral trochanter BMD (g/cm²)	0.79 \pm 0.1	0.77	0.51 – 1.32
Total femoral BMD (g/cm²)	0.97 \pm 0.16	0.95	0.66 – 1.42
Broadband ultrasound attenuation BUA (db/MHz)	69.61 \pm 15.86	66.0	41.0 – 120.0
Velocity of sound VOS (m/s)	1611.61 \pm 28.54	1611.0	1554.0 – 1675.0
Lumbar spine t-score	- 0.98 \pm 1.32	- 1.10	- 4.40 – 3.10
Total femoral t-score	- 0.23 \pm 1.33	- 0.40	- 2.80 – 4.0
BUA t-score	- 1.19 \pm 0.96	- 1.390	- 2.93 – 1.84
VOS t-score	- 2.1 \pm 0.66	- 2.120	- 3.45 – (-0.63)
Lumbar spine z-score	- 0.86 \pm 1.13	- 0.800	- 3.90 – 3.0
Total femoral z-score	- 0.127 \pm 1.13	- 0.30	- 2.10 – 3.50
BUA z-score	- 1.02 \pm 8.67	- 0.440	- 9.0 – 2.67
VOS z-score	- 0.91 \pm 0.64	- 0.910	- 2.41 – 0.73

Table 3.4: Bone indices data for 100 premenopausal Saudi women

	Mean \pm SD n=100	Median	Range
Lumbar spine BMD (g/cm²)	1.13 \pm 0.12	1.13	0.81- 1.41
Femoral neck BMD (g/cm²)	0.96 \pm 0.1	0.94	0.62 - 1.32
Femoral ward BMD (g/cm²)	0.92 \pm 0.15	0.93	0.53 - 1.40
Femoral trochanter BMD (g/cm²)	0.77 \pm 0.12	0.76	0.46 - 1.10
Total femoral BMD (g/cm²)	0.97 \pm 0.13	0.978	0.61 – 1.34
Broadband ultrasound attenuation BUA (db/MHz)	78.36 \pm 13.49	78.0	46.0 – 114.0
Velocity of sound VOS (m/s)	1802.71 \pm 1508.6	1655.0	1531.0 – 16811.0
Lumbar spine t-score	- 0.57 \pm 1.01	- 0.50	- 3.20 – 1.70
Total femoral t-score	- 0.20 \pm 1.11	- 0.20	- 3.20 – 2.90
BUA t-score	- 0.68 \pm 0.81	- 0.7	- 2.62 – 1.50
VOS t-score	- 1.14 \pm 0.69	- 1.1	- 3.98 – 0.28
Lumbar spine z-score	- 0.43 \pm 0.9	- 0.50	- 3.0 – 1.60
Total femoral z-score	- 0.16 \pm 0.9	- 0.10	- 2.06 – 1.90
BUA z-score	- 0.93 \pm 3.71	- 0.6	- 37.0 – 1.54
VOS z-score	- 1.11 \pm 1.25	- 1.0	- 11.67 – 0.38

3.4.3. Effect of body weight on bone health indices in postmenopausal women

The data were examined for any association existed between body weight and bone mineral density (BMD) at five skeletal sites, the lumbar spine, the femoral neck, the femoral ward, the femoral trochanter, and the calcaneal, in postmenopausal women. There was a significant positive association between body weight and BMD at all five sites ($P < 0.001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.003$) respectively (Fig. 3.1, 3.2, 3.3, 3.4, 3.5).

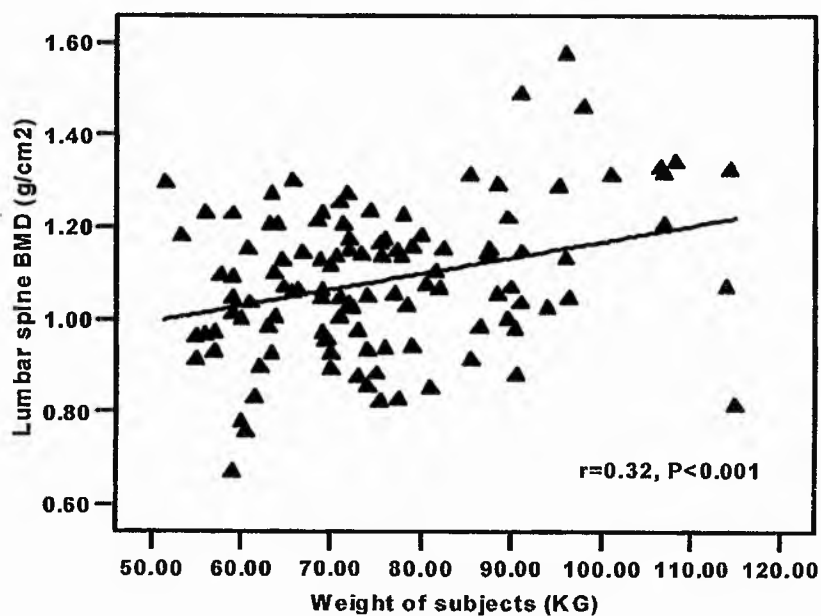


Figure 3.1: Effect of body weight on lumbar spine BMD in postmenopausal women

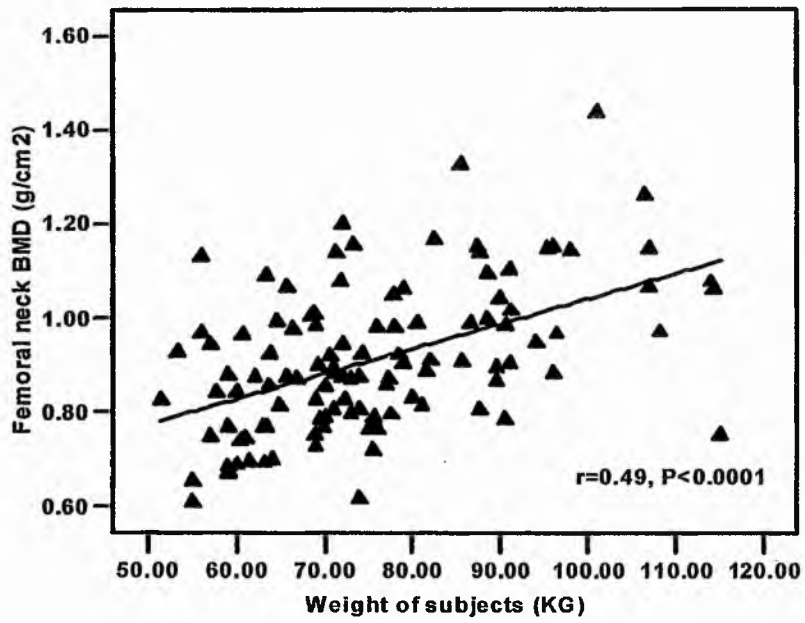


Figure 3.2: Effect of body weight on Femoral neck BMD in postmenopausal women

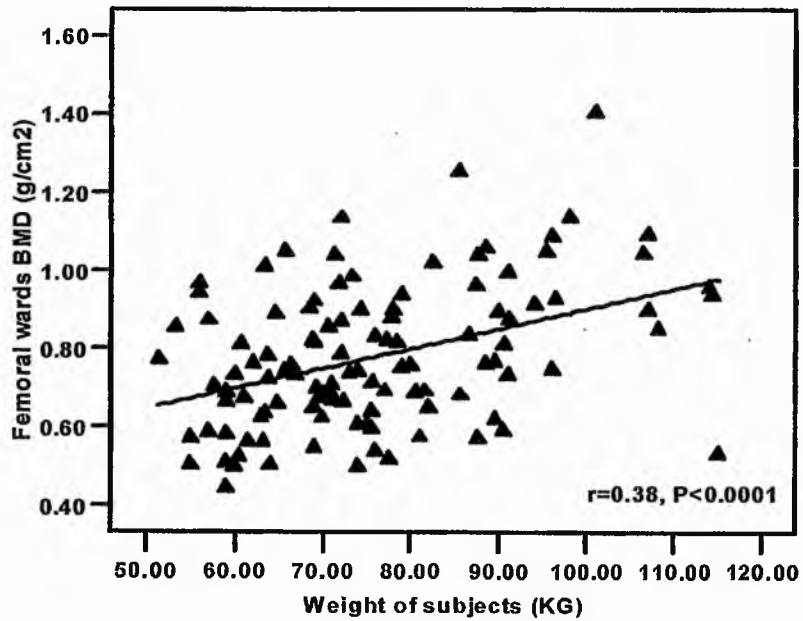


Figure 3.3: Effect of body weight on Femoral ward BMD in postmenopausal women

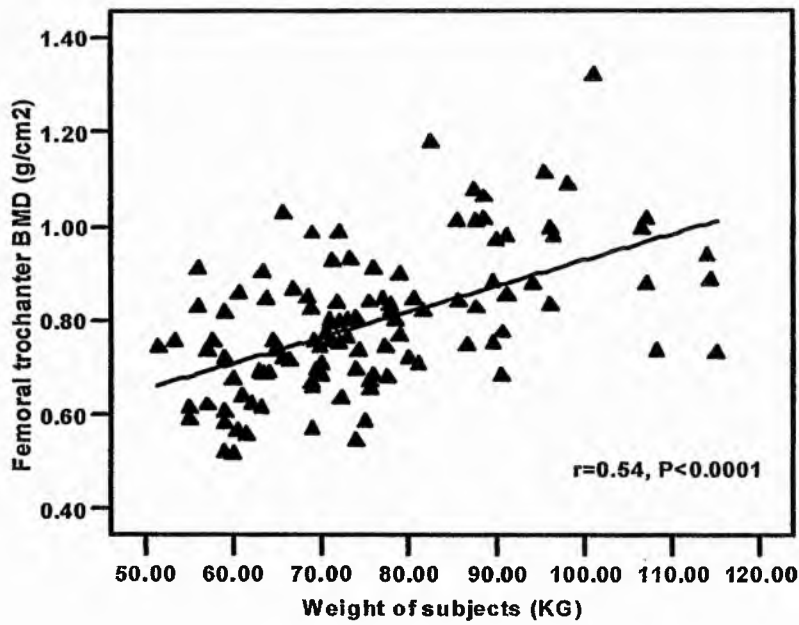


Figure 3.4: Effect of body weight on femoral trochanter BMD in postmenopausal women

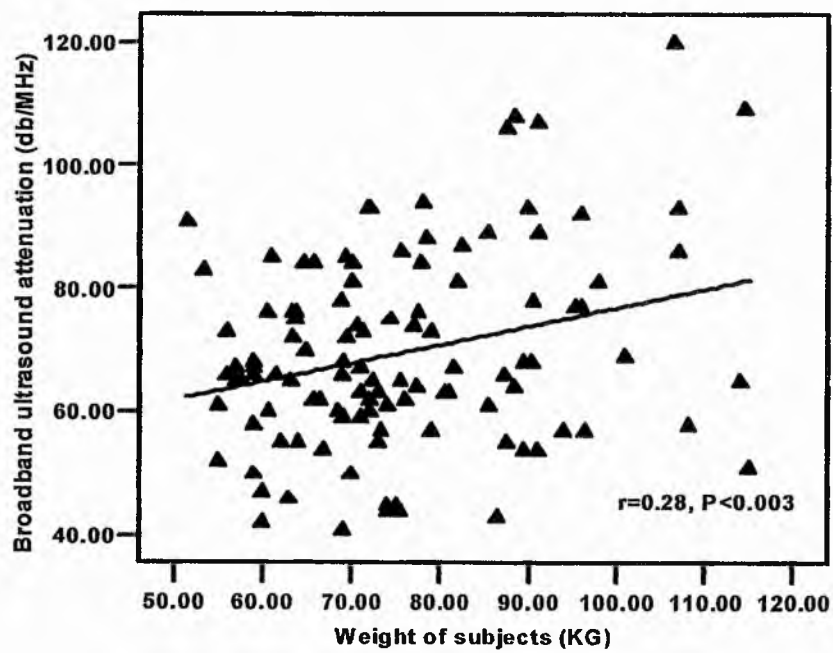


Figure 3.5: Effect of body weight on BUA in postmenopausal women

3.4.4. Effect of body weight on bone health indices in premenopausal women

The data were examined for the effects of weight on bone mineral density (BMD) at five skeletal sites, the lumbar spine, femoral neck, femoral ward, femoral trochanter, and calcaneal, in premenopausal women. There was a significant positive correlation between body weight and BMD at all five sites ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.003$) respectively (Fig. 3.6, 3.7, 3.8, 3.9, 3.10).

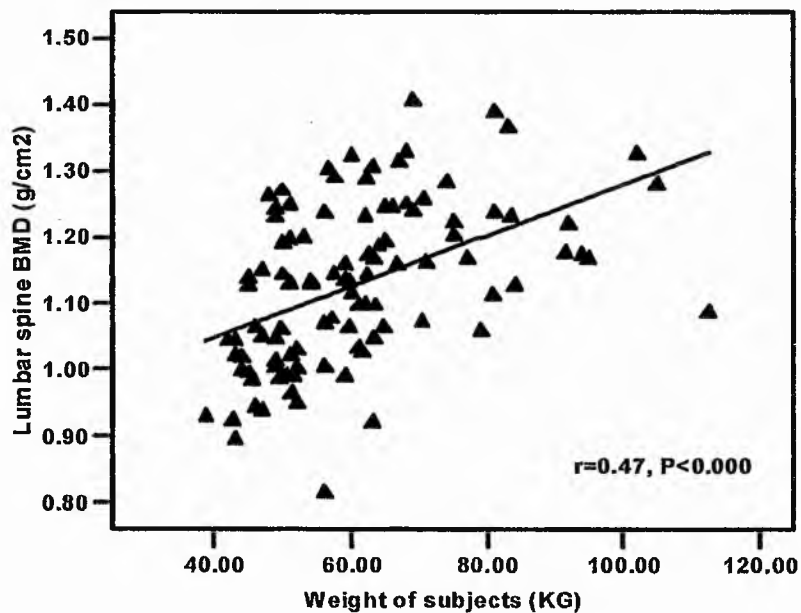


Figure 3.6: Effect of body weight on lumbar spine BMD in premenopausal women

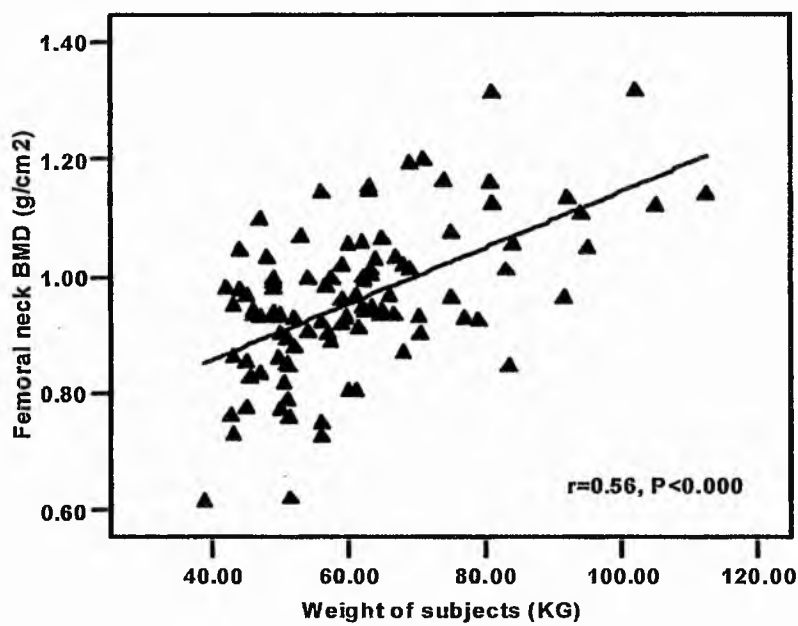


Figure 3.7: effect of body weight on femoral neck BMD in premenopausal women

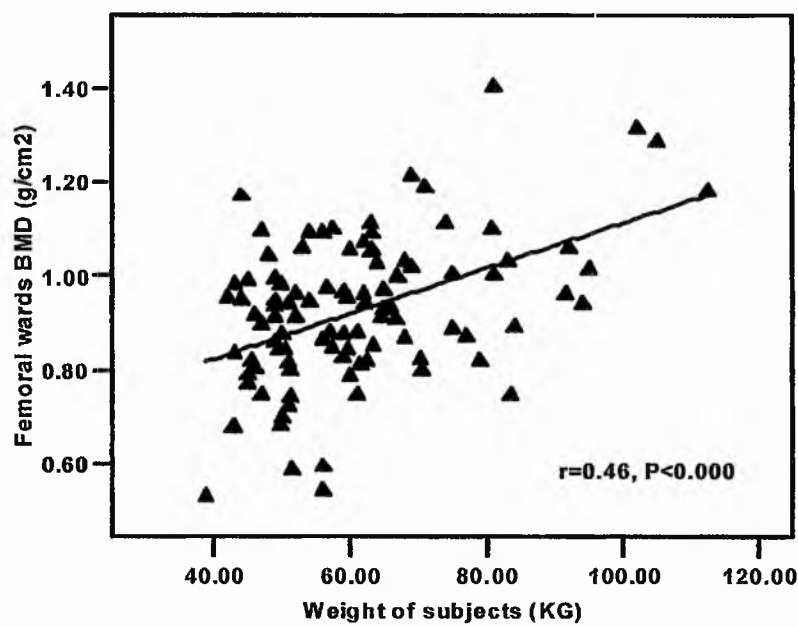


Figure 3.8: Effect of body weight on femoral ward BMD in premenopausal women

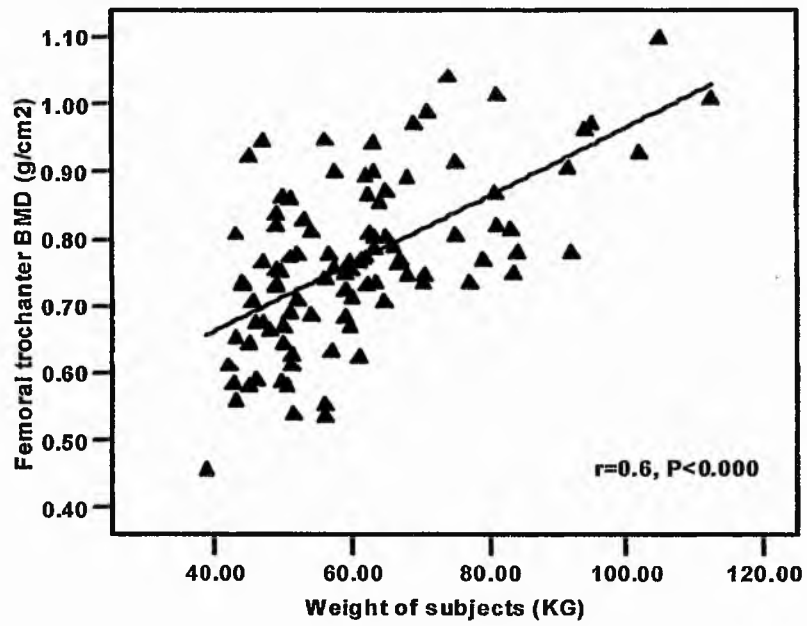


Figure 3.9: Effect of body weight on femoral trochanter BMD in premenopausal women

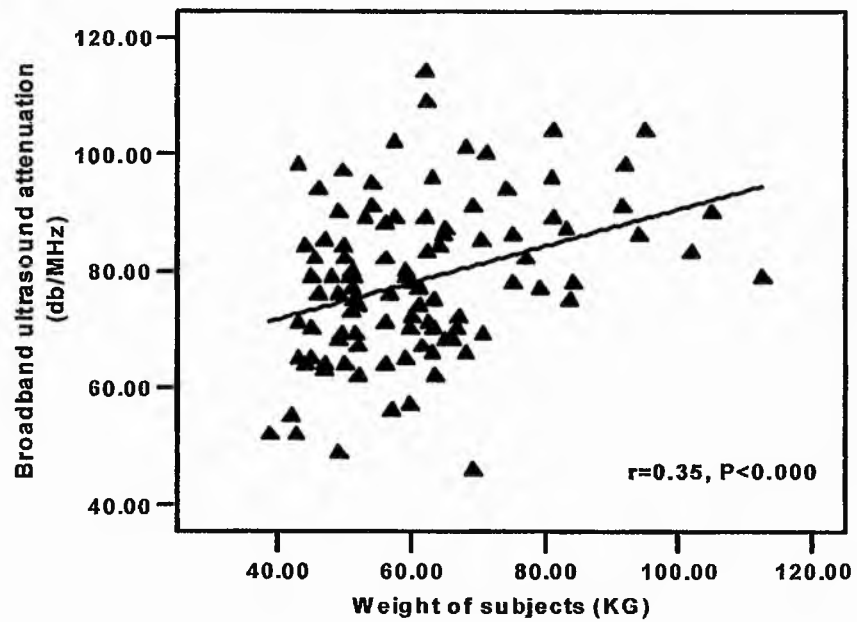


Figure 3.10: Effect of body weight on BUA in premenopausal women

3.4.5. Effect of body height on bone health indices in postmenopausal women

The data were examined for an association between body height and bone mineral density at four skeletal sites, the lumbar spine, the femoral neck, the femoral ward, and the femoral trochanter, in postmenopausal women. Pearson product-moment correlation demonstrated that, there was a significant positive association between body height and BMD at all four sites ($P < 0.001$, $P < 0.004$, $P < 0.02$, $P < 0.008$) respectively (Figure 3.11, 3.12, 3.13, 3.14).

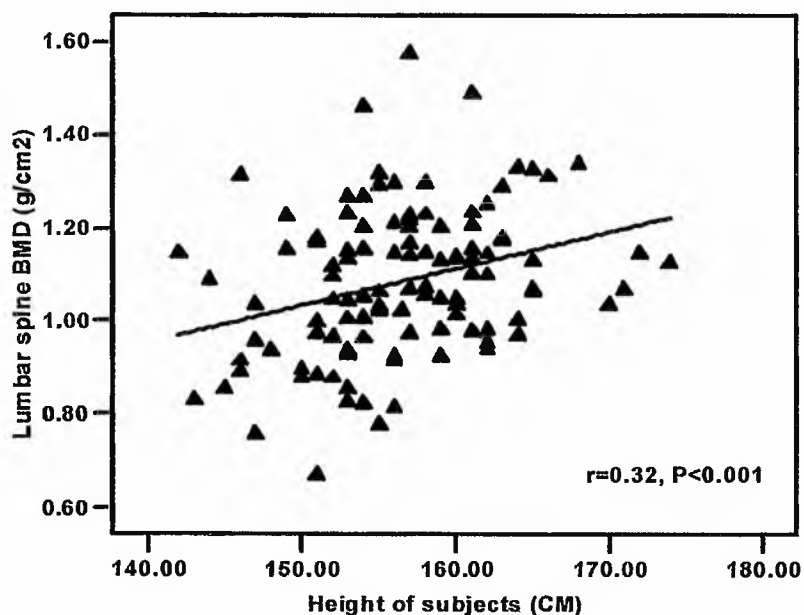


Figure 3.11: Effect of body height on lumbar spine BMD in postmenopausal women

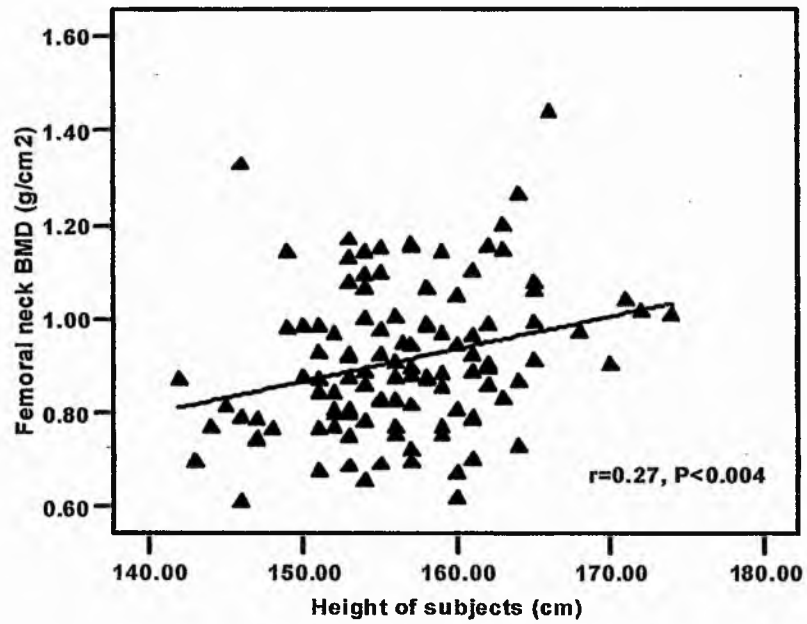


Figure 3.12: Effect of body height on femoral neck BMD in postmenopausal women

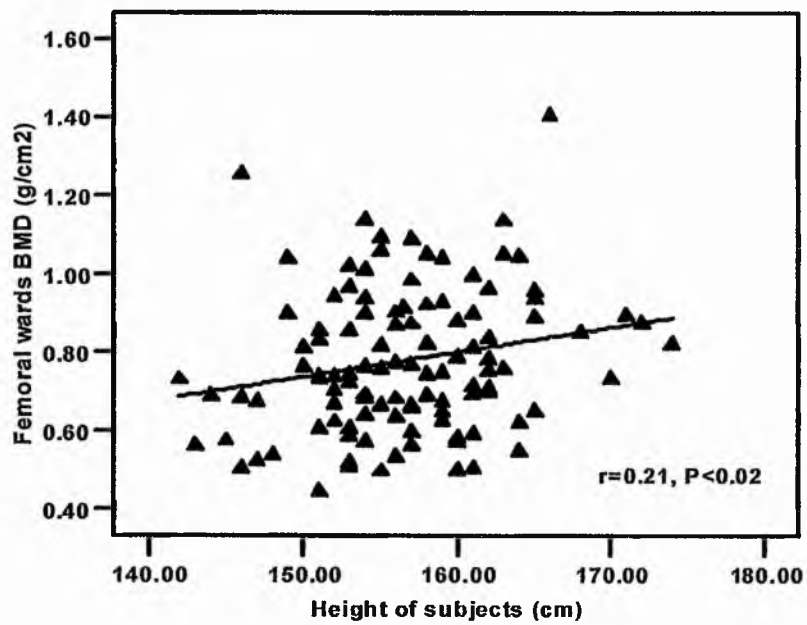


Figure 3.13: Effect of body height on femoral ward BMD in postmenopausal women

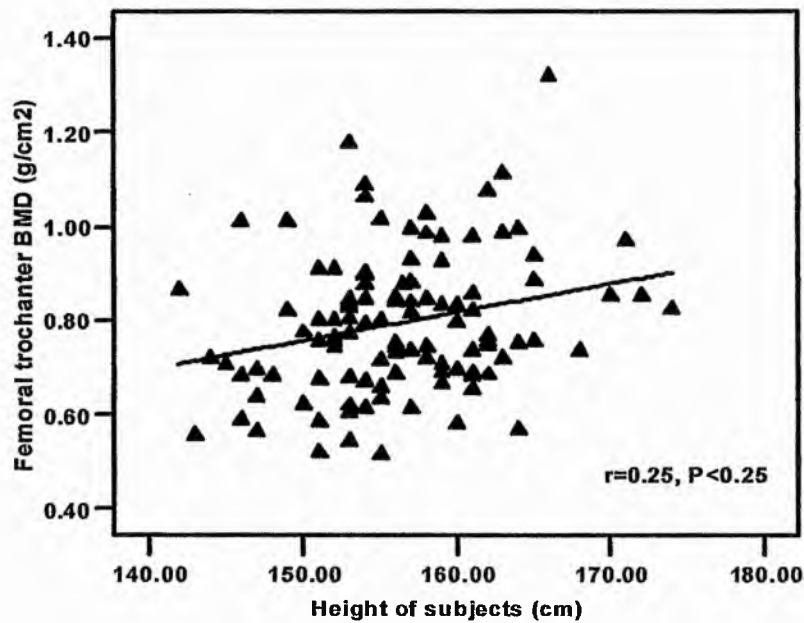


Figure 3.14: Effect of body height on femoral trochanter BMD in postmenopausal women

3.4.6. Effect of body height on bone health indices in premenopausal women

The data were examined for the effects of body height on bone mineral density (BMD) at five skeletal sites, the lumbar spine, femoral neck, femoral ward, femoral trochanter, and calcaneal, in premenopausal women. Pearson product-moment correlation demonstrated that, there was a significant positive correlation between body height and BMD only at the lumbar spine, femoral neck and positive trend for the calcaneal site ($P < 0.0001$, $P < 0.03$, $P < 0.08$) respectively (Fig. 3.15, 3.16).

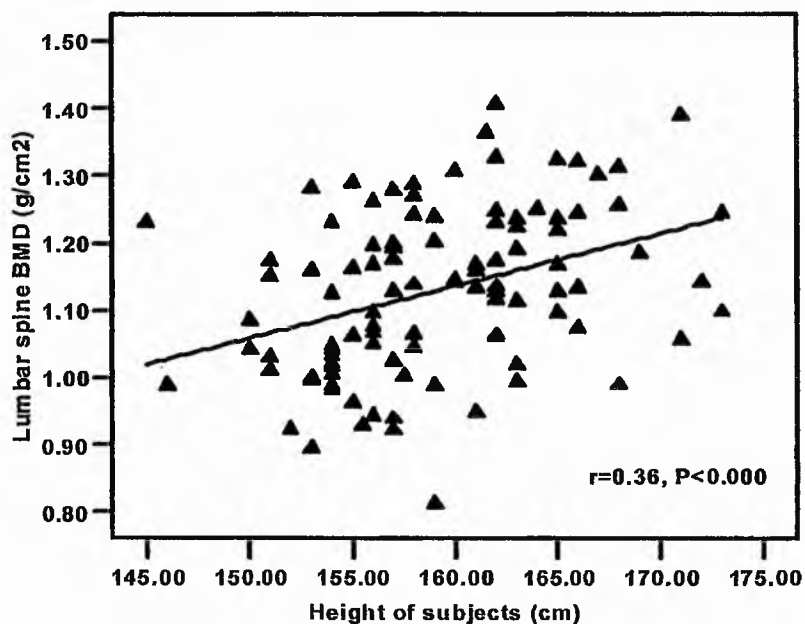


Figure 3.15: Effect of body height on lumbar spine BMD in premenopausal women

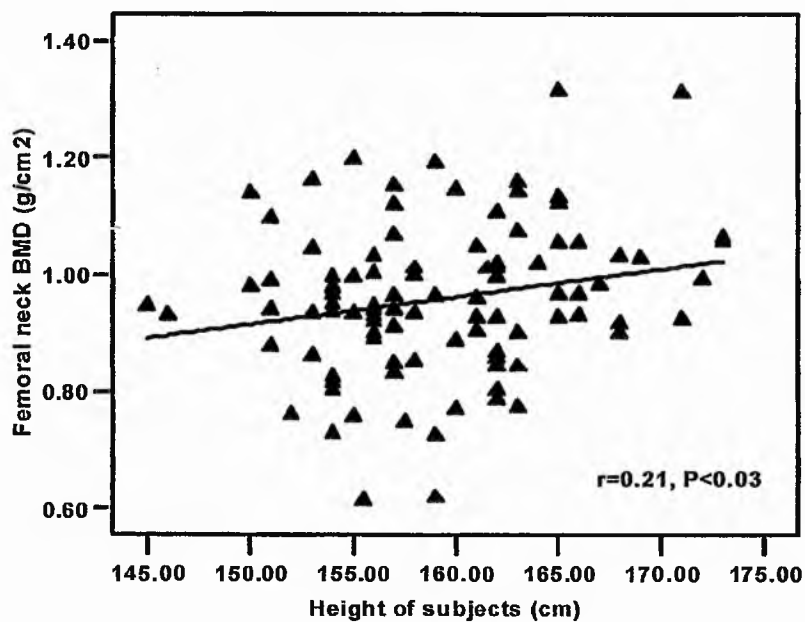


Figure 3.16: Effect of body height on femoral neck BMD in premenopausal women

3.4.7. Influence of age on bone health indices in postmenopausal women

The influence of age on postmenopausal bone indices was examined. There was association between age and bone mineral density at all five skeletal sites, the lumbar spine, femoral neck, femoral ward, femoral trochanter, and calcaneal. Pearson product-moment correlation demonstrated that, there was a significant negative correlation between age and BMD at four sites except for calcaneal there was negative trend with age ($P < 0.0001$, $P < 0.02$, $P < 0.002$, $P < 0.05$, $P < 0.2$) respectively (Fig. 3.17, 3.18, 3.19, 3.20). There was a significant negative correlation between age and velocity of sound (VOS) ($P < 0.007$).

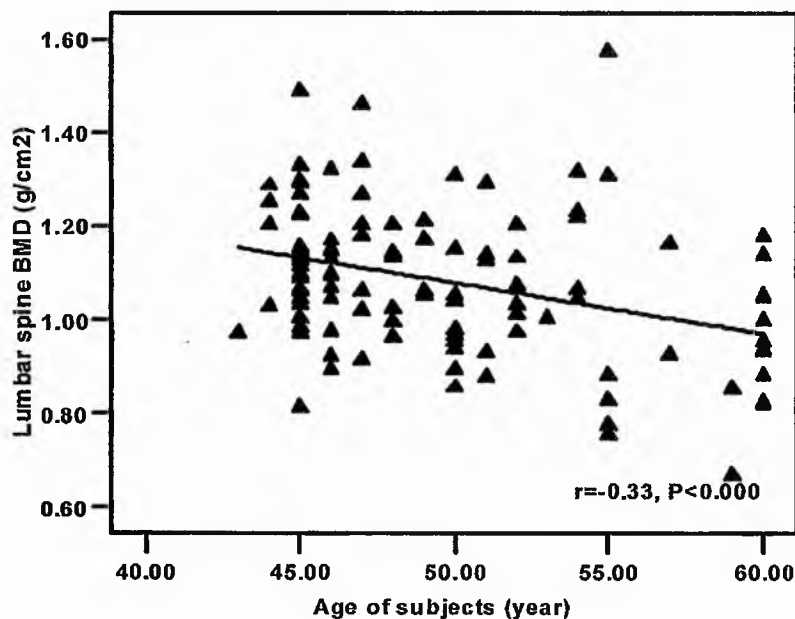


Figure 3.17: Effect of age on lumbar spine BMD in postmenopausal women

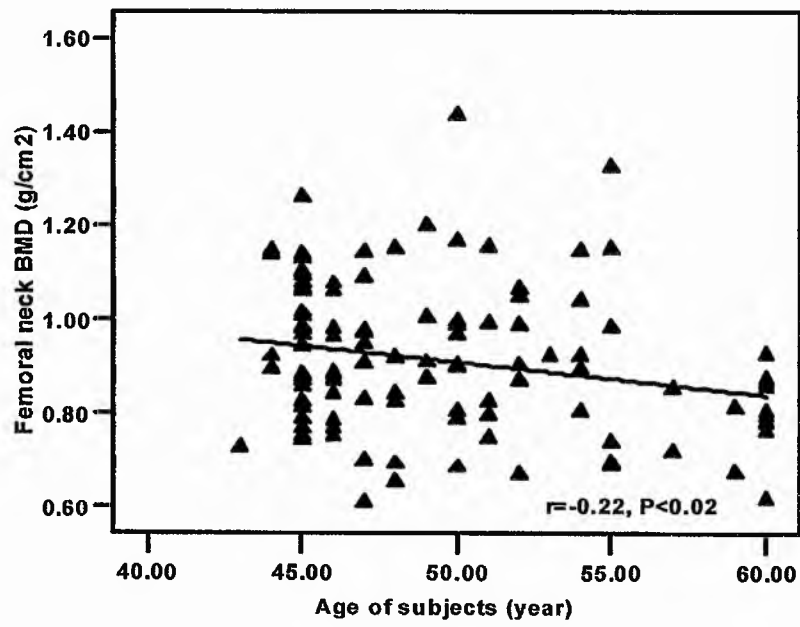


Figure 3.18: Effect of age on femoral neck BMD in postmenopausal women

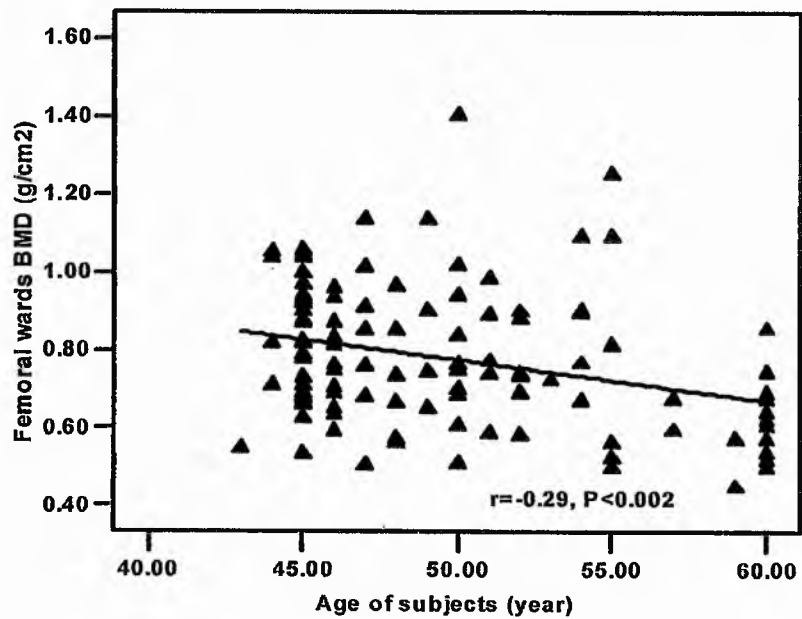


Figure 3.19: Effect of age on femoral ward BMD in postmenopausal women

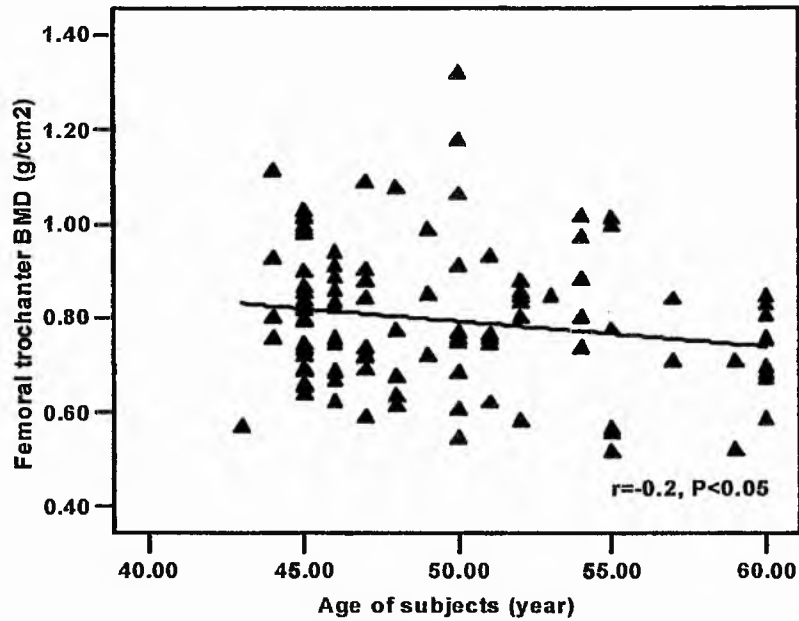


Figure 3.20: Effect of age on femoral trochanter BMD in postmenopausal women

3.4.8. Influence of age on bone health indices in premenopausal women

The data were examined for the effects of age on bone mineral density (BMD) at five skeletal sites, the lumbar spine, femoral neck, femoral ward, femoral trochanter, and calcaneal, in premenopausal women. Pearson product-moment correlation demonstrated that, there wasn't any correlation between age and BMD at the lumbar spine, femoral neck only negative trend at the femoral ward site ($P<0.07$). There was a significant negative correlation between age and velocity of sound (VOS) ($P<0.003$).

3.4.9. Relationship between physical activity and bone indices in postmenopausal women

The data were analyzed for an association between physical activity as (hr/week) and bone mineral density (BMD) in the postmenopausal women group. A physical activity was significantly associated (Spearman correlation) with BMD at five skeletal sites: the lumbar spine, femoral neck, femoral ward, femoral trochanter and calcaneum ($P < 0.0001$, $P < 0.002$, $P < 0.001$, $P < 0.0001$, $P < 0.01$) respectively. (Fig. 3.21, 3.22, 3.23, 3.24, 3.25).

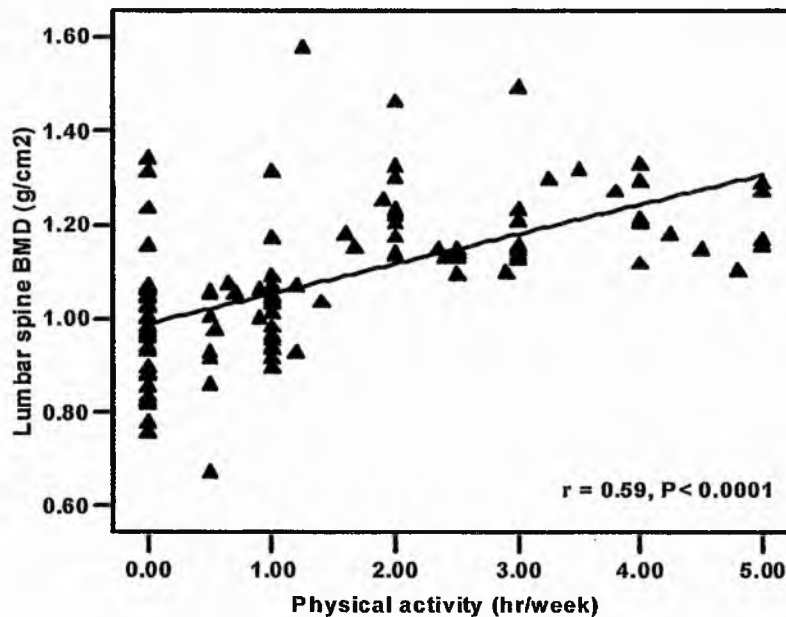


Figure 3.21: Impact of physical activity on lumbar spine BMD in postmenopausal women

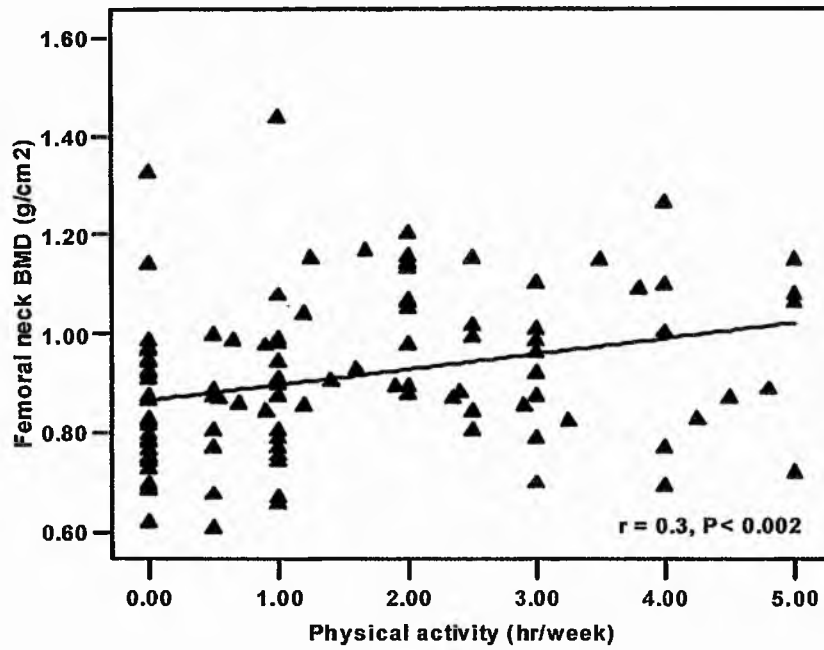


Figure 3.22: Impact of physical activity on femoral neck BMD in postmenopausal women

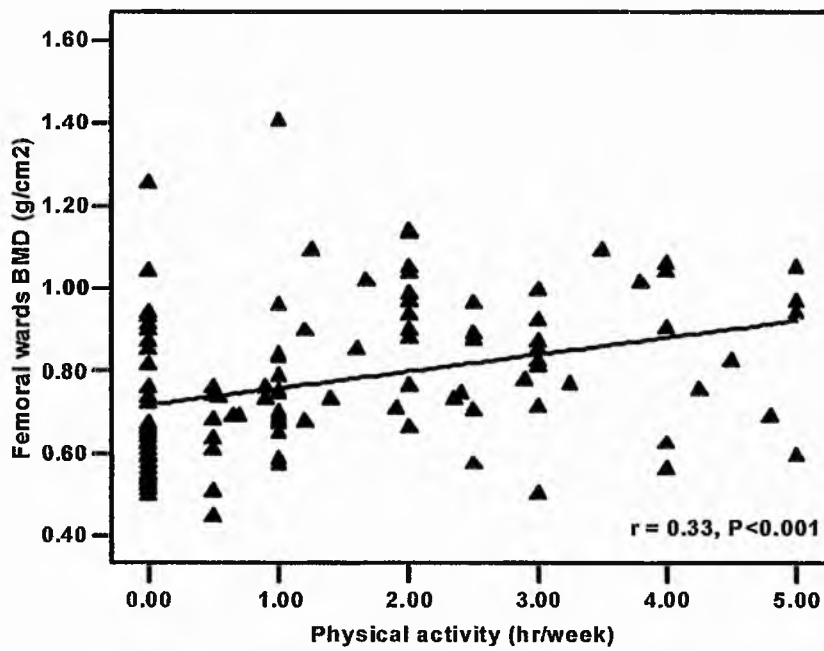


Figure 3.23: Impact of physical activity on femoral ward BMD in postmenopausal women

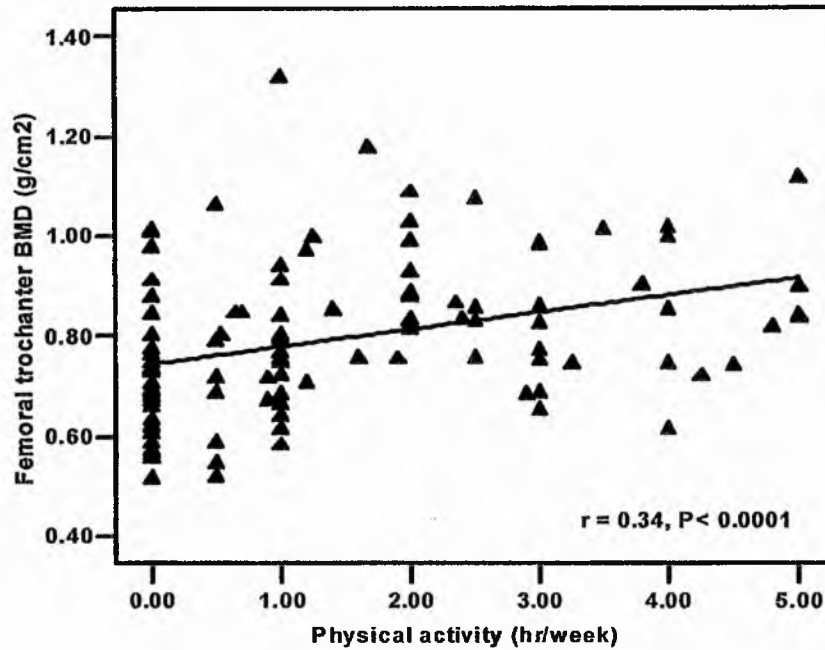


Figure 3.24: Impact of physical activity on femoral trochanter BMD in postmenopausal women

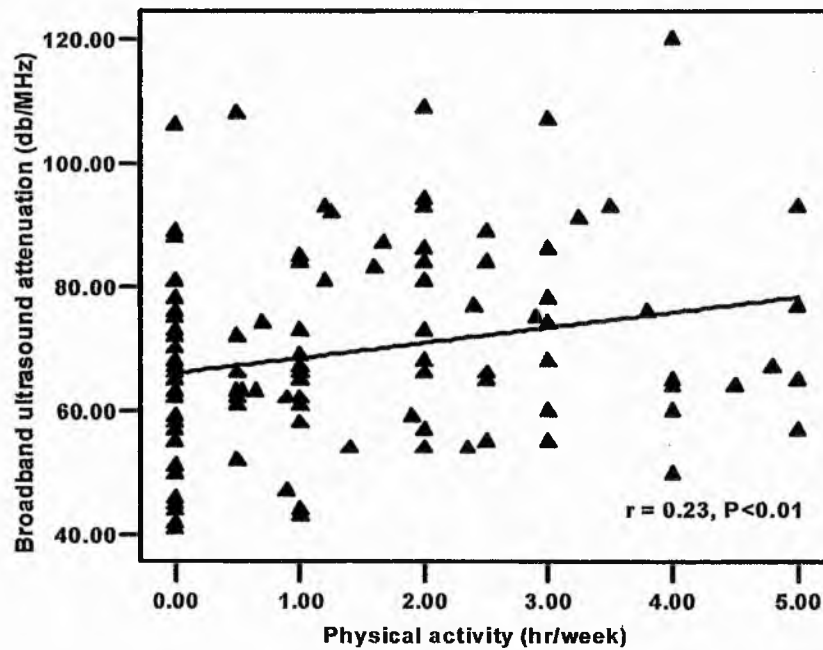


Figure 3.25: Impact of physical activity on calcaneal BUA in postmenopausal women

3.4.10. Relationships between physical activity and bone indices in premenopausal women

The data had been analyzed for an association between physical activity as (hr/week) and bone mineral density (BMD) in premenopausal women group. Physical activity was significantly associated with BMD at five skeletal sites, the lumbar spine, the femoral neck, the femoral ward, the femoral trochanter, and the calcaneal ($P < 0.001$, $P < 0.01$, $P < 0.002$, $P < 0.01$, $P < 0.01$) respectively. (Fig. 3.26, 3.27, 3.28, 3.29, 3.30)

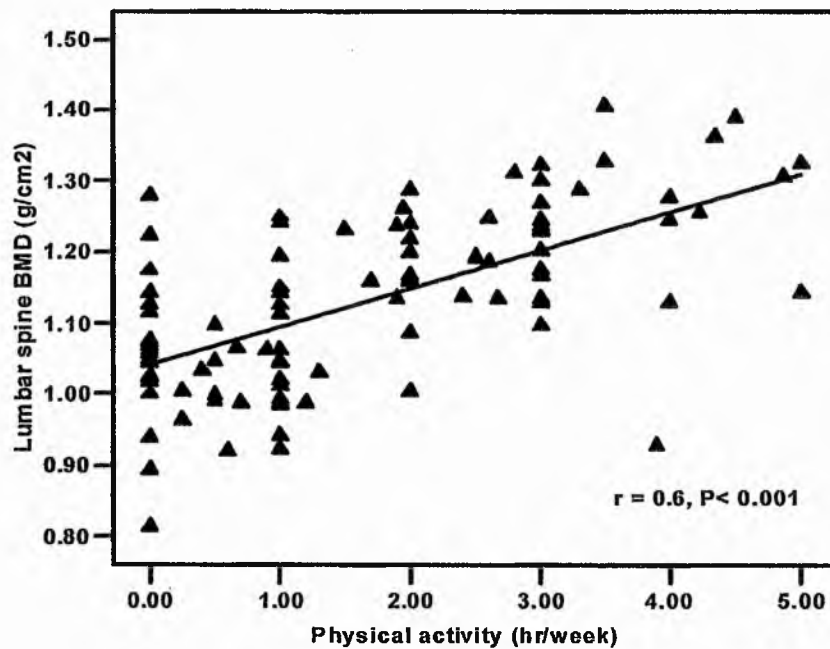


Figure 3.26: Impact of physical activity on lumbar spine BMD in premenopausal women

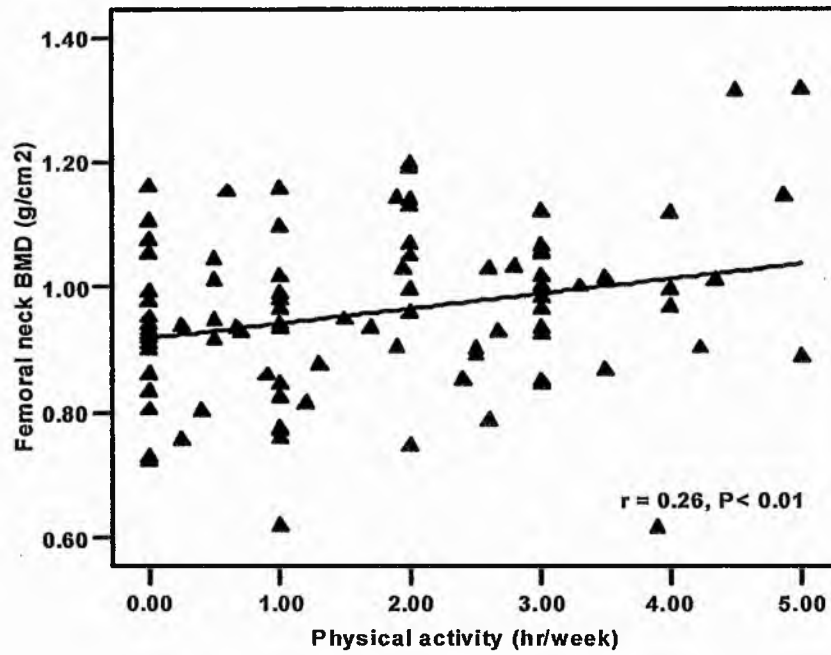


Figure 3.27: Impact of physical activity on femoral neck BMD in premenopausal women

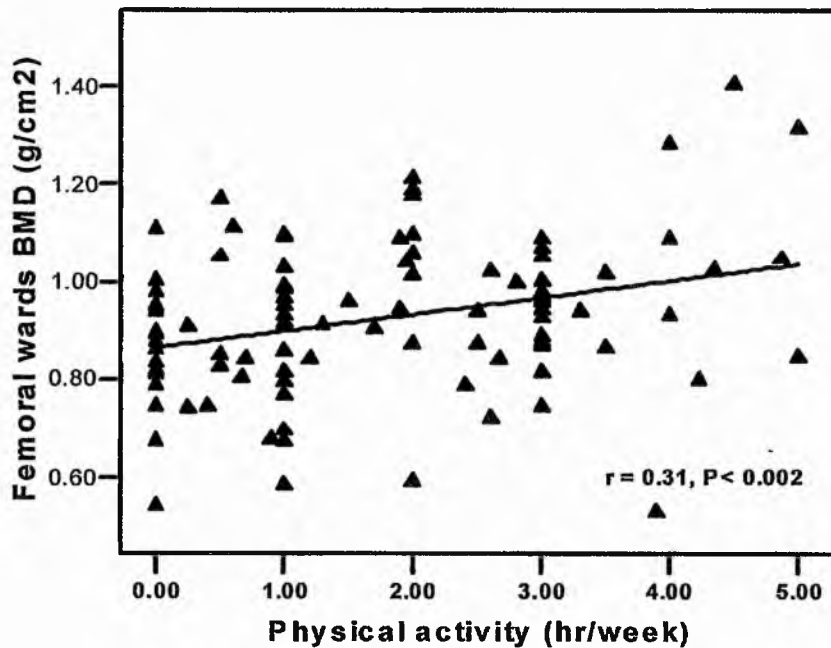


Figure 3.28: Impact of physical activity on femoral wards BMD in premenopausal women

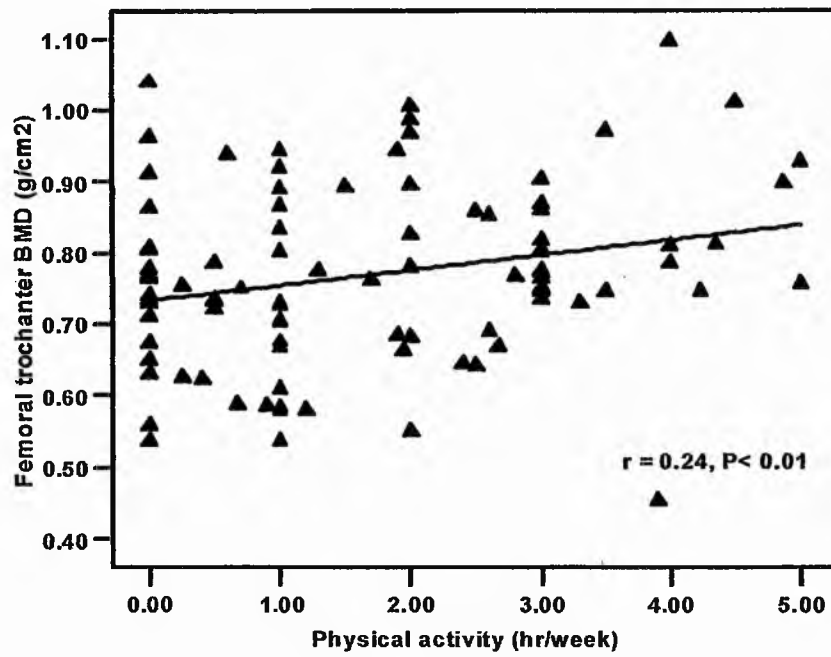


Figure 3.29: Impact of physical activity on femoral trochanter BMD in premenopausal women

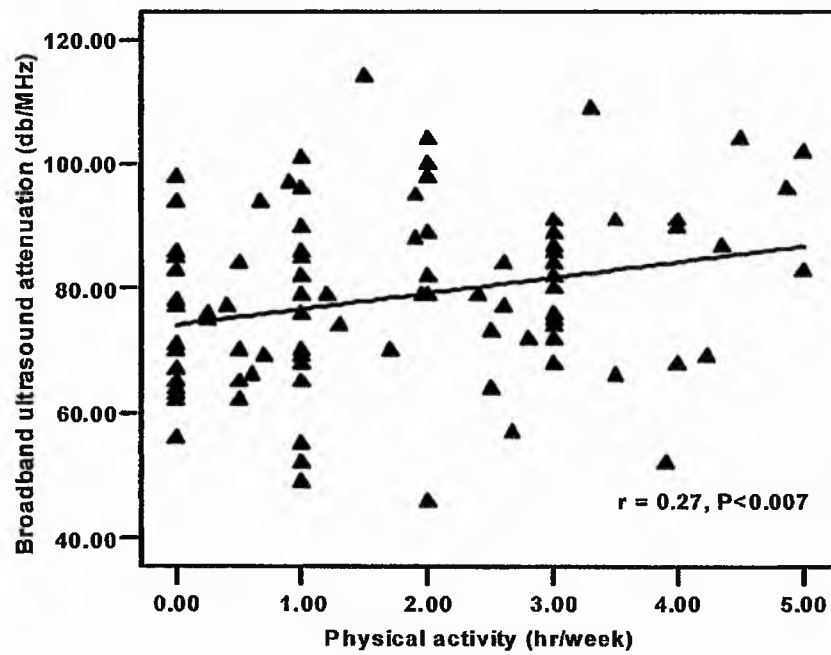


Figure 3.30: Impact of physical activity on BUA in premenopausal women

3.4.11. Multilinear regression analysis in postmenopausal women

Multiple variables that could possibly affect bone mineral density had been examined by regression analysis. Age, body weight, height and physical activity remained independent risk factors at all four skeletal sites (Lumbar spine, femoral neck, femoral ward and femoral trochanter) in postmenopausal women Table 3.5, 3.6, 3.7, and 3.8 respectively show the effect of age, weight, height and physical activity in postmenopausal group of Saudi women. Age-related changes in bone mineral density were evident. There was a decreased in BMD values by about 0.5% per year (lumbar spine), 0.5% per year (femoral neck), 0.8% per year (femoral wards) and did not come o as a significant predictors for femoral trochanter. The influences of body weight on BMD values in all four skeletal sites are nearly the same it exhibited increases of 0.5% per kilogram of the body. Height did not come out as a significant predictor of bone mineral density at any site measured. Physical activity showed significant increases in all four skeletal sites (% per hour): 5.4% (lumbar spine), 2.2% (femoral neck), 3.0% (femoral wards) and 2.8% (femoral trochanter).

Table 3.5: Regression coefficients of BMD for lumbar spine site on age, Weight, height and physical activity in postmenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.842	.347		.017
	Age of subjects (year)	-.005	.002	-.165	.034
	Weight of subjects (kg)	.003	.001	.259	.001
	Height of subjects (cm)	.001	.002	.051	.526
	Physical activity (hr/week)	.054	.008	.503	.000

Dependent Variable: Lumbar spine BMD (g/cm²)

$$\text{L.S. BMD} = 0.842 - 0.005 \times \text{Age} + 0.003 \times \text{Weight} + 0.054 \times \text{physical activity}$$

Multiple correlation coefficient (R)= 0.671

Model significant = P<0.0001

Table 3.6: Regression coefficients of BMD for femoral neck site on age, Weight, height and physical activity in postmenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.676	.380		.078
	Age of subjects (year)	-.005	.003	-.148	.084
	Weight of subjects (kg)	.005	.001	.473	.000
	Height of subjects (cm)	.000	.002	.012	.896
	Physical activity (hr/week)	.022	.009	.205	.017

Dependent Variable: Femoral neck BMD (g/cm²)

Femoral neck BMD = 0.676 – 0.005 x Age + 0.005 x Weight + 0.022 x physical activity.

Multiple correlation coefficient (R)= 0.574

Model significant = P<0.0001

Table 3.7: Regression coefficients of BMD for femoral wards site on age, Weight, height and physical activity in postmenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.942	.450		.039
	Age of subjects (year)	-.008	.003	-.214	.015
	Weight of subjects (kg)	.005	.001	.405	.000
	Height of subjects (cm)	-.001	.003	-.044	.628
	Physical activity (hr/week)	.030	.011	.239	.007

Dependent Variable: Femoral wards BMD (g/cm²)

Femoral wards BMD = 0.942 – 0.008 x Age + 0.005 x Weight + 0.030 x physical activity.

Multiple correlation coefficient (R)= 0.544

Model significant = P<0.0001

Table 3.8: Regression coefficients of BMD for femoral trochanter site on age, Weight, height and physical activity in postmenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.641	.346		.067
	Age of subjects (year)	-.003	.002	-.097	.236
	Weight of subjects (kg)	.005	.001	.532	.000
	Height of subjects (cm)	-.001	.002	-.041	.635
	Physical activity (hr/week)	.028	.008	.275	.001

Dependent Variable: Femoral trochanter BMD (g/cm²)

Femoral trochanter BMD = 0.641 – 0.003 x Age + 0.005 x Weight + 0.028 x physical activity.

Multiple correlation coefficient (R)= 0.623

Model significant = P<0.0001

3.4.12. Multilinear regression analysis in premenopausal women

In premenopausal women multilinear regression was performed using independent variables age, body weight, height and physical activity. Regression analysis indicated the influence of these variables on the bone mineral density at four skeletal sites (Lumbar spine, femoral neck, femoral ward and femoral trochanter) Table 3.9, 3.10, 3.11, and 3.12 respectively. In this group of women age-related changes in bone mineral density were also evident. Every skeletal site has a different rate of bone loss. Age did not come out as significant predictors of BMD at lumbar spine site, while in femoral neck, femoral wards and femoral trochanter were significant, it observed that age decreases bone mineral density values by about 0.9 % per year (femoral neck), 1.4 % per year (femoral wards) and 0.8 % per year (femoral trochanter). Lumbar spine, femoral neck, femoral words and femoral trochanter BMD exhibited increases from of 0.3% to 0.6 % per kilogram of the body weight. Height did not come out as a significant predictor of bone mineral density at any site measured. Physical activity showed significant increases in BMD values only at lumbar spine and femoral wards sites (% per hour): 4.3% and 2.2%. While at femoral neck and femoral trochanter come out as not significant.

Table 3.9: Regression coefficients of BMD for lumbar spine site on age, Weight, height and physical activity in premenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.635	.261		.017
	Age of subjects (year)	-.003	.003	-.090	.233
	Weight of subjects (kg)	.003	.001	.308	.000
	Height of subjects (cm)	.002	.002	.101	.209
	Physical activity (hr/week)	.043	.007	.501	.000

Dependent Variable: Lumbar spine BMD (g/cm²)

Lumbar spine BMD = 0.635 – 0.003 x Age + 0.003 x Weight + 0.043 x physical activity.

Multiple correlation coefficient (R)= 0.703

Model significant = P<0.0001

Table 3.10: Regression coefficients of BMD for Femoral neck site on age, Weight, height and physical activity in premenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	.793	.302		.010
	Age of subjects (year)	-.009	.003	-.250	.004
	Weight of subjects (kg)	.005	.001	.598	.000
	Height of subjects (cm)	.000	.002	.014	.877
	Physical activity (hr/week)	.008	.008	.091	.300

Dependent Variable: Femoral neck BMD (g/cm2)

Femoral neck BMD = 0.793 – 0.009 x Age + 0.005 x Weight.

Multiple correlation coefficient (R)= 0.620

Model significant = P<0.0001

Table 3.11: Regression coefficients of BMD for Femoral wards site on age, Weight, height and physical activity in premenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	1.315	.380		.001
	Age of subjects (year)	-.014	.004	-.319	.000
	Weight of subjects (kg)	.005	.001	.522	.000
	Height of subjects (cm)	-.003	.002	-.101	.270
	Physical activity (hr/week)	.022	.010	.202	.027

Dependent Variable: Femoral wards BMD (g/cm2)

Femoral wards BMD = 1.315 – 0.014 x Age + 0.005 x Weight - 0.003 x height + 0.022 x physical activity.

Multiple correlation coefficient (R)= 0.592

Model significant = P<0.0001

Table 3.12: Regression coefficients of BMD for Femoral trochanter site on age, Weight, height and physical activity in premenopausal women

Model		Unstandardized Coefficients		Standardized Coefficients	Sig.
		B	Std. Error	Beta	
1	(Constant)	1.089	.283		.000
	Age of subjects (year)	-.008	.003	-.213	.009
	Weight of subjects (kg)	.006	.001	.683	.000
	Height of subjects (cm)	-.003	.002	-.147	.086
	Physical activity (hr/week)	.009	.007	.096	.256

Dependent Variable: Femoral trochanter BMD (g/cm²)

Femoral trochanter BMD = 1.089 – 0.008 x Age + 0.006 x Weight - 0.003 x height.

Multiple correlation coefficient (R)= 0.658

Model significant = P<0.0001

3.4.13. Sample size and power in regression

There are several rules regarding the minimum sample size required to achieve a particular power, which is dependent on the number of predictor variables. One rule of thumb attributed to Green (1991) is $50+8*k$, where k is the number of predictors. Thus for 5 variables, one needs a minimum sample of 90. Another factor that comes in the equation is the effect size. Miles and Shevlin (2001) produced a series of graphs to determine the sample size required to achieve a certain power for different effect sizes, as the numbers of predictors vary. We used the simplified graphs provided by Field (2005), which showed to achieve a power level of 80%, for three predictors model, one would need a sample size of 80 to detect a medium size effect (40 for large effects and 600 for small effects). Our sample size is about 100, and the effect is medium size, so we have a power exceeding 80%. From the same graphs one finds, for single variable model the required sample sizes are 35, 60 and 400 for large, medium, and large effects respectively.

3.5. Discussion

The anthropometric parameter shows that the BMI for the premenopausal women was (24 kg/m^2) which is at the higher limit of the normal range ($18\text{-}24 \text{ kg/m}^2$), whereas in the postmenopausal women was (30kg/m^2) which is higher than the normal range and indicate that these women are considered to be overweight (obese class 1). The bone health indices for the study groups of premenopausal (20-30 y) and postmenopausal (45-60y) Saudi women in the city of Jeddah indicated that all the BMD and QUS measurement variables were significantly lower in postmenopausal women compared with the premenopausal women. The prevalence of osteopenia in the premenopausal women at the lumbar spine site was 37%, the percentage of postmenopausal women that is 52%. Similarly at the femoral neck site the osteopenia was 23% in premenopausal women and 32% in postmenopausal women. Higher percentage of osteopenia, were found for calcaneal bone mass being 62% for postmenopausal. A comparison of our study with a previous study by Ghannam *et al* (1999) in the city of Riyadh (The capital of the kingdom) revealed that the osteopenia percentage in postmenopausal women was 45% in the lumbar spine whereas the osteoporotic was around 17%. It obvious that the prevalence of osteopenia in postmenopausal women is higher in Jeddah than the Riyadh city, but the prevalence of osteoporotic is higher in Riyadh. Similarly, the comparison in the femoral neck of premenopausal women between the two cities showed that the prevalence of osteopenia is slightly lower in the city of Jeddah (23%) than the Riyadh city (27%), where as the prevalence of osteoporosis is higher in Jeddah (4%) compared with Riyadh (2%)

Numerous studies have demonstrated the importance role of physical activities in developments and maintenance of bone mineral density. Various studies have reported the beneficial affects of physical activity on growing bone (Boot *et al.*, 1997; Grimston *et al.*, 1992; Slemenda *et al.*, 1991; Slemenda *et al.*, 1993). Regular exercise could be a valuable way to retard bone loss in postmenopausal women (Pruitt *et al.*, 1992; Welsh and Rutherford, 1996; Shimegi *et al.*, 1994; Krall and Dawson-Hughes, 1994; Hartard *et al.*, 1996; Bravo *et al.*, 1996). However, the impact of lifetime physical activity on bone status has not been well established. In this study, the influence of physical activity on bone health has been examined by survey questionnaire, which includes questions of the amount of time spent and type of exercise including walking, jogging, cycling, swimming and aerobic per week of both groups. The results show that, higher physical activity levels were associated with high BMD. This finding is consistent with postmenopausal and premenopausal women. It is also consistent with five skeletal sites, the lumbar spine, the femoral neck, the femoral ward, the femoral trochanter, and the calcaneal. Our results provide clear evidence of the benefits of increased levels of physical activity on sites of the skeleton.

Body weight is recognized as an important risk factor of osteoporosis. A positive association between body weight and bone density has been documented in epidemiological studies (Felson *et al.*, 1993; Edelman *et al.*, 1993). In this study, our results show a strong relation between body weight and bone density at five skeletal sits, the lumbar spine, the femoral neck, the femoral ward, the femoral trochanter, and the calcaneal. Significant positive association found between body weight and bone density in our postmenopausal and premenopausal women. Women with the highest body weights tended to have higher bone density. Our results

confirm the finding from other studies that reported that high body weight appeared to protect against low bone density (Macdonald *et al.*, 2005).

Our results show that there was a significant relationship between body height and bone density in postmenopausal women at four skeletal sites, the lumbar spine, the femoral neck, the femoral ward, and the femoral trochanter. While in premenopausal women only at two skeletal sites, the lumbar spine, and the femoral neck. The taller the women the greater the bone density. Our results are in line with the finding from many studies that height loss was significantly associated with fragility fractures that indicate the presence of an already osteoporotic condition (Gunnes *et al.*, 1996; Hunt, 1996). A recent study indicate that loss of height may be an important factor in detecting osteoporosis of the hip, implying that evaluation of height loss should be routine in the outpatient setting (Kantor *et al.*, 2004).

This study found that bone mineral density was inversely related to age in postmenopausal women. BMD continue to fall with age at all of the five sites. Cross-sectional and longitudinal studies also have reported age-related bone loss (Ensrud *et al.*, 1995; Burger *et al.*, 1994; Steiger *et al.*, 1992). In premenopausal women, we did not see any association between age and bone density except at velocity of sound VOS, there were significant inverse relationship.

CHAPTER 4

Limited sunlight exposure is associated with reduced bone mass in Saudi women

4.1 Introduction

Saudi Arabia is a sunny country where abundant sunlight is available all year round. In spite of the data suggesting that vitamin D deficiency is common in Saudi Arabia (Sedrani *et al.*, 1983; Al Arabi *et al.*, 1984; Fonseca *et al.*, 1984). Although, the relative contributions of vitamin D are known to vary widely between different geographical areas, Saudi Arabia at latitude of (20°N) there is no evidence of seasonal variation, 7-dehydrocholesterol conversions in winter is similar to the summer months (Ghannam *et al.*, 1999; Loveridge, 2000).

Despite the assumption that abundant sunshine ensures adequate vitamin D supply, people living in those sunny climates are not ensured of desirable vitamin D concentration. Culture, clothing, and shelter minimize the natural production of vitamin D by the skin. Sensible sun exposure (usually 5-10 min of exposure of the arms and legs or the hands, arm, and face for two or three times per week) is reasonable to guarantee vitamin D sufficiency (Holick, 2004). It had been estimated that in a young adult there is approximately 1 µg of 7-dehydrocholesterol per each centimeter of epidermis. When an exposure to one minimal erythemal dose of sunlight it will convert 5-10% of the 7-dehydrocholesterol into previtamin D₃ this will increase the concentration of vitamin D closer to ingesting 10,000-25,000 IU of vitamin D (Holick, 1994). Sunlight contributes 80% to the body's store of vitamin D while the smaller dietary component is also very important but their influence on bone mass attainment is still undetermined (Heaney *et al.*, 2001). The implication of period of sunlight exposure on indices of bone health remains undefined.

4.1.1. Factors that regulate the cutaneous production of vitamin D₃

1) Sunlight

During skin exposure to sunlight, solar ultraviolet beta photons (290-315 nm) will photolyze 7-dehydrocholesterol to previtamin D₃. Prolonged exposure to sunlight cannot cause the over production of previtamin D₃. It is maintained in quasi-photostationary state whereby no more than 10 to 15 % of the initial concentration of 7-dehydrocholesterol can be converted to previtamin D₃ (Adams *et al.*, 1982).

2) Environment

Air pollution contains ozone that can be quite substantial. When the ozone concentration in the atmosphere is increased, the ozone will efficiently absorb ultraviolet B photons which it will reduce the cutaneous photosynthesis of previtamin D₃ (Holick, 1994).

3) Melanin pigmentation

Melanin is produced in the skin in response to sunlight. It effectively absorb ultraviolet B and ultraviolet A radiation. Increased melanin pigmentation in the skin will decrease the efficiency in the photosynthesis of previtamin D₃.

4) Sunscreen

Sunscreen use for the prevention of sunburn, skin cancer and skin damage, it has been shown that sunscreen can prevent the beneficial effect of sunlight, the production of previtamin D₃. The mechanism for this is that sunscreens absorb

ultraviolet B radiation, and thereby, compete with 7-dehydrocholesterol for the previtamin D₃ producing photons (Holick, 2004)

5) Clothing

Clothing absorbs solar ultraviolet B radiation. A study by Matsuoka had show when human volunteers wore fabrics made out of white or black cotton, wool or polyester followed by exposure to sunlight for up 40 minutes, there was no elevation in circulating concentrations of vitamin D (Matsuoka *et al.*, 1992).

6) Latitude, season and time of day

Ultraviolet photons quantity that penetrates the earth's surface is dependent on many factors such as path length which sunlight penetrates through the atmospheric ozone layer and the solar radiation distance that must travel through the atmosphere as a function of solar zenith angle which depends on latitude, season and time of day (Holick, 2004).

7) Glass and plastics

Window glass and many plastics made from polycarbonate. Fiberglass and polystyrene all absorb solar ultraviolet B radiation. Exposure to sunlight through one of these filters will completely prevent the cutaneous production of vitamin D₃ (Holick, 1994).

4.1.2 Study aim

The aim of the analysis was to determine if bone health indices and bone turnover varied according to different levels of sunlight exposure.

4.2. Study design

4.2.1. Assessment of sunlight exposure

Sunlight exposure was assessed by questionnaire relating to the amount of daily exposure during days, weekends and holidays as minute per day (section 2.3.1). All women were veiled or partially veiled

4.2.2. Groups information

Postmenopausal and premenopausal women each were divided into three groups according to the time they spent in the sun (min/day). Groups were as follows:

- Low: minimal exposure to sun (0min/day)
- Medium: medium exposure to sun (15 min/day)
- High: high exposure to sun (30-60min/day)

The percentage of postmenopausal women in these sunlight classification groups was: low 54.6%, medium 30.5%, and high 14.8%. Premenopausal sunlight classification groups were: low 39.0%, medium 28%, and high 33%.

4.3. Statistical analysis

Results for the effect of sunlight exposure time on bone indices presented in three groups are given as mean values and their standard deviation. The relationship between the extent of sunlight exposure and bone indices was investigated using analysis of variation (ANOVA). Preliminary analysis was performed to ensure normality (K-S test). Since age, weight and height were shown to be positively correlated with bone indices in Chapter 3, analysis of covariance (ANCOVA) was used to adjust for these factors in the sunlight analysis.

4.4. Results

4.4.1. The effect of sunlight exposure on bone indices in postmenopausal women

As shown in the Table 4.1, values of bone health indices were mean and S.D. Differences in lumbar spine and femoral neck BMD were examined. Differences in bone ultrasound and bone turnover were also examined. Lumbar spine BMD was significantly lower in group one compared with group three ($P<0.04$). Non-significant differences were seen at femoral neck, femoral wards and femoral trochanter BMD ($P<0.2$, $P<0.2$, $P<1.5$). Calcaneal bone mass as assessed by BUA and velocity of sound VOS was non-significantly lower in group one compared to group three ($P<0.08$, $P<0.14$) (Fig. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6). In Table 4.3, both the lumbar spine and total femoral t-scores were significantly different between the groups (LS t-score Grp 1= -1.21 (1.41) vs. Grp 2 =-0.65 (1.04) vs. Grp3 =-0.78(1.43); TF t-score Grp 1= -0.41 (1.4) vs. Grp 2 = -0.17 (1.1) vs. Grp 3=0.19 (1.3). A non-significant correlation was found between period of sunlight exposure (min/d) and axial BMD and calcaneal bone mass for those women. Results remained significant at lumbar spine and lumbar spine t-score after adjustment for age, physical activity, weight and height.

4.4.2. Effect of sunlight exposure on bone indices in premenopausal women

Table 4.2 shows values of bone health indices as mean and S.D. Differences in lumbar spine and femoral neck BMD assessed by DXA were examined in premenopausal women. Differences in bone ultrasound and bone

turnover were also examined in those women. Lumbar spine BMD was significantly lower in group one compared with group three ($P < 0.05$). Non-significant differences were seen at femoral neck, femoral wards and femoral trochanter BMD ($P < 0.4$, $P < 0.1$, $P < 0.1$). Calcaneal bone mass as assessed by BUA and velocity of sound VOS was non-significantly lower in group one compared to group three ($P < 0.13$, $P < 0.6$) (Fig. 4.7, 4.8, 4.9, 4.10, 4.11, 4.12). In Table 4.4, lumbar spine t-scores were significantly different between the groups (LS t-score Grp 1 = -1.83 (0.99) vs. Grp 2 = -0.64 (1.04) vs. Grp3 = -0.23 (0.94). Total femoral t-scores were non-significantly different between the groups (TF t-score Grp 1 = -0.50 (1.1) vs. Grp 2 = -0.19 (1.1) vs. Grp 3 = -0.09 (1.0). A non-significant correlation was found between period of sunlight exposure (min/d) and axial BMD and calcaneal bone mass for those women. Results remained significant at lumbar spine and lumbar spine t-score after adjustment for age, physical activity, weight and height.

Table 4.1: Impact of sunlight exposure groups on bone indices of postmenopausal women

	Low	Medium	High	P value	Linearity
Age (y)	50.67±5.2	50.67±5.2	47.75± 4.3	P< 0.01	P< 0.007
Weight (kg)	75.71±15.5	75.71±15.5	83.31±15.0	P< 0.04	P< 0.2
Height (cm)	156.68±5.5	155.75±7.2	158.0± 6.1	P< 0.4	P< 0.7
BMI (kg/m²)	30.76±5.68	29.83± 4.56	33.41± 5.96	P< 0.9	P < 0.2
LS BMD (g/cm²)	1.05 ± 0.17	1.12 ± 0.12	1.1 ± 0.17	¹ P<0.12 ² P<0.04	P<0.09
FN BMD (g/cm²)	0.89 ± 0.16	0.92 ± 0.14	0.96 ± 0.16	¹ P<0.3 ² P<0.2	P<0.13
FWBMD (g/cm²)	0.75 ± 0.19	0.80 ± 0.15	0.81± 0.18	¹ P<0.3 ² P<0.2	P<0.18
FTBMD (g/cm²)	0.78± 0.15	0.79 ± 0.12	0.84± 0.14	¹ P<0.3 ² P<0.5	P<0.19
LS t-score	-1.21 ± 1.41	-0.65 ± 1.04	-0.78±1.43	¹ P<0.12 ² P<0.04	P<0.09
TF t-score	- 0.41 ±1.4	-0.17± 1.1	0.19±1.3	¹ P<0.2 ² P<0.2	P<0.09
BUA (db/MHz)	67.11±14.7	69.53±14.5	78.87±20.6	¹ P<0.03 ² P<0.08	P<0.01
VOS (m/s)	1607.61±29.4	1617.87±27.5	1618.56±25.6	¹ P<0.16 ² P<0.14	P<0.08

1. ANOVA
2. ANCOVA

Table 4.2: Impact of sunlight exposure groups on bone indices of premenopausal women

	Low	Medium	High	P value	Linearity
Age (y)	24.43±4.1	22.6±3.1	21.69± 2.2	P< 0.003	P< 0.001
Weight (kg)	58.64±14.0	61.1±13.3	62.6±16.5	P< 0.5	P< 0.25
Height (cm)	158.7±5.7	159.6±7.0	159.1± 4.7	P< 0.81	P< 0.71
BMI (kg/m²)	23.3±5.96	23.89± 4.4	24.6± 6.0	P< 0.63	P < 0.34
LS BMD (g/cm²)	1.09 ± 0.11	1.12 ± 0.12	1.17 ± 0.11	¹ P<0.03 ² P<0.05	P<0.01
FN BMD (g/cm²)	0.93 ± 0.11	0.96 ± 0.14	0.98 ± 0.12	¹ P<0.2 ² P<0.4	P<0.11
FWBMD (g/cm²)	0.88 ± 1.5	0.93 ± 0.17	0.96± 0.13	¹ P<0.70 ² P<0.12	P<0.02
FTBMD (g/cm²)	0.73 ± 0.12	0.77 ± 0.12	0.8 ± 0.11	¹ P<0.1 ² P<0.1	P<0.03
LS t-score	- 0.83 ± 0.99	-0.64 ± 0.14	-0.23 ± 0.94	¹ P<0.04 ² P<0.05	P<0.01
TF t-score	- 0.50 ± 1.1	-0.19± 1.1	0.09±1.0	¹ P<0.07 ² P<0.13	P<0.025
BUA (db/MHz)	75.5±13.9	79.28±14.2	80.72±12.2	¹ P < 0.2 ² P<0.13	P<0.1
VOS (m/s)	1652.51±29.8	1657.64±28.4	1653.0±22.6	¹ P<0.7 ² P<0.6	P<0.9

1. ANOVA
2. ANCOVA

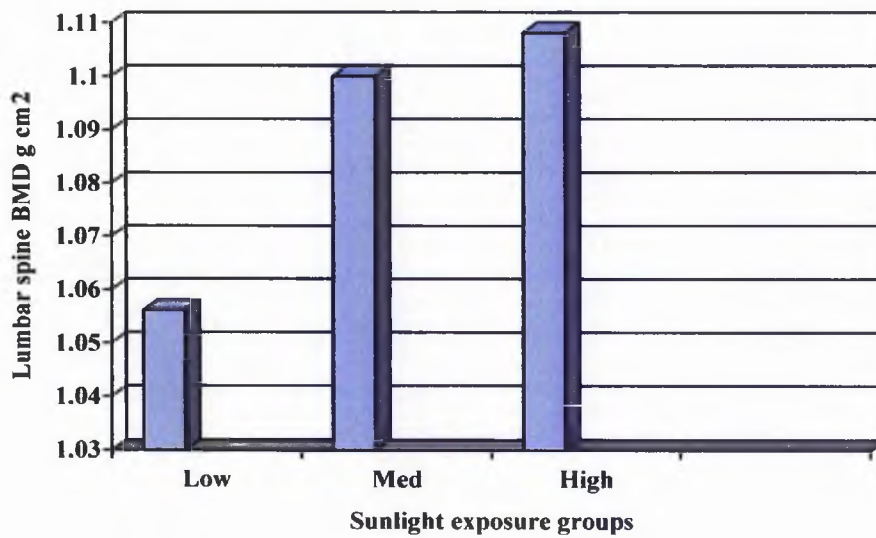


Figure 4.1: Impact of sun exposure time as groups on lumbar spine BMD in postmenopausal women

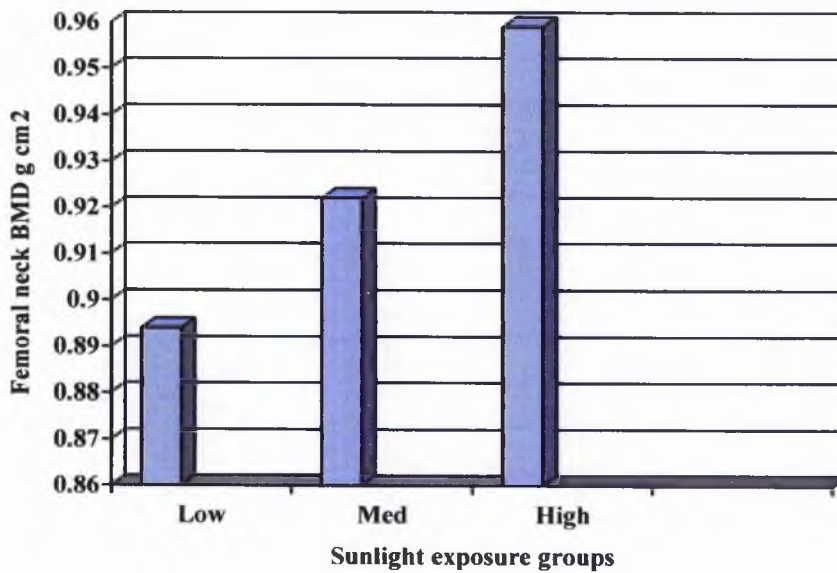


Figure 4.2: Impact of sun exposure time as groups on femoral neck BMD in postmenopausal women

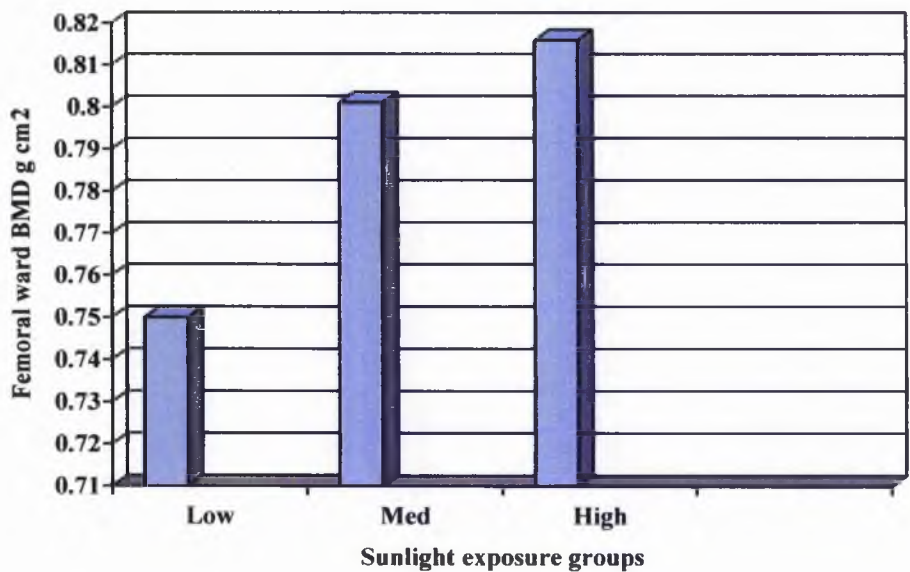


Figure 4.3: Impact of sun exposure time as groups on femoral ward BMD in postmenopausal women

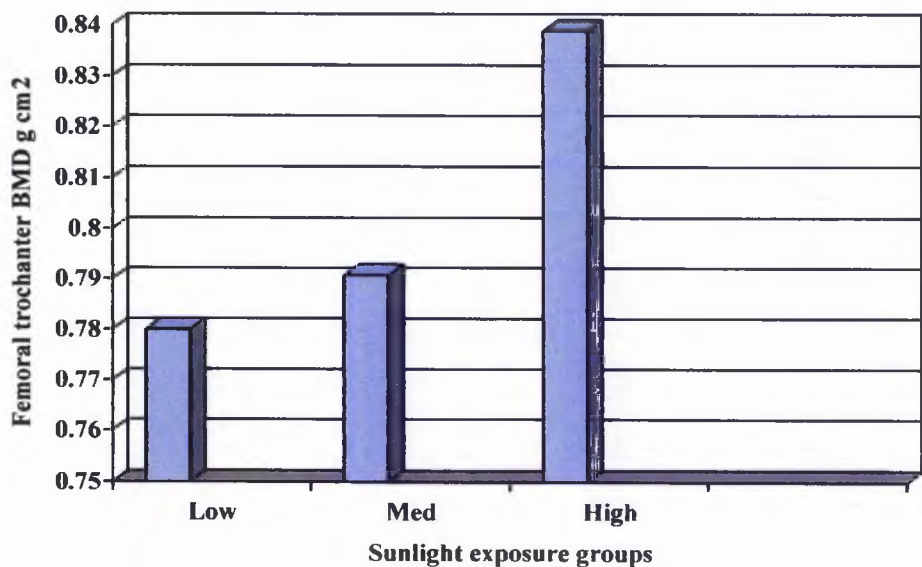


Figure 4.4: Impact of sun exposure time as groups on femoral trochanter BMD in postmenopausal women

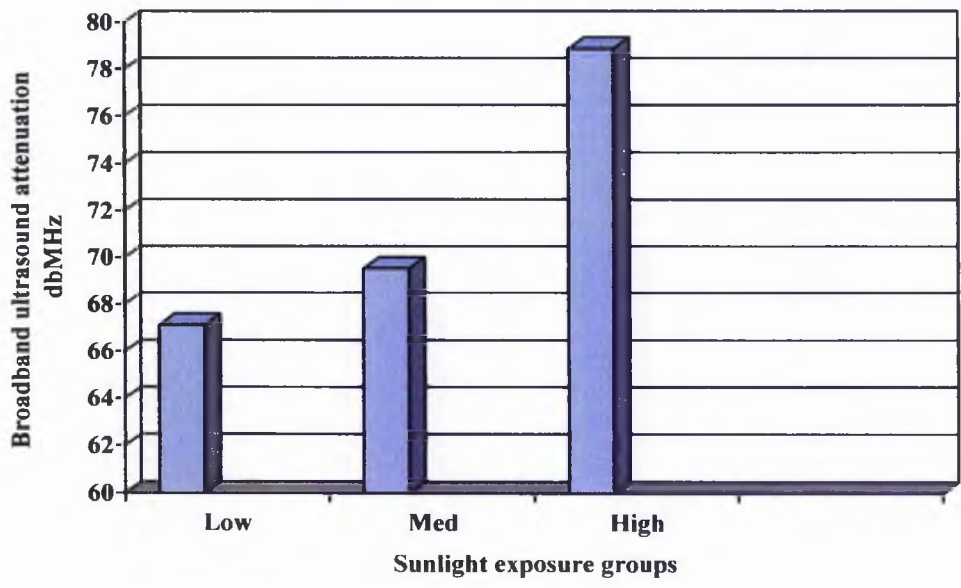


Figure 4.5: Impact of sun exposure time as groups on BUA in postmenopausal women

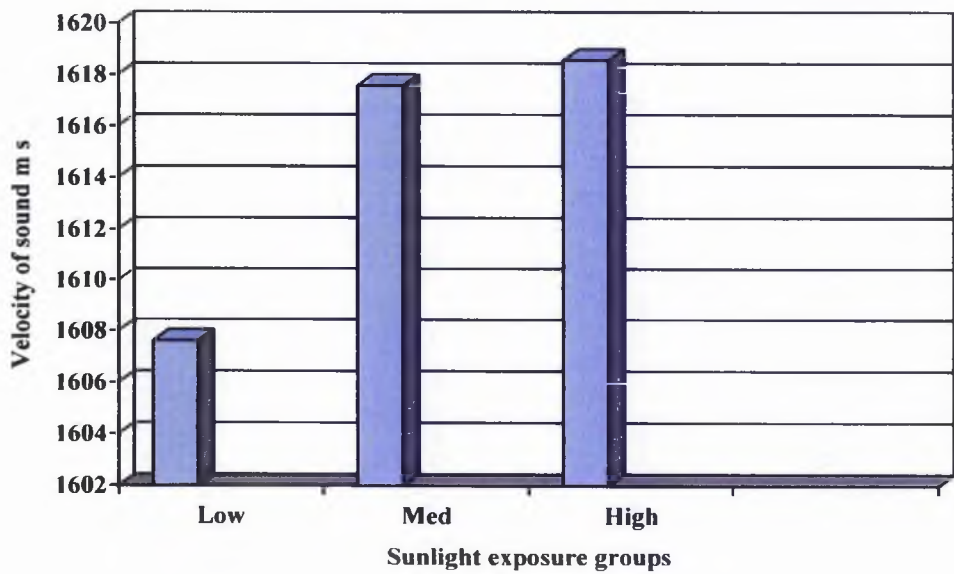


Figure 4.6: Impact of sun exposure time as groups on VOS in postmenopausal women

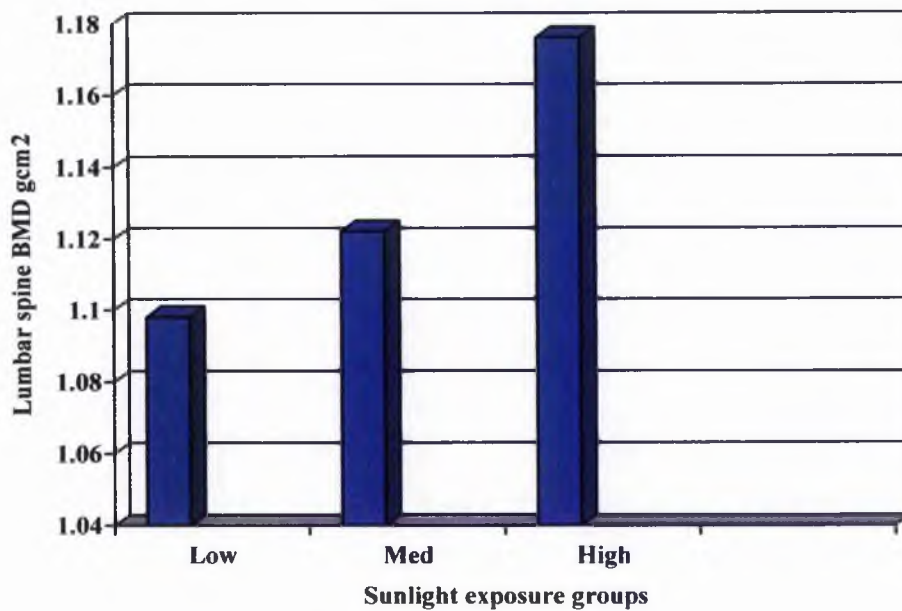


Figure 4.7: Impact of sun exposure time as groups on lumbar spine BMD in premenopausal women

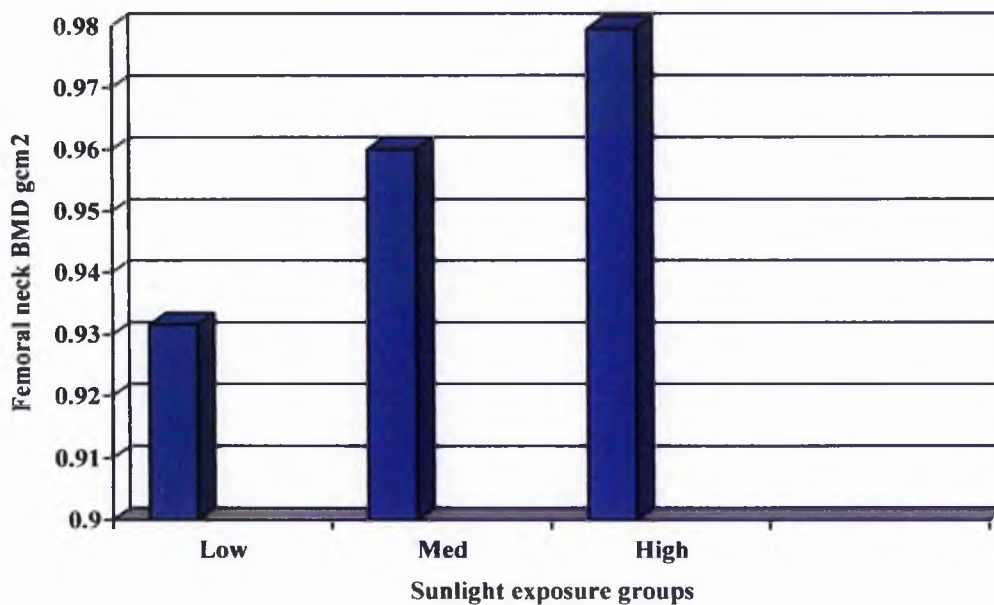


Figure 4.8: Impact of sun exposure time as groups on femoral neck BMD in premenopausal women

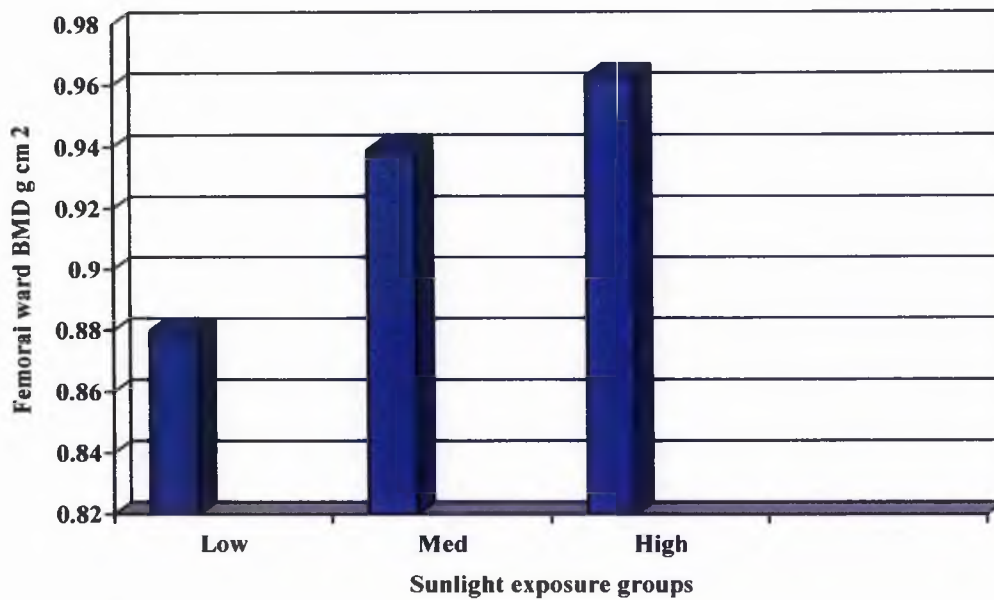


Figure 4.9: Impact of sun exposure time as groups on femoral ward BMD in premenopausal women

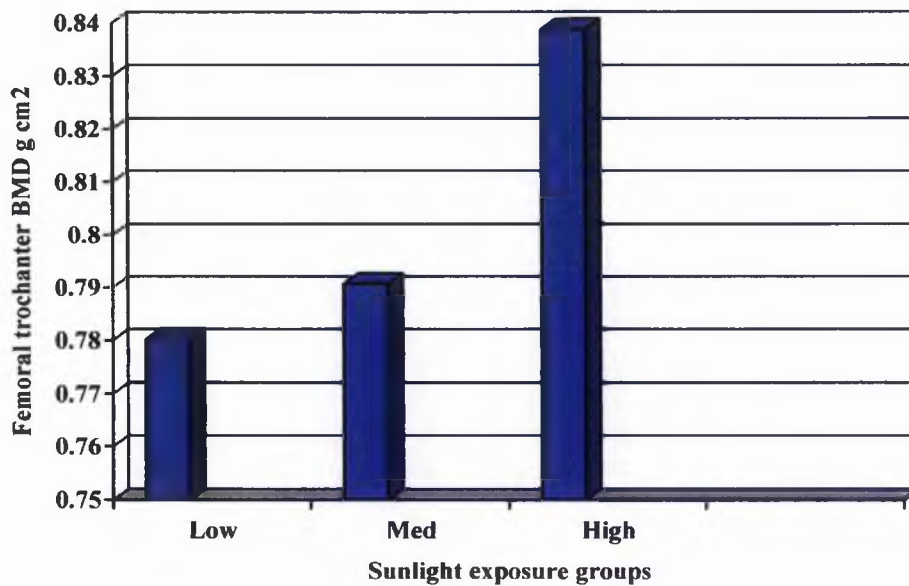


Figure 4.10: Impact of sun exposure time as groups on femoral trochanter BMD in premenopausal women

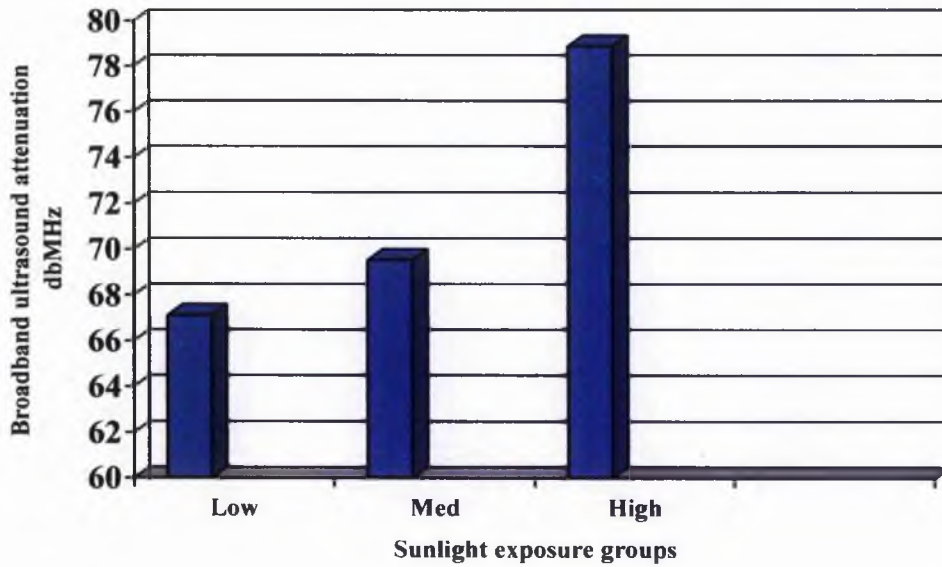


Figure 4.11: Impact of sun exposure time as groups on BUA in premenopausal women

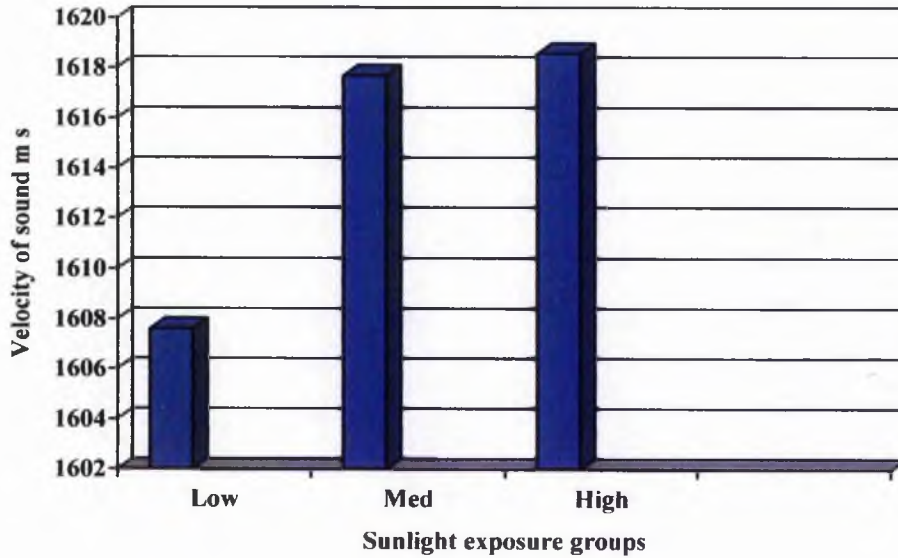


Figure 4.12: Impact of sun exposure time as groups on VOS in premenopausal women

Table 4.3: Values of t-score between groups in postmenopausal women

	Low Group 1	Medium Group 2	High Group 3	P value
Lumbar spine t-score	- 1.21 ± 1.41	- 0.65 ± 1.04	- 0.78± 1.43	P< 0.04
Total femoral t-score	- 0.41 ± 1.4	- 0.17 ± 1.1	- 0.19 ± 1.3	P< 0.2

Table 4.4: Values of t-score between groups in premenopausal women

	Low Group 1	Medium Group 2	High Group 3	P value
Lumbar spine t-score	- 1.83 ± 0.99	- 0.64 ± 1.04	- 0.23± 0.94	P< 0.05
Total femoral t-score	- 0.50 ± 1.1	- 0.19 ± 1.1	- 0.09 ± 1.0	P< 0.13

4.4.3. Effect of sunlight exposure on bone turnover markers in postmenopausal women

As Table 4.5 shows, mean and standard deviation values of bone turnover markers of bone resorption and bone formation. We examined these data to see if there any association existed between bone turnover markers in postmenopausal women and the sunlight exposure time. There was a significant positive association between the bone formation markers (OC and BSAP) and the sunlight exposure groups ($P < 0.04$; $P < 0.002$) respectively. For the bone resorption markers there were no significant association between PYD and DPD and the sunlight exposure. A trend was observed between groups ($P < 0.2$; $P < 0.2$) respectively.

4.4.4. Effect of sunlight exposure on bone turnover markers in premenopausal women

As shown in Table 4.6, mean and standard deviation of bone turnover markers of bone resorption and bone formation. Data were examined to see if there any association existed between bone turnover markers in premenopausal women and the sunlight exposure time. Associations between bone resorption makers (PYD and DPD) and sunlight exposure was positive trend ($P < 0.3$, $P < 0.8$) respectively. Association between bone formation markers (OC and BSAP) and sunlight exposure were also positive trend ($P < 0.6$, $P < 0.5$) respectively.

Table 4.5: Effect of sunlight exposure groups on bone turnover markers in postmenopausal women

	Low Group 1	Medium Group 2	High Group 3	P value
PYD (nmol/l)	39.38± 14.2	34.43 ± 12.15	37.93± 11.56	P< 0.2
DPD (nmol/l)	10.6 ± 3.9	9.4 ± 3.5	9.55 ± 3.15	P< 0.2
OC (ng/ml)	10.74 ± 4.0	8.0 ± 2.31	10.3 ± 2.5	P<0.04
BSAP (U/l)	21.6±7.4	18.16±8.5	23.47±5.3	P<0.002

Table 4.6: Effect of sunlight exposure groups on bone turnover markers in premenopausal women

	Low Group 1	Medium Group 2	High Group 3	P value
PYD (nmol/l)	35.16 ± 11.45	34.23 ± 10.86	45.40± 56.45	P< 0.3
DPD (nmol/l)	10.68 ± 5.3	10.18 ± 3.3	10.39 ± 4.25	P< 0.8
OC (ng/ml)	12.26 ± 4.8	13.25 ± 3.7	12.69 ± 3.1	P<0.6
BSAP (U/l)	17.12± 5.47	17.99± 5.72	18.58±5.45	P<0.5

4.5. Discussion

The effect of sunlight exposure on bone health indices were studied in this population of postmenopausal and premenopausal women after divided into three groups according to the time spent under the sun. There is clearly an association between the time of exposure to the sun and bone health indices. The association with sunlight was stronger at lumbar spine site in both groups of women the postmenopausal and premenopausal. For the other three sites, femoral neck, femoral Ward, and femoral trochanter there was a trend in the postmenopausal and premenopausal women. A study published by Rosen in women demonstrated that exposure to sunlight plays important role in maintaining circulating conc. of 25(OH)D and bone mineral density presumably because of the effect of casual exposure to sunlight in providing these women with their vitamin D requirement (Rosen *et al.*, 1994).

Free-living healthy young adults would be at risk of vitamin D deficiency. However, they have several risk factors for vitamin D deficiency, such as working long hours indoors with little exposure to sunlight, they are more likely wearing sun protection on sun exposed areas such as clothing because of their worry from increased risk of skin cancer and wrinkles as a result, when they exposed to sunlight they make little vitamin D₃ in their skin. Tangpricha observed that 32 % of medical students and young doctors aged 18-29 years were vitamin D deficient (Tangpricha *et al.*, 2002). It has been recognized for more than three decades that elders are at high risk of developing vitamin D deficiency (Gloth *et al.*, 1995; Lips, 2001; Chapuy *et al.*, 1997). Vitamin D deficiency is extremely common in older adults in Europe because essentially no foods are fortified with vitamin D. In the United States and Canada, vitamin D deficiency is also more common than

expected. Gloth *et al* reported 54 % of community dwellers and 38 % of nursing home residence in the Baltimore area were severely vitamin D deficient. Inpatients are at high risk of vitamin D deficiency (Thomas *et al.*, 1998). It was reported that 57 % of middle aged and older adults were vitamin D deficient.

The principal cause of vitamin D deficiency is lack of adequate exposure to sunlight. The skin has a large capacity to produce vitamin D₃. Exposure of an adult in a bathing suit to stimulated sunlight that mimicked the amount of time that would be one minimal erythermal dose (1 MED) that is, cause a minimum pinkness to the skin, resulted in an increase in blood levels of vitamin D₃ comparable to ingesting between 10,000 and 25,000 IU of vitamin D. Although aging substantially reduces the amount of 7- dehydrocholesterol in the skin, it still has an adequate capacity to make vitamin D (Holick, 2002; Chel *et al.*, 1998; Chuck *et al.*, 2001).

The second cause of vitamin D deficiency is that it is very few foods naturally contain vitamin D. To satisfy the vitamin D requirement by drinking milk, would require ingesting two, four, and six glasses a day for children and adults up to the age of 50, and adults aged 51-70, 70+ years, respectively. Because the vitamin D content in milk is highly variable and often contains less than 50 % of what is stated on the label, it may not provided an adequate amount of vitamin D (Holick *et al.*, 1992). Intestinal malabsorption syndromes, especially of the small intestine where vitamin D is absorbed, can lead to severe vitamin D deficiency (Lo CW *et al.*, 1985).

Sunlight exposure, however, must be of value in protecting against vitamin D deficiency. Vitamin D increases intestinal absorption of calcium favoring the ideal environment for mineralization of bone and via its osteoclastic action it prevents hypocalcaemia (Gannage-Yard *et al.*, 2001).

CHAPTER 5

Dietary 'quality' and 'quantity' in Saudi Arabian premenopausal and postmenopausal women

5.1. Introduction

5.1.1. General

A healthy diet must provide sufficient energy and nutrients to maintain normal physiological function, permit growth and replacement of body tissues, and offer protection against disease (Barasi, 1997). The main reasons for obtaining information about the food intake of individual, groups or population are to assess the adequacy of the diet; to study the relationships between dietary intake and disease, or risk factors or markers associated with disease. Nutrient data obtained from dietary assessments can be broken down into: total energy intake, total macronutrient intakes (protein, fats) and total micronutrient intakes (calcium, vitamin D, phosphorus, potassium, vitamin C). The major factors influencing what is eaten in different cultures are the foods available in that particular culture, traditional practices and beliefs and any religious proscriptions. The importance of these foods to health forms the basis of many of the published pyramid shape food guides (Figure 5.1). The basis of the diet for most people is:

1. A core food, referred to as the 'staple', around which the majority of meals are constructed. They are generally cereals, or roots and tubers.
2. Secondary foods are those that enhance the meal, but are not an essential part of it only at particular life stages. They have specific properties of their own; for example they may promote strength (protein- rich food, such as meat) or good health (fruit and vegetables).
3. The third category of foods is peripheral foods. These are non-essential, but pleasant to eat. Examples include biscuits, cakes, confectionery, preserves, sauces, puddings and beverages.

In general, a greater part of the diet comes from the staple in the poorer parts of the world, and the peripheral foods make an excessively large contribution in richer countries. The core and secondary foods in any national or culture diet must supply the essential nutrients in appropriate amounts to sustain life and promote health.

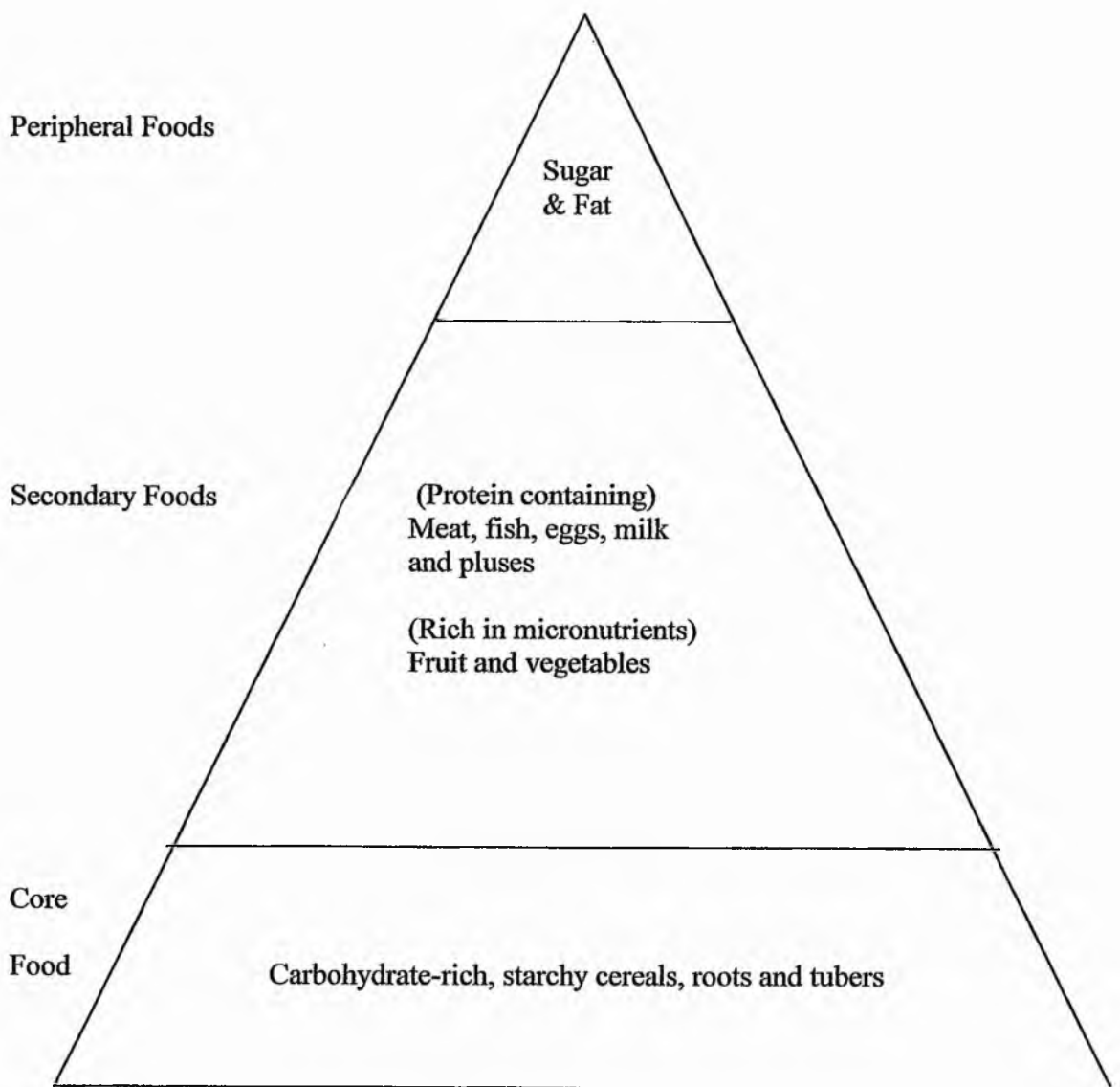


Figure 5.1: A pyramid food guide

5.1.2. Food guide pyramid

In 1992, the US Department of agriculture (USDA, 1992) published a new guide to food selection which is a development of the food groups that has been designed to reflect the dual aims of selecting a diet that is not only adequate, but also meet the nutritional guidelines aimed at reducing the risk of chronic disease. These food groups and an outline of their essential nutrient profiles are listed below.

- The bread and cereals group (bread, rice, pasta, breakfast cereals and products made from flour) at the base of the food pyramid that should contribute more serving than any other single group to the ideal diet 6-11 servings, depending upon total energy intake (in the range 1600-2800 Kcal or 6.7-11.8 MJ). The whole-grain cereals are good sources of B vitamins, some minerals and fiber. White flour still provides reasonable amounts of fiber and it is often fortified with vitamin and minerals.
- The fruit and vegetable groups- a total of 5-9 servings from these groups, depending upon energy intake. They provide carotene, vitamin C, folate, riboflavin, potassium and fiber.
- The milk group (milk, cheese, yoghurt and other milk products) these should contribute from 2-3 servings. They provide good amounts of energy, good-quality protein, vitamin A, calcium and riboflavin.
- The meat group (meat, fish, eggs, and also meat substitutes such as pulses and nuts) these should contribute from 2-3 servings. They provide protein, vitamin A, B vitamins and iron.
- The fats, oils and sweets (salad dressings, cream, butter, margarine, soft drinks, sweets (candies) and sweet desserts). These foods provide few

nutrients but are high in energy, sugars and fats and should be used sparingly in the ideal diet.

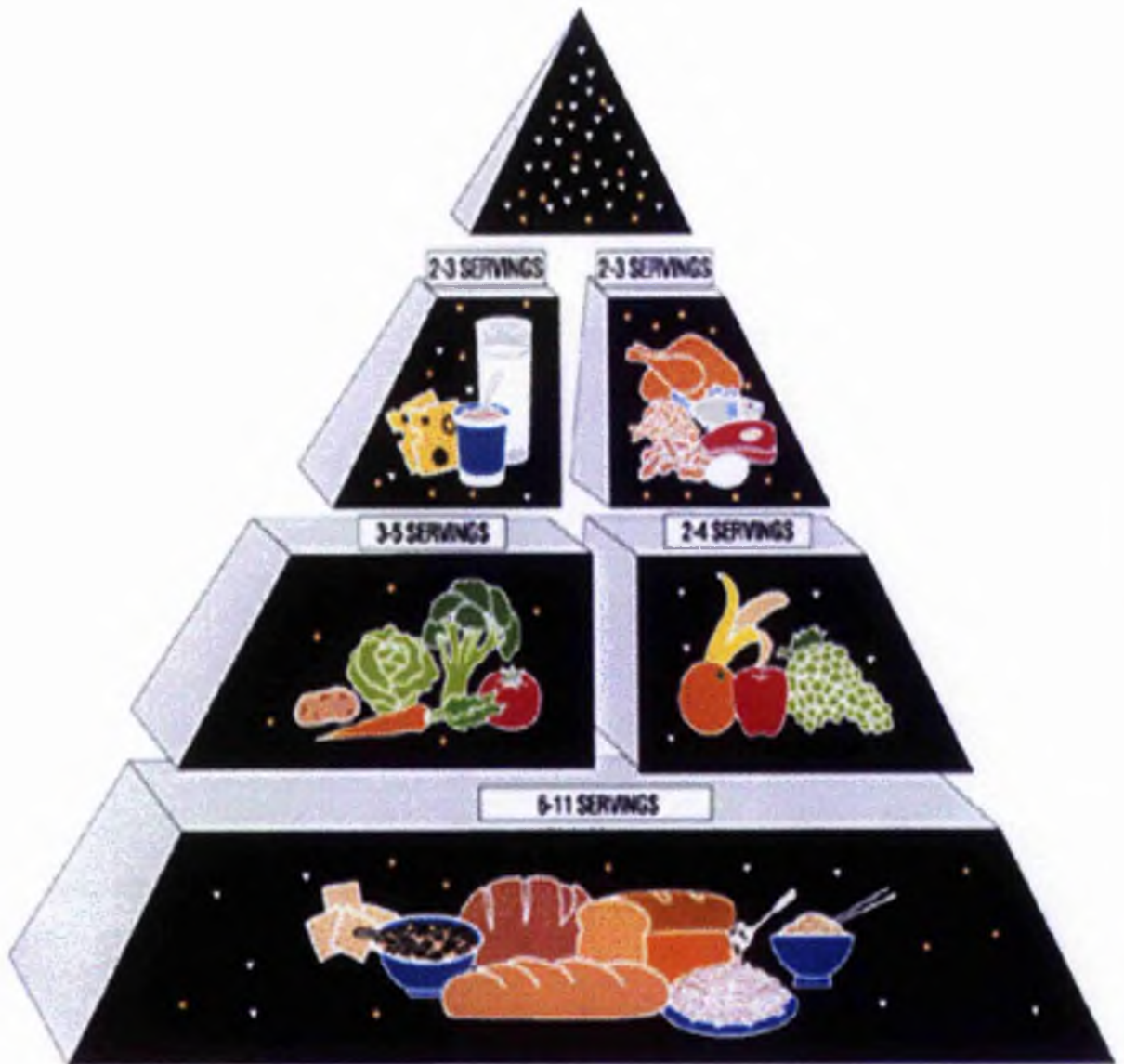


Figure 5.2: Recent food pyramid guide

In Figure 5.2, a triangle symbol is distributed lightly within the cereal, fruit and milk groups to show that some foods from these categories may contain added sugars, e.g. sweetened breakfast cereals, fruit canned in syrup, some flavored yoghurts, milk shake and ice cream. Also in Figure 5.2, a circle symbol is distributed lightly throughout the cereal and vegetable groups to show that these may contain added fats and more densely in the meat and milk groups to show that many of the foods in these groups contain substantial amounts of naturally occurring fat. The food pyramid thus indicates a general dietary structure that should ensure nutritional adequacy and helps to prevent chronic disease (Webb, 2002).

5.1.3. Tilted-plate model

In Britain a tilted-plate model is used Figure 5.3. Two large sectors of the plate are occupied by starchy foods (cereals and potatoes) and the fruit and vegetable group, and two moderate-sized sectors by the meat and alternatives group and the milk group. The last, small section of the plate is occupied by fatty and sugary foods. The general dietary structure indicated in the tilted-plate model is essentially the same as that suggested by the Americans pyramid, except that potatoes were included with the cereals (rather than with the other vegetables). The plate model contains no recommended number of portions but does contain advice for selection within in each group.

- Choose low-fat options from the meat and milk group where possible
- Eat a variety of fruits and vegetables
- Eat all types of starchy foods and choose high fiber varieties where possible.

There is a version of this tilted –plate model that contain recommended

numbers of portions of food. These portions are similar to those suggested on the Americans pyramid guide:

- 5-14 measures of bread cereals and potatoes
- 5-9 measures of fruit and vegetables
- 2-3 measures of meat, fish and alternatives
- 2-3 measures of milk and dairy foods
- 0-4 measures of fatty and sugary foods.

The Balance of Good Health

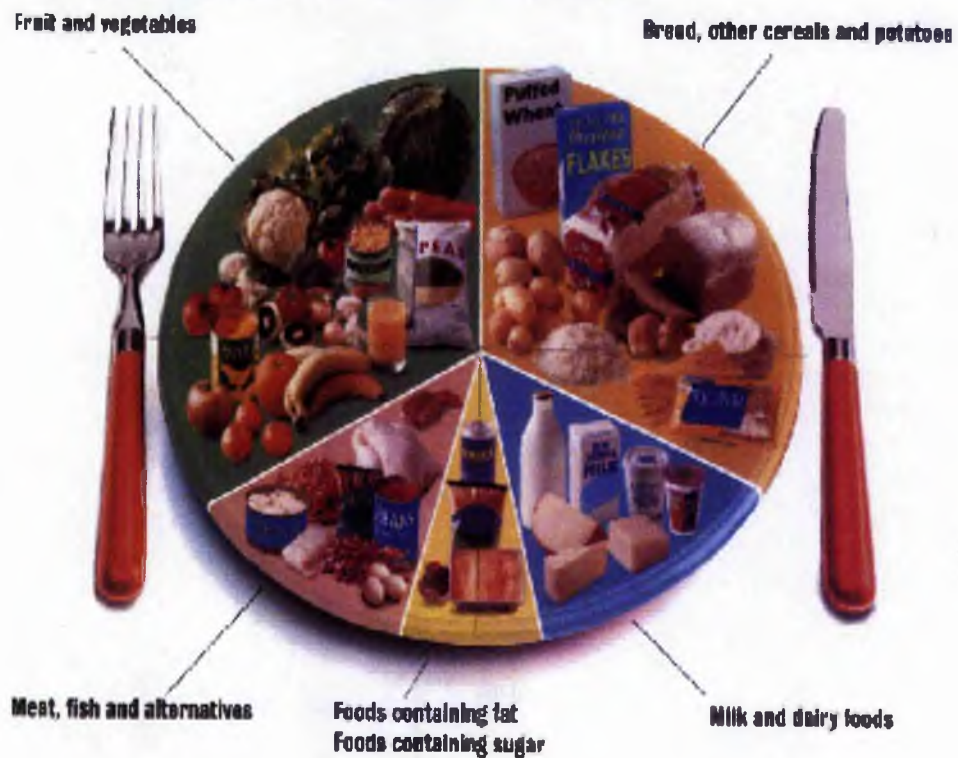


Figure 5.3: The tilted plate model used as the national food guide in Britain

5.1.4. Diet in Saudi Arabia

The desert environment and Islam have influenced the Saudi diet. It consists mainly of dates together with milk from goats, sheep or camels, rice, meat and sometimes wheat. Arabic coffee and tea are favorite drinks. Atypical Saudi meal consists of rice with mildly flavored and spiced lamb or chicken. Food is traditionally eaten with the fingers while sitting cross-legged on the floor. The most common meats are lamb and chicken. Beef is rare and pork is proscribed under Islamic law. The main meat meal of the day is lunch. Saudi law prohibits the sale or use of alcohol. Over the last two decades the diet in Saudi Arabia has changed and becoming more 'Westernized'. The diets have become higher in fat, animal products and refined foods and lower in vegetables, fruit, fiber and these lower in essential micronutrient content such as vitamins and minerals.

5.1.5. Study aims

The problem in many Middle-Eastern countries, including Saudi Arabia, is the lack of any food composition tables, which would enable accurate estimates of nutrient intake to be made with these limitations. The aims of this study were three fold:

- i. To determine the dietary 'quality' patterns in Saudi Arabian using the plate/pyramid model
- ii. To calculate intakes of key macronutrient and micronutrient using dietary conversion factors from the existing Middle Eastern food composition tables and the total intake of the five food groups identified in (i)
- iii. To determine the extent of nutrient intake 'insufficiency' in Saudi Arabian premenopausal and postmenopausal women

5.2 Design of dietary ‘quality’ study

5.2.1. Subject information

As part of our on-going bone health study in 212 Saudi Arabian women. A total of 71 premenopausal and 109 postmenopausal women living in the city of Jeddah were involved in this dietary sub-study. They were aged between 20-30 years and 45-60 years respectively and had not suffered from any known condition and were not taking any medication likely to affect bone metabolism.

5.2.2. Collection of dietary ‘quality’ information

The food diaries collected for this dietary study have been described (2.4.2.2). In brief, women estimated their dietary intake over a 3-d period. Full instructions were provided to subjects and the recording period included a weekend day. Regular contact was also made to ensure compliance. The amount of food consumed (in grams) for the five food groups was calculated by hand for each subject: (I) bread, cereals and potatoes; (II) fruit and vegetables; (III) meat, fish and protein alternatives; (IV) milk and dairy products; (V) fatty and sugary foods. Intakes were converted to frequency of consumption (times per day) by dividing the food groups by average portion sizes. The average portion sizes were taken from the Food Portion Size Manual (MAFF, 2001).

5.2.2.1 Calculation of the amount of food consumed using the five food group concept

For each subject (109 postmenopausal; 71 premenopausal), the 3 days food diary was examined in detail, by hand. Calculations were made on the amount of food consumed, for each of the days recorded. The calculation was undertaken for each of the five groups listed in section 5.2.2. The data was then transferred into the Stats package, SPSS. The total amounts (in grams) were recorded and then divided by 3 to give an estimation of daily amounts.

Subject completed an estimated 3 days food diary. Thus, in order to be able to calculate total amounts, the standard portion size book was used (MAFF, 2001). It was not possible to use a standard portion size publication from the Middle East since this does not exist. Hence, UK values were used.

5.2.2.2 Calculation of the number of portions consumed using the five food group concept

For each of the five food groups, an average portion size was calculated. The portion size was taken from two sources: (i) those provided by subjects in the food diaries; (ii) those published in the standard portion size book. The foods and portion sizes subsequently used are shown in Table 5.1.

For Group 1 (Bread and cereals), portion sizes were used for cereals, breads, rice, pasta and potatoes.

For Group 2 (Vegetable and fruit), portion sizes were used fruit (melon, orange, banana and dates) and vegetable (Salad, mixed vegetables, okra and tomatoes).

For Group 3 (Milk and dairy) portion sizes were used for milk, hard cheese, soft cheese, yoghurt [plain], yoghurt [fruit], ice-cream and cream.

For group 4 (Meat, fish and alternatives) portion sizes were used for red meat, white meat, whitefish, tuna, eggs, beans, nuts, seeds and legumes.

For Group 5 (Fats and sugars) portion sizes were used for butter, margarine, oils, cakes, biscuits, sweets, puddings and chocolates.

The total amount (gram) of food consumed per day for each subject as calculated as detailed in (Section 5.2.2.1).

Table 5.1: Calculation of the number of portions consumed using the five food group concept

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
	Breads & Cereals	Fruits & Vegetables	Milk & Dairy	Meat & Products	Fats & Sugar	Beverages
	Cereal 30	<u>Fruit:</u> Melon 150	Milk 200	Red meat 90	Butter 10	Tea 190
	Bread 36	Orange 120	Hard cheese 40	White meat 100	Margarine 10	Coffee 190
	Rice 100	Bananas 100	Soft cheese 30	White fish 100	Cakes 60	Cola 347
	Pasta 100	Dates 50	Yoghurt (plain) 60	Tuna 92	Biscuits 20	Others (Fruit drinks) 160
	Potato 100	<u>Vegetables:</u> Salad 200	Yoghurt (fruit) 125	Eggs 60	Sweets 45	
		Mixed Vegetables 90	Icecream 75	Beans 60	Oils 25	
		Okra 25	Cream 30	Nuts + Seeds 20	Puddings 110	
		Tomato 85		Legumes 60	Chocolates 56	
Average Portion use	$366/5=$ 73.25	$820/8=$ 102.5	$582/8=$ 72.7	$625/7=$ 89.3	$380/8=$ 47.5	$878/4=$ 221.75

5.3 Design of dietary 'quantity' study

5.3.1 Identification of nutrient values from Middle Eastern food composition table

Using the only existing Food Composition Table for the Middle East (Pellet and Shadarevian, 1970), the nutrient values for energy, protein, fat, fibre, calcium, phosphorus, iron, vitamin C, vitamin D and potassium were chosen for the five food groups identified in (Section 5.1.2.). The food composition table was very limited. Hence, the number of foods, which could be used, was restricted. Table 5.2 shows the nutrient values per 100g for each of the five food groups.

5.3.2 Calculation of nutrient values using the five food group concept

The nutrient values identified in section 5.3.1 (Table 5.2) were tabulated into the statistics package, SPSS. The values for energy, protein, fat, fiber, calcium, phosphorus, iron, vitamin C, vitamin D and potassium are presented per 100g. Using the calculations undertaken in Section 5.2, the total amount of food consumed for each of the five food groups, per day, was divided by 100g to enable appropriate calculation of nutrients per food group. Hence the energy, protein, fat, fiber, calcium, phosphorus, iron, vitamin C, vitamin D and potassium for each of the five food groups were calculated. These were then added together to provide total values, for each subject, for each nutrient per day.

**Table 5.2: Nutrient values used for each of the five food groups as identified
1970 Middle Eastern Food composition table**

Food groups					
Macro and micronutrients	Group 1	Group 2	Group 3	Group 4	Group 5
Energy	174.75	71.15	204.0	351.44	692.50
Protein	3.98	1.27	13.93	19.52	3.18
Fat	0.75	0.30	14.73	24.04	68.43
Fiber	0.65	0.90	0.00	1.50	0.20
Calcium	10.50	25.84	27.55	119.78	11.00
Phosphorus	47.00	29.46	269.50	320.11	56.83
Iron	0.50	0.92	0.43	4.9	0.78
Vitamin C	4.0	18.1	0.5	0.5	0.0
Vitamin D	0.0	0.0	0.15	0.96	1.55
Potassium	154.5	234.50	152.50	504.11	36.50

5.3.3. Statistical analysis

Intakes in grams per day were calculated and data are presented as mean, SD and range for the postmenopausal and premenopausal groups. Comparisons were made between the nutrient intakes values achieved in this dataset compared with recommended values.

5.4. Results: Dietary food 'quality' study

5.4.1. Amount of food consumed (grams/day) using the five food group concept

Results of the calculation for the foods consumed (gram/d) for each of the five groups are shown in Table 5.3 for postmenopausal women and Table 5.4 for premenopausal women. Intakes of the Bread and Cereals groups were low in both postmenopausal and premenopausal women. Similarly fruit and vegetable were also low for both groups. Intakes of Group 3 and 4 were similar to those recommended but higher for group5. As shown in the Figure 5.4 and 5.5 detailed the differences between postmenopausal and premenopausal women.

5.4.2. Number of portions consumed (time/day) using the five food group concept

Results for the calculation of the number of portions consumed per day for each of the five food groups are also shown in Tables 5.3 and 5.4 for postmenopausal and premenopausal women respectively. Both groups of women are below the recommendable for bread, potatoes, cereals (group1) and fruit and vegetable (group2) and above the recommendable for fats and sugar (group5). These results are shown graphically in Figure 5.6 and 5.7

Table 5.3: Amount of food consumed (gram / day) and the number of portions (time/day) for postmenopausal women using the five food group concept. (n=109)

Postmenopausal Women					
Amount of food consumed			Number of portions		
Food Group	Mean SD	Range	Mean SD	Range	Recommended Portions
Breads and cereals	207.7 81.6	50.0–480.0	2.8 1.1	0.7 – 6.6	6 – 11
Fruits and vegetables	250.9 138.4	16.6–870.0	2.4 1.3	0.2 – 8.5	5 – 9
Milk and dairy foods	186.3 107.9	0.00–502.3	2.1 1.2	0.0 – 5.6	2 – 3
Meat and alternatives	152.4 59.4	0.00–300.0	2.1 0.81	0.0 – 4.1	2 – 3
Fat and sugary foods	73.27 42.0	0.00–236.0	1.5 0.88	0.0 – 5.0	Springly

Table 5.4: Amount of food consumed (gram / day) and the number of portions (time/day) for premenopausal women using the five food group concept (n=71)

Premenopausal Women					
Amount of food consumed			Number of Portions		
Food Group	Mean SD	Range	Mean SD	Range	Recommended Portions
Breads and cereals	234.6 85.2	70.7-452.0	3.2 1.2	0.97 - 6.2	6-11
Fruits and vegetables	188.8 130.5	13.3-641.7	1.8 1.3	0.13 - 6.3	5-9
Milk and dairy foods	182.1 106.1	5.7 - 491.7	2.0 1.2	0.06 - 5.5	2-3
Meat and alternatives	135.0 57.4	0.0 - 306.7	1.8 0.8	0.00 - 4.2	2-3
Fat and sugary foods	82.9 47.8	14.7-211.7	1.7 1.0	0.31 - 4.5	Springly

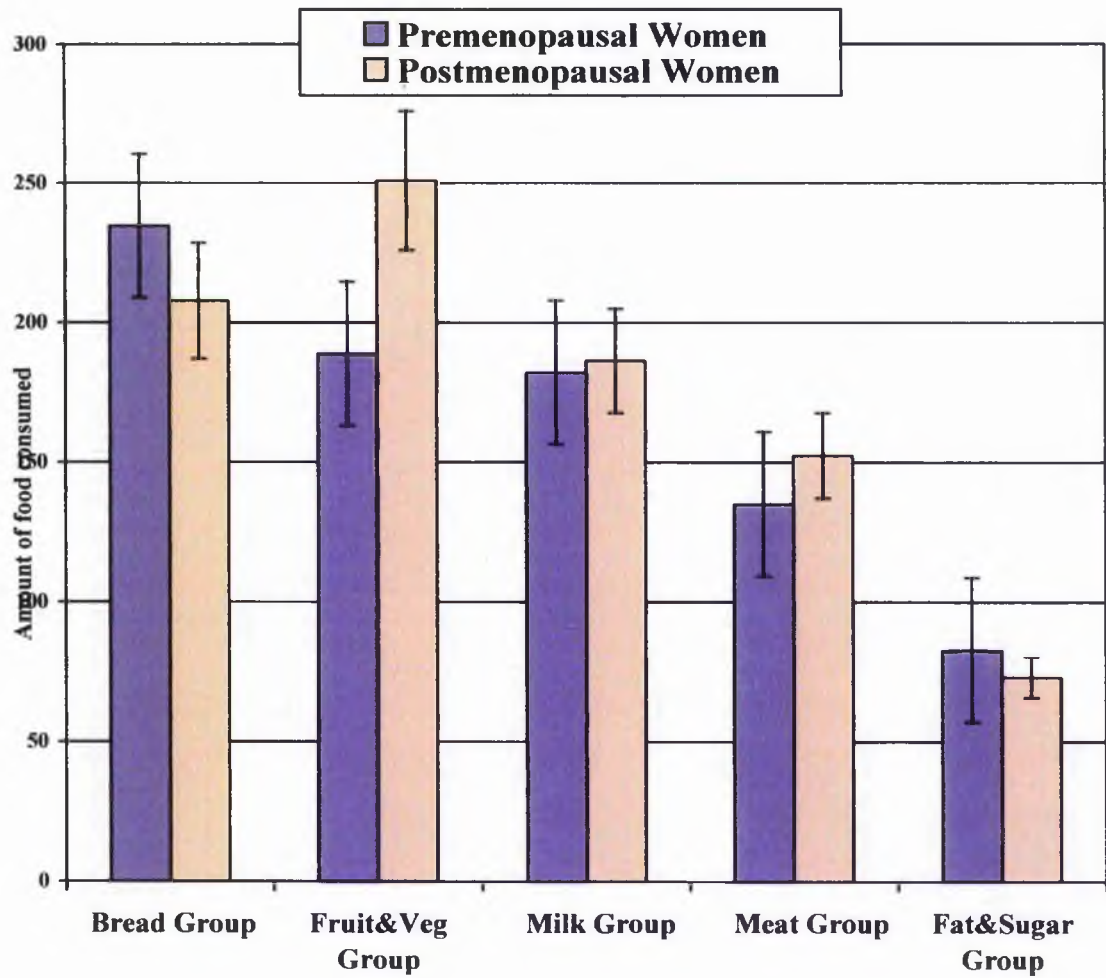


Figure 5.4: The difference in amount of food consumed in grams for the five food groups between postmenopausal and premenopausal women

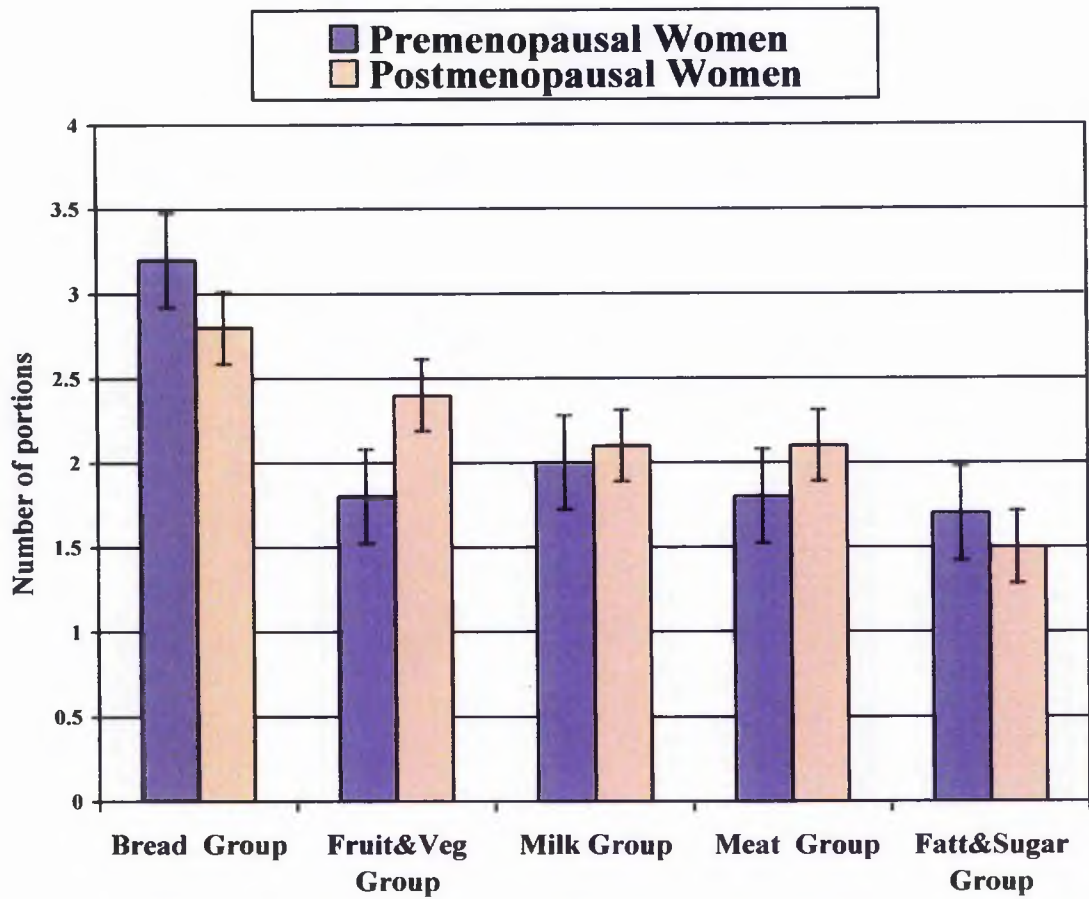


Figure 5.5: The difference in number of portion consumed times/day for the five food groups between postmenopausal and premenopausal women

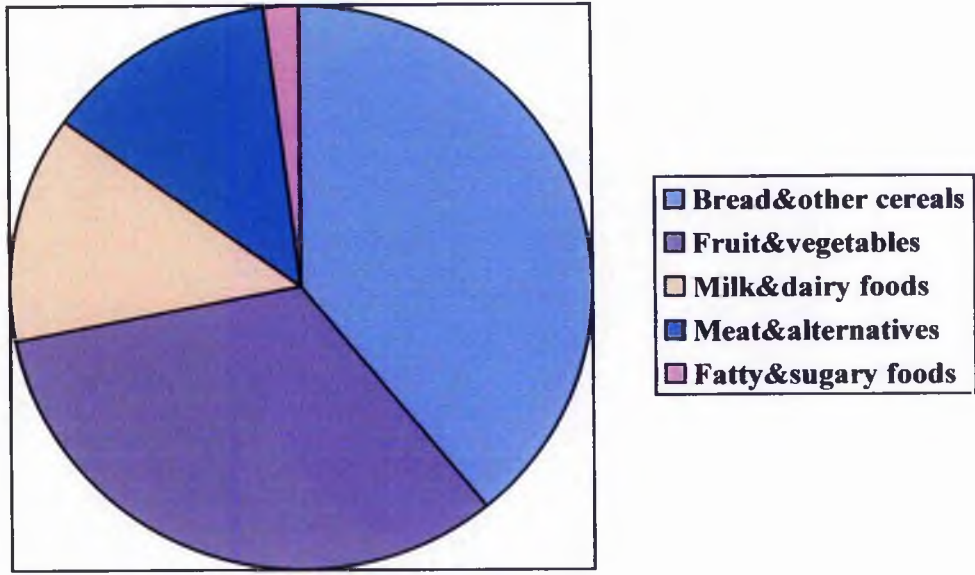


Figure 5.6: The AUS/UK recommended food guide

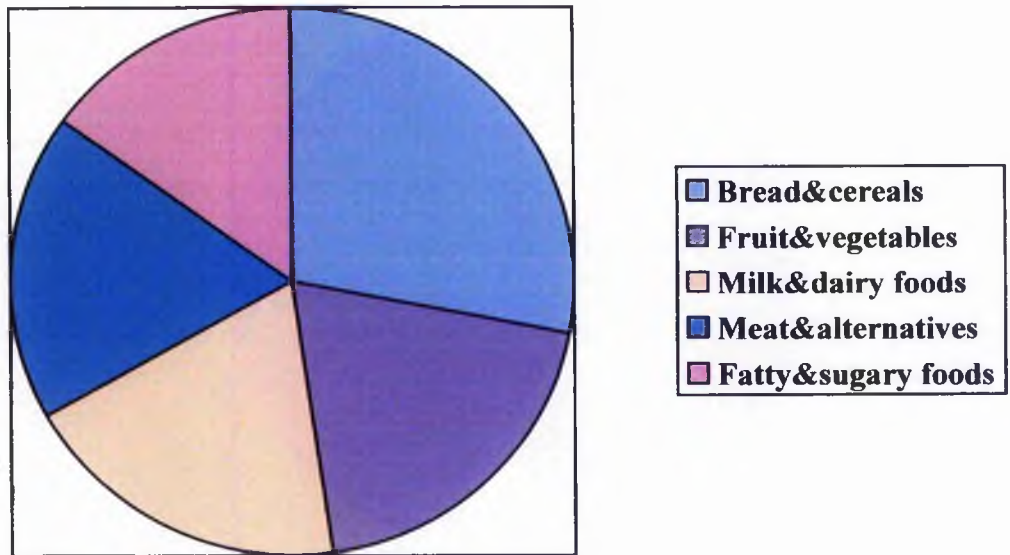


Figure 5.7: The Saudi Arabian diet

5.5. Results: Dietary food 'quantity' study

5.5.1. Macro and micronutrient intakes in Saudi Arabian

postmenopausal women

The nutrient intakes of energy, protein, fat, fibre, calcium, phosphorus, iron, vitamin C, vitamin D and potassium for Saudi Arabian postmenopausal women are shown in Table 5.5. Results indicate that intakes of energy, fibre and potassium in postmenopausal women were lower than those recommended in Western population but intakes of phosphorus are somewhat higher. Calcium, vitamin C and iron were around recommended levels. There is no dietary recommendation for vitamin D in the age groups of women they were studying in the UK as sunlight exposure is believed to be suffice. However in the USA, the recommendation for vitamin D in postmenopausal women is 10µg/d. For postmenopausal women percentage of women of underreporting was 38% (EI: BMR <1.2 n=43).

5.5.2. Macro and micronutrient intakes in Saudi Arabian

premenopausal women

The nutrient intakes of energy, protein, fat, fiber, calcium, phosphorus, iron, vitamin C, vitamin D and potassium for Saudi Arabian premenopausal women are shown in Table 5.6. Results indicate that intakes of Calcium, vitamin C and iron are around recommended levels. Energy, fiber and potassium in premenopausal women are lower than those recommended in western population but intakes of phosphorus are somewhat higher. There is no dietary recommendation for vitamin D in the age groups of women they were studying in the UK as sunlight exposure is

believed suffices. However in the USA, the recommendation for vitamin D in premenopausal women is 5µg/d. and for premenopausal women underreporting was 34.3% (EI: BMR <1.2 n=24). Underreporting was found to be in one third of our women.

Table 5.5 Nutrient intakes of macro and micronutrient for postmenopausal women (n=109)

Postmenopausal Women						
Macro and micronutrient intakes			Recommendations			
Dietary variable	Mean	SD	Range	USA	EU	UK EAR/RNI
Energy (MJ/d)	8.2	± 2.3	3.2 – 13.7			8.00
Energy (Kcal/d)	1964	±552	768 - 3278.0	2200		1900
EI: BMR	1.36	± 0.41	0.52 - 2.41			
Protein (g/d)	69	±21	26.7 - 122	50		46.5
Fat (g/d)	116.4	±40.3	28.5 – 230	73		63
Fibre (g/d)	5.9	±1.7	2.2-10.8	12		18
Calcium (mg/d)	791	±318.3	201.3 - 1745	1200	700	700
Phosphorus (mg/d)	1203	±382.1	473 - 2222	700	800	550
Iron (mg/d)	12.1	±3.5	3.47 – 20.6	8	14	8.7
Vitamin C (mg/d)	55.5	±25.4	6.4 – 165.2	60	45	40
Vitamin D (µg/d)	2.9	±0.98	0.32 – 5.65	10	0-10	—
Potassium (mg/d)	1988	±537.9	950.5 - 3424.2	2000	3100	3500

Table 5.6 Nutrient intakes of macro and micronutrient for premenopausal women (n=71)

Premenopausal Women						
Macro and micronutrient intakes			Recommendations			
Dietary variable	Mean	SD	Range	USA	EU	UK EAR/RNI
Energy (MJ/d)	8.2	±2.33	3.5 – 14.9			8.10
Energy (Kcal/d)	1968	±558	849.8 – 3569	2200		1940
EI: BMR	1.45	±0.45	0.62–2.86			
Protein (g/d)	66.2	±20	31.5 – 117.2	50		45
Fat (g/d)	118.5	±42.2	44.3 – 253	73		63
Fibre (g/d)	5.2	±1.6	1.9 – 9.56	12		18
Calcium (mg/d)	747	± 303.9	211.3–15551.3	1000	700	700
Phosphorus (mg/d)	1139	±361.3	516.9 - 2025.9	700	550	550
Iron (mg/d)	11	±3.3	2.9 – 19.4	18	14	14.8
Vitamin C (mg/d)	45	±23.4	14.7 – 123.4	60	45	40
Vitamin D (µg/d)	2.8	± 0.9	0.7 – 5.7	5	0-10	—
Potassium (mg/d)	1799.7	±386.3	799.4 – 3208.4	2000	3100	3500

5.6 Discussion

The data showed that the amount of food consumed (gram/d) by postmenopausal women compared with premenopausal women in the five groups, that the postmenopausal women have had vegetables and fruits (group 2) much higher than the premenopausal women which indicates that they do care to have a significant nutrients for their health from the vegetables and fruits, whereas the other groups (1, 3, 4 and 5) does not showed a significant differences. In comparison of number of portions for both group of women with a recommended portion, the results revealed that the number of portions for food group 1 and 2 for postmenopausal women and premenopausal women are much lower than the recommended portion, while group 3 and 4 are quite similar to the recommended portions. In contrast, in food group 5 is much higher than the recommended portions.

Although, the major factors influencing the food intakes in every culture are different from one culture to another and it depends to the traditional practices and religions proscriptions, the food consumption for food group 3 and 4 are quite similar for both Middle Eastern and Western countries as well as for postmenopausal and premenopausal women. Surprisingly, although the number of portions had been calculated in this study for food intakes of group 3 (milk and dairy food) for postmenopausal and premenopausal women are in the range of the international recommended portion.

The analysis of quantity study for the macro and micronutrient intakes of energy, fat, fiber, calcium, phosphorus, iron, vitamin C, vitamin D and potassium for postmenopausal and premenopausal women showed no significant variation between them which is similar to the quality study, there is no any dietary guidelines

for Middle Eastern countries can we refer our results to the recommended dietary allowances for macro and micro nutrients. A comparison of our finding results for energy with the UK Estimated Average Requirements (EARs) and Americans Dietary Guidelines, recommended dietary allowance (RDA) was found that Saudi postmenopausal and premenopausal women average energy allowance is about the same as the average energy allowance for UK and less than the average energy allowance of American women. The average energy allowance for American women at age of 25-50 years is 2200 kcal/day, which is quite close to the average ages of Saudi postmenopausal and premenopausal women. The difference of energy allowance between two women society, American and Saudi could be referred to the individual variation in body size and physical activities as well as to the difference of dietary intakes.

The UK Reference Nutrient Intake (RNI) and USA recommended dietary allowances for protein of age up to 50 years is 46.5-45 and 50 g/d respectively, (Baker and Benevenaga, 2005) whereas our finding is 69.4 ± 12.0 g/d for postmenopausal women and 66.2 ± 20 for premenopausal women which is much higher in both women groups than the RNI and the RDA which indicated that the Saudi females consumed protein more than adequate amounts. The higher amount of protein intakes by Saudi females could be characterized as traditional dish and good food intakes especially if it is an excellent in both quality and quantity. The higher percentage of protein intakes in Saudi population is animal proteins (meat, milk and eggs).

The 1995 Dietary Guideline for Americans recommends that the intake of total fat should be no more than 30 percent of total calories, the results in this study showed that postmenopausal and premenopausal women have much higher fat

intakes 116.4 ± 40.3 and 118.5 ± 42.2 gram respectively, than UK and USA recommended values for fat intakes which is 63 and 73 gram respectively. This may refer to the nature of food consumed in Saudi Arabia. According to the total calories the intakes of fat for postmenopausal and premenopausal women should be 65.46 and 65.6 gram respectively. This high excess of fat intakes by Saudi women is a growing problem of overweight and obesity.

The Dietary Reference Values (DRVs) and the Recommended Dietary Allowance (RDA) for fiber intake is estimated 13-18 and 12 gram/day respectively, whereas many health organizations recommend 20-35 gram/day. The fiber intakes in postmenopausal and premenopausal women were approximately 5.9 ± 1.7 and 5.2 ± 1.6 gram/day respectively, which is obvious much less than the western current values. Consumption of diets high in fiber may prevent a variety of conditions including obesity, diabetes, coronary artery disease and colon cancer (CSEDRI, 1997).

The amounts of calcium intake in Saudi women diets in both groups postmenopausal and premenopausal are quite similar in the range of 791 ± 318.3 and 747 ± 303.9 mg/day respectively. This value is the same as the UK Reference Nutrient Intake (RNI) for women at age 19-50 and >50 years (700 mg/d) whereas much lower than the Recommended adequate intake (AI) of 1200 mg/d for women at age 51-70 years and 1000 mg/d for women at age 19-30years. According to data (CNPP.1996) 73% of calcium is from milk, 9% is from fruits and vegetables, 5% is from grain products and the remaining 12% is from all other sources. Inadequate intake of calcium is one of several important causes of reduced bone mass and osteoporosis (NIH, 1994).

Phosphorus (P) as an essential mineral that is found in all cells within the body. About 85% of the phosphorus is found in bones as a calcium salt and 14% in the muscles. The intakes amount of this mineral by our postmenopausal women (1203 ± 382.1 mg/d) is a little higher than the premenopausal women (1139 ± 361.3 mg/d) and both groups are much higher than the western recommended values (RNI, 550 mg/d; RDA, 700 mg/d). The relative contributions of food groups to phosphorus intake are: 60% from milk, meat, poultry, fish and eggs; 20% from cereals and legumes; 10% from fruits and fruit juices; 3% from soft drinks and other beverages (Allen and Wood, 1994). Dietary P intake have risen 10% to 15% over the past 20 years because of the increased use of phosphate salts in food additives and cola beverages (Ilich and Kerstetter, 2000). An excess amount of dietary phosphorus absorbed is readily excreted in the urine.

Iron dietary intakes of our postmenopausal and premenopausal women are almost similar 12.1 ± 3.5 and 11 ± 3.3 mg/day respectively. The western recommended values, Reference Nutrient Intake (RNI) and Recommended Dietary Allowance (RDA) for women at age 50+ years was 8.7 and 8.0 mg/day respectively and at age 19-50 years was 14.8 and 18 mg/day respectively (Whitney, 2002). In comparisons, our postmenopausal women iron intake are higher than the (RNI) and the (RDA) iron values for women at age 50+ years, but the premenopausal women iron intake value was lower than both the (RNI) and (RDA) iron values for women at age 19-50 years. The major contributors of iron are Meats, fish and poultry other protein rich foods such as legumes and eggs are also good sources. Milk group is poor source of iron.

Vitamin C intakes in postmenopausal and premenopausal women in this study are 55.5 ± 25.4 and 45 ± 23.4 mg/day respectively which are in between the

Recommended Dietary Allowance for adults 60mg/day and the Reference Nutrient Intakes 40 mg/day for women at age 19-50 and >50 years. In other western countries it may range from 30-75 mg/day (CNPP.1996). Recent kinetic analyses suggest that intakes of 150-200 mg/day, but below 400mg/day, obtained from the diet, may benefit healthy individuals (Weber *et al.*, 1995). The best food sources of vitamin C are citrus fruits, berries, melons, tomatoes, potatoes, green peppers and leafy green vegetables.

Vitamin D is important vitamin for the body. The major functions of vitamin D are to increase the efficiency of intestinal calcium absorption and to mobilize calcium stores from bone in order to maintain the serum calcium and phosphorus concentration within the normal physiological range. The dietary intakes of vitamin D for both Saudi postmenopausal and premenopausal women groups are quite similar in the range of 2.8 ± 0.9 and 2.9 ± 0.98 $\mu\text{g/day}$ (112 and 116 IU) respectively. There is no dietary recommendation for vitamin D in the UK as sunlight exposure is believed sufficient. The Institute of Medicine (IOM) for the National Academy of Science issued a report in 1997 recommend an Adequate Intake (AI) rather than Recommended Dietary Allowance for vitamin D. It been recognized that vitamin D insufficiency and deficiency are prevalent in adults over the age of 50 years and set the Adequate Intake (AI) for adults 51-70 years as $10\mu\text{g/day}$ (400IU) and for ages 0-50 years as $5\mu\text{g/day}$ (200IU) (Holic, 1994). According to those values, the daily intakes of vitamin D by Saudi postmenopausal and premenopausal women are very low which explain the prevalent of osteopenia and osteoporosis among these groups. Good food sources are milk properly fortified with vitamin D, fatty fish, cod liver oil, some bread and some cereals and some egg yolks.

Potassium intakes by postmenopausal and premenopausal women are 1988 ± 537.9 and 1799.7 ± 386.3 mg/day respectively. The UK Dietary Reference values for women at age 19-50 and >50 years is 3500 mg/day. The USA Estimated Minimum Requirement for adults is 2000mg/day but the usual dietary intake of potassium for adults is about 4000mg/day. A comparison of potassium dietary intake of our groups of women with the western values showed a quite similarity.

Analysis of these nutrient data obtained from dietary assessments, total energy intakes, total macronutrient intakes (Protein and fat) and total micronutrients intakes (Ca, P, Fe, vitamin C, D and K) for both postmenopausal and premenopausal Saudi women. The accuracy of this comparison could not be of that much legitimate for a simple reason that the finding results in this study was compared with USA/UK recommended portions which is definitely profoundly differences between the Middle Eastern countries habitual food intakes and western countries and this unfortunately due to inexistence of standard portion size book for Middle Eastern countries. The limitation of the comparison results can be sight also from the side of the food composition table book available for Middle Eastern which is the only and very old one (Pellet and Shadarevian, 1970) besides very limited of number of food. Calculation was done by hand, which induces error; women may not have recorded accurately and under reporting in existence. But this is the first time we are able to show what the Saudi Arabian diet looks like and with all the given limitations expressed above, this data is first in the country of Saudi Arabia.

There are some limitation associated with dietary assessments which is:

- 1- For estimated dietary records procedures there is underestimation of the quantification of amounts of food (e.g. Mixed dishes), which contain many ingredients.

- 2- Quantification of portion sizes, the ability of a person to estimate portion sizes will be poor without the aid of measuring devices.
- 3- A tendency of to select foods that are more socially acceptable or considered to be healthier.
- 4- For ease of recording, subjects may choose simple foods that are easy to record.
- 5- Inaccuracies in the coding of food intake data are a major source of error in nutrient calculation that is associated with manual coding.
- 6- Errors related to selecting incorrect matches for items listed on records are difficult to control.

CHAPTER 6

Influence of food consumption and nutrient intakes on indices of bone health

6.1. Introduction

Bone is constantly undergoing a metabolic process called remodelling. This includes a degradation process or bone resorption, which is mediated by the action of osteoclasts. Next step is bone formation or a building process, which is mediated by the action of osteoblasts. Remodeling is required for the maintenance of bone and is tightly coupled where resorption and formation are in balance. In abnormal states of bone metabolism this process becomes uncoupled when resorption exceeds formation, which results in a net loss of bone. The analysis and measurement of specific degradation products of bone matrix provides analytical data of the rate of bone metabolism (Baron R., 1996). Biochemical markers of bone turnover are substances found in blood and urine that reflect rates of bone resorption or bone formation. Typically, these biochemical tests measure proteins or other products of osteoblasts that reflect bone formation, such as osteocalcin or bone-specific alkaline phosphatase, or collagen breakdown products that reflect bone resorption, such as pyridinolines or Deoxypyridinoline (Looker *et al.*, 2000).

The modifiable dietary factors are particularly important for bone health. The extensive data available on calcium and vitamin D nutrition as it relates to bone has led to successful interventions to prevent bone loss and fractures (Heaney, 1993; Dawson-Hughes *et al.*, 1990; Chapuy *et al.*, 1994). Based largely on experimental studies, most agree that calcium and vitamin D are important nutrients to bone health (Dawson-Hughes *et al.*, 1990; Dawson-Hughes, 1991). A number of papers have contributed of the influence of other nutrients on bone, although effects have been evaluated for protein, magnesium, potassium, and vitamin K (Heaney, 1993; Tucker *et al.*, 1999; Sellmeyer *et al.*, 2001).

6.1.1. Effect of food groups on bone health

An alternative approach of examining the relationship between diet and bone loss was to examine different food groups and their effect on indices of bone health.

A. Milk and milk products intake

Milk is a preferred source of calcium considering its absorption and bioavailability. It provides over 50% of the total calcium in the diet (New, 2001). Milk is an important source of a number of nutrients including minerals and factors such as milk basic protein, which enhances bone strength by the stimulation of bone formation and collagen synthesis (Toba *et al.*, 2000). Calcium intake, often assessed as milk intake, has been related to bone mass (Welton *et al.*, 1995). Calcium is a substrate for bone formation and it is also an antiresorptive agent. Dietary calcium at high levels, usually 1000 mg per day or more, can lower the bone-remodelling rate by 10-20% in older adults (Yergey *et al.*, 1994). Data from the EVOS study suggest that milk consumption during growth and adulthood is associated with a reduced risk of vertebral fractures in women (Lunt *et al.*, 2001). Some studies have distinguished between the effect of milk and calcium as supplements in adolescences. Of these trials that calcium was given as supplement (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lambert *et al.*, 2000). In other trial milk was given (Chan *et al.*, 1995) another study calcium derived from milk extract was given (Bonjour *et al.*, 1997). The results of these trials provided evidence that calcium positively influence bone mineralization during growth and that the mechanism for its action is likely due to a suppression of bone remodeling.

Observational studies provided an indication that past and present calcium intake was associated with current BMD (Nieves *et al.*, 1995; Parsons *et al.*,

1997). The association was stronger at lower level of calcium intake than higher intakes (Kardinaal *et al.*, 1999). Heaney and Matkovic have proposed a threshold for calcium intake during adolescence. Increasing intakes of calcium up to approximately 1500 mg/day has a positive effect on the skeleton, but no further benefit is observed above this level (Matkovic and Heaney, 1992). The finding in the MEDOS was that if men and women with the lowest milk consumption had started consuming enough milk, then 4% of the hip fractures are avoided (Johnell *et al.*, 1995; Kains *et al.*, 1999). Permanent effect of calcium and milk on bone mineralization has not yet been resolved. The follow up studies completed to date indicate that the beneficial effects of calcium do not persist beyond the period of supplementation (Slemenda *et al.*, 1997; Lee *et al.*, 1997; Lloyd *et al.*, 1996; Ghatge *et al.*, 2001). This reversed effect may not be the case for milk and milk-based supplements (Barker *et al.*, 1998; Bonjour *et al.*, 1999).

B. Fruit and vegetable intakes

Observational studies have demonstrated a beneficial effect of fruit and vegetable on indices of bone health in premenopausal and postmenopausal women. Aberdeen prospective osteoporosis screening study (APOSS) has shown an association between nutrients found in fruit and vegetables (such as potassium, magnesium, fiber, and vitamin C) and bone mass and markers of bone resorption. Women in the lowest quartile of fruit and vegetables intakes had lower femoral neck, lumbar spine and forearm BMD (New *et al.*, 1997; New *et al.*, 2000). A longitudinal study demonstrated that fruit and vegetable intake might protect against premenopausal bone loss, possibly because of their beneficial effect on acid-base balance (Macdonald *et al.*, 2004).

A recent observational study shows that high intakes of fruit may be important for bone health in girls (McGartland *et al.*, 2004). In early pubertal girls, high fruit and vegetable consumption have a beneficial effects on bones such as radius and whole body also it decrease urinary calcium excretion (Tylavsky *et al.*, 2004).

C. Other food groups

Little is known concerning the remaining food groups with respect to their effect on bone. Studies examining tea and coffee consumption and bone density suggest that a positive association between tea drinking and bone mass in postmenopausal women which may be due to the effect of flavonoids found in tea on bone health (Hegarty *et al.*, 2000), and a negative association only with high caffeine intake is accompanied by very low calcium intakes (Barrett-Connor *et al.*, 1994). Many of food groups associated with bone loss at the femoral neck in perimenopausal and early postmenopausal at women are fried foods, cakes, processed meat and pudding (Macdonald *et al.*, 2001).

6.1.2. Effect of macronutrients and micronutrients on bone health

Carbohydrates, fat and protein are called macronutrients because they are relatively large molecules. In contrast, vitamins and minerals are micronutrients. They are considered having some effect on indices of bone health.

- **Protein:** Dietary protein intake influences both bone mass acquisition and bone loss. A positive association between dietary protein intake and bone mass has been detected (Michaelsson *et al.*, 1995; Cooper *et al.*, 1996). While other studies, have not find any link between bone mineral mass and

protein intake (Mazess and Barden, 1991; Wang *et al.*, 1997). In contrast, there is study showing negative correlation between bone mass and dietary protein intake, in which higher protein intake was accompanied with lower BMD (Metz *et al.*, 1993). Increased protein intake has been associated with an increase in urinary calcium excretion. Thus, high protein intake would induce a negative calcium balance, which is favor bone loss (Heaney and Recker, 1982).

- **Fiber:** Are the structural parts of plants such as vegetables, fruits, grains and legumes. Most fibers are polysaccharides. Fibers are often described as nonstarch polysaccharides. Increased intake of fiber is associated with decreased in Ca absorption (Reid and New, 1997). Presence of phytic acid in the grains, legumes and seeds is capable of binding minerals such as calcium, iron, magnesium and copper in insoluble complexes in the intestine, which the body excretes unused.
- **Vitamin C:** is an essential micronutrient. It is a water-soluble vitamin. Human are not able to synthesise vitamin C. Deficiency of vitamin C will result in scurvy disease which is appear as tooth loss, joint pains, poor wound healing and bone and connective tissue disorders (Benzie, 1999). Vitamin C acts as a cofactor for the hydroxylation of lysyl to hydroxylysyl residues in collagen. Pyridinoline (PYD) and deoxypyridinoline (DPD) are pyridinium cross-links, which provide a stable matrix. PYD formed from three hydroxylysine residues and DPD consist of two hydroxylysine and one lysine residue. Bone collagen PYD: DPD ratio is about 3:1. A deficiency of vitamin C will alter this ratio, resulting in a decrease in the number hydroxylated lysine residues (Munday, 2003). Epidemiological studies

showed a positive relationship between vitamin C intakes and BMD. In a study of New *et al.*, women in the lowest quartile of vitamin C intakes had significant lower lumbar spine and femoral neck bone mineral densities (New *et al.*, 1997; Hall and Greendale, 1998). Low Vitamin C intakes may adversely affect bone formation and resorption by the synthesis of abnormal collagen that may degrade before maturation (Munday, 2003).

- **Phosphorus (P):** is a major component of bone mineral as is calcium and low intakes could limit adequate bone growth and mineralization (Matkovic *et al.*, 2003). However, it is rare for diets to be deficient in phosphorous. Despite concerns that increase intakes of phosphorous would be harmful to bone health (Eastell and Lambert, 2002). Studies showed negative associations of phosphorous intake and bone size (Hoppe *et al.*, 2000).
- **Potassium (K):** Potassium is the major intracellular cation of the body. It is essential for cellular integrity and the maintenance of fluid, electrolyte and acid-base balance (Barasi, 1997). Lifetime acid load from the ingestion of mixed diets leads to gradual bone loss. Bone mineral functions as buffer base. Studies suggested that using a diet favoring alkaline ash might decrease the rate of bone loss. This type of diet emphasizes eating more fruits, vegetables, vegetable protein and milk (Tucker *et al.*, 1999). Potassium is one of few nutrients that may have such buffering effects. It is found in all whole foods: meats, milk, fruits, vegetables, grains and legumes. Early and recently studies demonstrated a positive effect of potassium intake on indices of bone health (Michaelsson, 1995; New *et al.*, 1997). Increase potassium intake was significantly associated with increase BMD at 3ites for women (Tucker *et al.*, 1999). Low potassium intake has been shown to increase rates

of calcium excretion (Leman *et al.*, 1993). The oral administration of potassium bicarbonate resulted in a decrease in urinary calcium and phosphorus, reduction in urinary excretion of hydroxyproline (bone resorption) and an increased excretion of serum osteocalcin (bone formation) (Sebastian *et al.*, 1994).

- **Vitamin D:** Dietary forms are principally cholecalciferol (vitamin D₃) and Ergocalciferol (vitamin D₂). Vitamin D₃ and D₂ consumed in foods are biologically inactive. Vitamin D is found naturally in a few foods. Mainly in fish and fish liver oil and lower amounts in eggs (yolk) and meat products (Ovesen *et al.*, 2003). Foods such as margarine and milk are fortified with vitamin D. In its active form, vitamin D plays an important role in maintaining calcium homeostasis. It acts on bone cells to mobilize calcium from stores and on intestinal cells to increase absorption of calcium from diet (Eastell and Lambert, 2002).
- **Iron (Fe):** The best sources of iron are red meats particularly liver and other meat organs. Iron may play a role in bone formation acting as a cofactor for enzymes involving in collagen synthesis. It is required for the hydroxylation of both proline and lysine in protocollagen. Iron overload may be detrimental to bone health. Iron deficiency may play a role in bone fragility (Walsh *et al.*, 2003).

6.1.3. Acid-base homeostasis and the skeleton

Acid base homeostasis is important to health. The body normally produces 50-100mEq of non-carbonic protons by metabolizing the ingested food (Rose, 1977). Extracellular fluid PH must be between 7.35 and 7.45. Each minor

decrease of the pH stimulates bone resorption and inhibits tubular reabsorption of calcium, while each increase of the pH has the opposite effect. Thus, it is vital for our metabolic system to ensure that H^+ concentrations are maintained between 0.035 and 0.045 mEq/l (Green & Kleeman, 1991). The body adaptive response to this particularly narrow limits involves three mechanisms: buffer systems, exhalation of CO_2 and the renal system. Human subjects eat on a daily basis substance that consume and generate H^+ . The more the diet contains acid precursors, the greater the systemic acidity (Kurtz *et al.*, 1983).

The skeleton has been considered as a source of alkali buffer to preserve the body pH and to defense the system against acid-base disorders, since 80% of body carbonate, 80% of body citrate, and 35% of body sodium are contained in solution in the hydration shell of bone (Barzel, 1995).

6.1.4. Net Endogenous Acid Production (NEAP)

The best way to quantify the link between acid-base balance and skeletal health is to estimate the content of acid-base of the diet consumed. Humans eat substances of the diet that generate and consume protons and as a net result; this diet will be considered as low or high grade metabolic acidosis. The severity of this metabolic acidosis is estimated in part by the net rate of endogenous noncarbonic acid production (NEAP), which varies with diet contents (Kurtz *et al.*, 1983). Protein-to-potassium ratio predicts net acid excretion, which, in turn, predicts calcium excretion. NEAP was estimated by examining the ratio of protein to potassium intake normalized to a diet of 8.2 MJ (1964 kcal). The concept of NEAP is based on the acidifying effect of protein, mainly through sulfate excretion, and the

alkalizing effect of potassium that results from the dietary intake of potassium as salts of weak organic acid (Frassetto *et al.*, 1998).

$$\{\text{Renal net acid excretion} = 54.5 \times (\text{protein intake} / \text{potassium intake}) - 10.2\}$$

6.1.5. Study aim

- 1) To determine if a low consumption of milk, dairy products (Food Group3); fruit and vegetables (Food Group2) or a high consumption of protein food (food group 4) result in a lower bone mass and high bone turnover.
- 2) To determine if there are any association between macro and micronutrients and indices of bone health in postmenopausal and premenopausal Saudi women.

6.2 Study design

6.2.1. Subject selection

For this dietary intake Total 109 postmenopausal and of 71 premenopausal women living in the city of Jeddah were studied. They were aged 45-60 years and 20-30 years respectively and had not suffered from any known condition and were not taking any medication likely to affect bone metabolism. Measurements were made on weight and height as well as spine, hip and calcaneal bone mass.

6.2.2. Collection of dietary information

6.2.2.1. Milk and dairy intake information

All subjects were interviewed through a food frequency questionnaire concerning their habitual milk and dairy intake as described in (section 2.4.2.1). In brief, women were asked how many glasses of milk they drunk during the day including milk used in tea or coffee, chocolate, cereals, sweet drinks etc. and

what kind of milk do they used (full fat, semi skimmed and skimmed milk). They also been asked if the milk they are using usually fresh, long life or dried and the frequency of consumption (number of times per day and number of days per week). Intake of milk products how many times a day / week do you eat the following milk products (yogurt, cream, ice-cream (all kinds), hard cheese, soft cheese and milk based pudding, e.g. Custard.

6.2.2.2. Dietary 'quality' intake information

Dietary intakes have been collected from food diaries as been described in (Section 2.4.2.2). In brief, women estimated their dietary intake over a 3-d period. They were asked to record all type of food / drink and the amount of food / drink actually eaten. The recording period included a weekend day. The amount of food intakes was calculated by hand for each woman as gram consumed for the five food groups. Frequency of consumption (times per day) were calculated as described in section (Section 5.2.2)

6.2.3. Bone metabolism measurements

In the assessment of bone turnover, laboratory tests are used. The basic indices of bone metabolism are the serum calcium, phosphate and alkaline phosphatase concentrations. For assessing bone resorption there are urinary hydroxyproline and urinary pyridinoline and deoxypyridinoline cross-links of collagen. For assessing the rate of bone formation, circulating level of serum bone alkaline phosphatase and serum osteocalcin can be employed. Urinary fasting collagen cross-links can be effectively measured by ELISA immunoassay.

6.2.3.1. Blood and urine sample collection

Fasting blood sample

Blood sample were collected following a 12-hour overnight fast in evacuated tubes with no additives. Serum was collected by allowing the blood sample to clot at room temperature and centrifuged at 3000 X g for 10 min and stored at -85°C . Analytical methods for the measurement of markers of bone formation as Bone specific alkaline phosphatase (BASP) and Osteocalcin (OC) is described in (Section 2.5.1).

SMV urine sample

Urine sample were collected following an overnight fast before 11.00 AM. It was the second morning void (SMV). Urine then centrifuged at 5000 X g.min for 10 min. and stored at -85°C . Analytical methods for the measurement of markers of bone resorption as pyridinium crosslinks (pyridinoline (PYD) and deoxy-pyridinoline (DPD) is described in (Section 2.5.2).

6.2.4. Analytical methods for assessment of bone formation

Bone specific alkaline phosphatase (BSAP) immunoassay is a microtiter strip format utilizing a monoclonal anti- BSAP antibody coated on the strip to capture BSAP in the sample. The enzyme activity of the captured BSAP is detected spectrophotometrically with p-nitrophenyl phosphate (pNPP) substrate (Price, CP. 1993)(Section 2.5.1.2). Osteocalcin (OC) was analyzed by a competitive immunoassay to measures intact (de novo) osteocalcin in serum. It uses a mouse anti-osteocalcin antibody (Section 2.5.1.1)

6.2.5. Analytical methods for assessment of bone resorption

Measurement of pyridinium crosslinks: Pyridinoline (PYD) and Deoxypyridinoline (DPD) was a competitive immunoassay in a microtiter stripwell format utilizing a monoclonal anti-pyridinium crosslinks coated on the strip. The PYD and Dpd concentration in the specimen is determined spectrophotometrically and calculated from a standard calibration curve. Assay values are corrected for urinary dilution by urinary creatinine analysis (Colwel *et al.*, 1993). These methods were described in chapter 2: -

- Analysis of Pyridinoline (PYD)(Section 2.5.2.2)
- Analysis of Deoxypyridinoline (DPD) (Section 2.5.2.1)

6.2.6. Statistical analysis

Statistical analysis was performed by using SPSS software (version 12; SPSS Inc, Chicago), and descriptive statistics (means, medians, SDs, and ranges) were determined for all variables. Results for the effect of milk consumption on bone markers presented in three groups are given as mean values and their standard deviation. In tables, data is presented as mean values with their standard deviation for each group. Pearson's correlations (with adjustment for age, weight, and height) were obtained between milk groups, nutrients (macro and micro), fruit vegetable group, net endogenous acid production (NEAP), and bone mass (BMD sites, BUA, and VOS) and bone metabolism (bone turnover markers PYD, BDP, BSAP and OC excretion). All these variables defined into quartiles, and the mean values for BMD, BUA and VOS at each site and for bone resorption and formation were calculated.

6.2.7. Subject information

Each group of women were divided into three groups according to their level of milk consumption: Non-Milk Group (NMG); Low milk group (LMG) (< 1 pint/d); High milk group (HMG) (>1 pint/d). Bone resorption was assessed by ELISA measurement of pyridinium crosslinks (pyridinoline (PYD) and deoxy-pyridinoline (DPD) using a second morning urine sample. Bone formation was assessed by Immunoassay of bone specific alkaline phosphatase (BSAP) and osteocalcin (OC) using a fasted blood (serum) sample.

6.3 Result

6.3.1. Effect of milk consumption on bone health indices and bone metabolism in postmenopausal women

Women were divided into three groups according to milk consumption as detailed in (Section 6.2.7). Differences in lumbar spine and femoral neck BMD assessed by DXA were examined. Differences in bone ultrasound and bone turnover were also examined. No differences were found in the lumbar spine or femoral neck BMD between none, low or high milk consumers. As shown in Table 6.1 there was a trend for lower bone ultrasound with low milk consumption and this trend demonstrated a linear association ($P < 0.17$) (Fig. 6.1) No significant changes were seen in markers of bone formation (BSAP or OC). DPD excretion was significantly higher in the non-milk group compared with either the low milk or high milk groups indicative of increased bone resorption in the non-milk group. This remained significant after adjustment for heights; menopause and weight ($P < 0.002$) see Figure 6.2.

6.3.1.1. Verification of milk consumption data

Using the three days food diaries, we checked to verify the milk intake data by looking at differences in Ca consumption between the three milk groups. Results shown in Table 6.2.

Table 6.1: Milk consumption groups and their response on bone ultrasound and markers of bone turnover in postmenopausal women.

Bone ultrasound & Turnover Markers	NMG Mean SD (N=29)	LMG Mean SD (N=79)	HMG Mean SD (N=4)
BUA(db/MHz)	66.2 ± 14.0	70.3 ± 16.1	81.2 ± 19.7
BUA t-score	-1.3 ± 0.8	- 1.1± 0.9	- 0.5 ± 1.2
DPD nmol/l	12.0 ^a ± 4.7	9.6 ^b ± 3.1	9.4 ^b ± 2.9
PYD nmol/l	40.8 ± 15.6	36.9 ± 12.2	39.4 ± 17.3
BSAP u/l	21.5 ± 8.1	20.0 ± 7.4	24.6 ± 8.7
OC ng/ml	9.5 ± 3.7	10.1 ± 3.6	8.9 ± 1.0

Unlike superscripts are significantly different, ANOVA P<0.05

Table 6.2: Differences in calcium intake (as assessed by 3 day food diaries) between the three milk groups (assessed by questioners) in postmenopausal women

	NMG Mean SD (N=29)	LMG Mean SD (N=79)	HMG Mean SD (N=4)	P value
Calcium intake mg/day	651.7 ± 305	827 ± 301.5	1054 ± 446	P<0.001

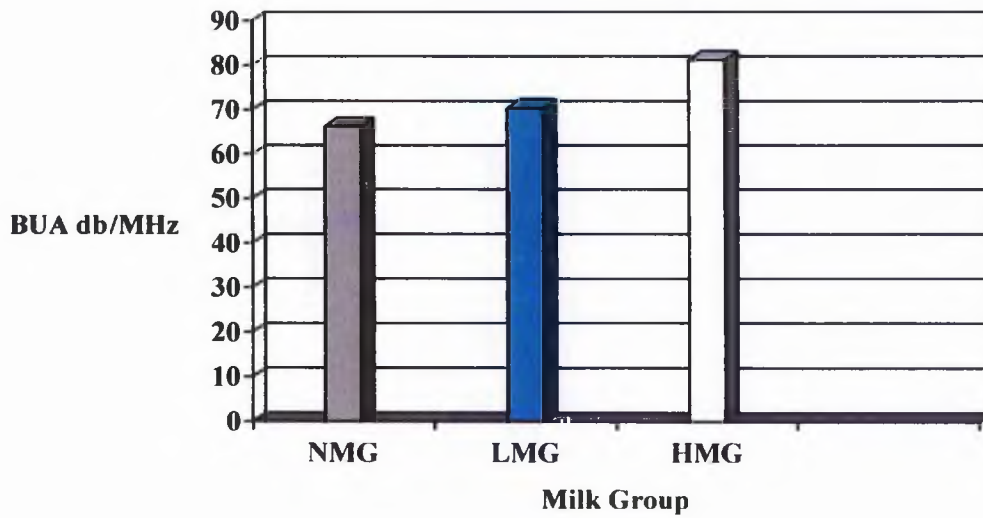


Figure 6.1: Effect of milk consumption groups on bone ultrasound BUA (db/MHz) in Postmenopausal Women. Mean BUA for (NMG: 66.2), (LMG: 70.3) and (HMG: 81.2). Low milk consumption associated with low bone ultrasound this trend demonstrated a liner association $p < 0.08$.

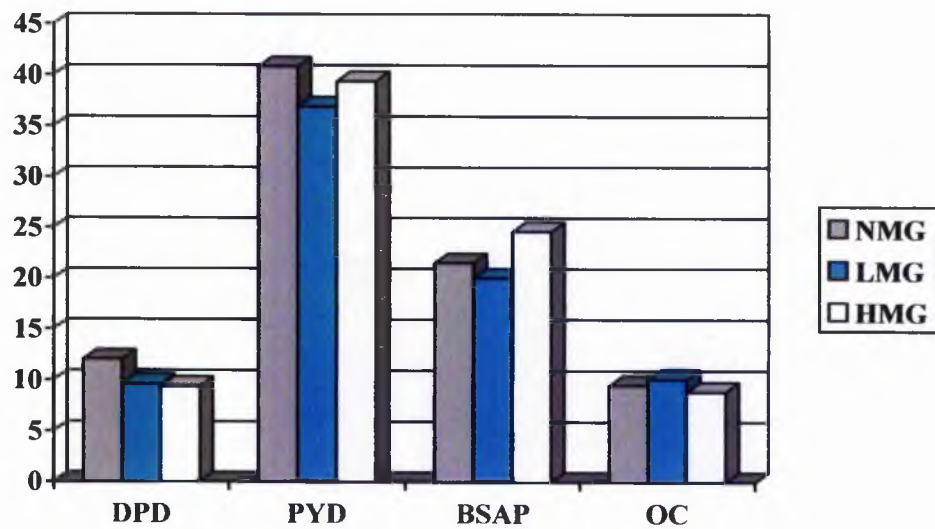


Figure 6.2: Comparisons between milk consumption groups and different markers of bone turnover in postmenopausal women.

6.3.2. Effect of milk consumption on bone health indices and bone metabolism in premenopausal women

Women were divided into three groups according to milk consumption as detailed in (Section 6.2.7). Differences in lumbar spine and femoral neck BMD assessed by DXA were examined. Differences in bone ultrasound and bone turnover were also examined. No differences were found in the lumbar spine or femoral neck BMD between none, low or high milk consumers. As shown in Table 6.3 there was a trend for lower bone ultrasound with low milk consumption and this trend demonstrated a linear association ($P < 0.10$) (Fig. 6.3). No significant changes were seen in bone formation markers (BSAP or OC). PYD + DPD excretion was significantly higher in the non-milk and low-milk groups compared with high milk group indicating of increased bone resorption in the non- milk group. This remained significant after adjustment for height, menopause and weight ($P < 0.003$) see Figure 6.4.

6.3.2.1. Verification of milk consumption data

Using the three days food diaries, we checked to verify the milk intake data by looking at differences in Ca consumption between the three milk groups. Results shown in Table 6.4.

Table 6.3: Milk consumption groups and their response on bone ultrasound and markers of bone turnover in premenopausal women.

Bone ultrasound & Turnover Markers	NMG Mean SD (N=20)	LMG Mean SD (N=70)	HMG Mean SD (N=4)
BUA(db/MHz)	76.4 ± 12.6	78.2 ± 13.4	90.0 ± 17.9
BUA t-score	- 0.8 ± 0.7	- 0.7 ± 0.8	0.03 ± 1.0
DPD nmol/l	10.4 ^a ± 4.1	10.6 ^b ± 4.4	9.2 ^b ± 5.5
PYD nmol/l	37.8 ± 8.2	39.2 ± 8.1	29.8 ± 7.1
BSAP U/L	18.9 ± 5.8	17.5 ± 5.5	17.7 ± 5.6
OC ng/ml	13.5 ± 5.3	12.5 ± 3.6	11.4 ± 3.0

Unlike superscripts are significantly different, ANOVA P<0.05

Table 6.4: Differences on calcium intake (as assessed by 3 days food diaries) between the three milk groups (assessed by questioners) in premenopausal Women

	NMG Mean SD (N=20)	LMG Mean SD (N=70)	HMG Mean SD (N=4)	P value
Calcium intake mg/day	646.1 ± 219.7	757.5 ± 303.6	1395.6 ± 67.1	P<0.001

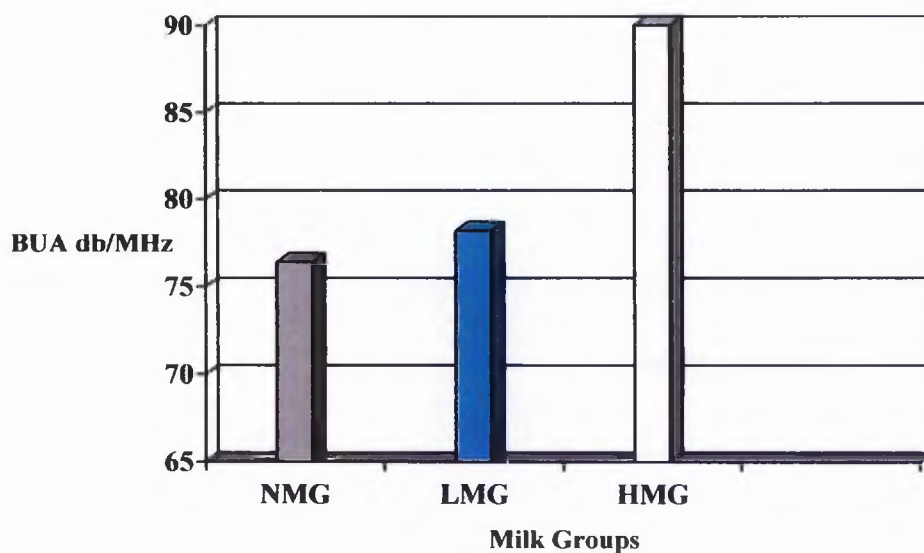


Figure 6.3: Effect of milk consumption groups on bone ultrasound BUA in premenopausal women. Low milk consumption with low bone ultrasound this trend demonstrated a liner association ($P < 0.10$)

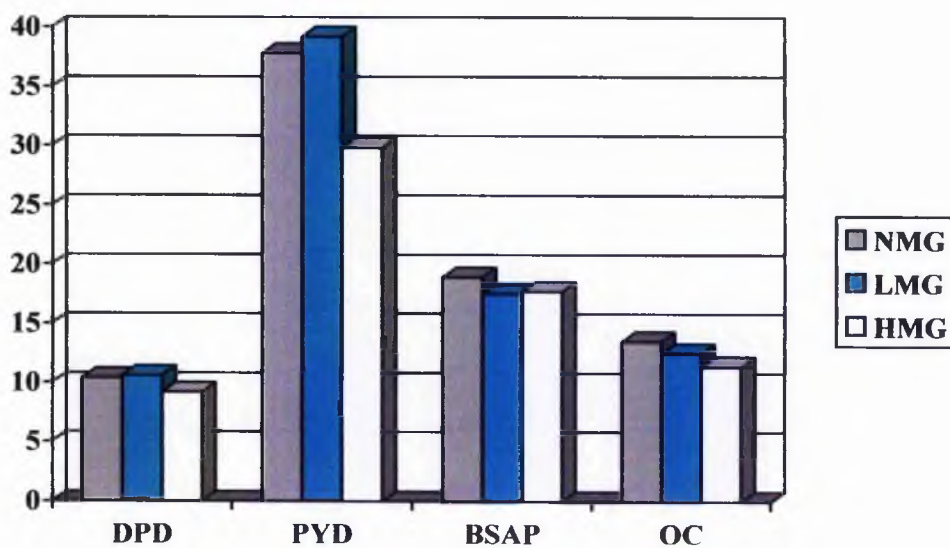


Figure 6.4: Comparisons between milk consumption groups and different markers of bone turnover in premenopausal women.

6.3.3. Influence of nutrient intake on bone health indices and bone metabolism in postmenopausal women

The data were examined to see if any association existed between nutrients intakes: proteins, fibre, vitamin C, vitamin D, phosphorus, calcium, potassium and bone mass/ bone metabolism in postmenopausal women. There was a negative trend for phosphorus intake and lumbar spine BMD; a higher phosphorus intake was non-significantly associated with low bone mass at the spine ($P < 0.1$). There was a negative trend for potassium intake and DPD excretion; a higher potassium intake was associated with lower bone resorption ($P < 0.09$). There was a negative trend for ferric intake and DPD excretion; a higher iron intake was associated with lower bone resorption ($P < 0.08$).

Vitamin C intake was negatively associated with BSAP. A higher intake of vitamin C associated with lower bone turnover ($P < 0.027$). Vitamin D intake was negatively associated (non-significantly) with DPD excretion. That is, a higher Vitamin D intake resulted in lower bone resorption ($P < 0.06$). There was a negative trend for protein intake and lumber spine BMD; a higher protein intake is associated with a lower lumber spine BMD ($P < 0.13$) differences reviewed the same after adjustment for age, weight and height.

6.3.4. Influence of nutrient intake on bone health indices and bone metabolism in premenopausal women

The data were examined to see if any association existed between nutrients intakes: proteins, fibre, vitamin C, vitamin D, phosphorus, calcium, potassium and bone mass/ bone metabolism in premenopausal women. There was a positive trend for calcium intake and VOS; a higher Ca intake was non-significantly

associated with higher value of velocity of sound VOS ($P < 0.12$). A negative trend was seen between Ca intake and PYD excretion; a higher Ca intake was associated with lower bone resorption ($P < 0.06$). There was a positive trend for phosphorus intake and velocity of sound VOS; the higher P intake was associated (non-significantly) with high bone quality ($P < 0.18$). A negative trend was seen for phosphorus and PYD, BSAP excretion; a higher P intake was associated with lower bone turnover ($P < 0.09$, $P < 0.12$) respectively.

There was a positive trend for iron intake and FN-BMD, FW-BMD and BUA; the higher iron intake was non-significant associated with high bone mass at the femoral neck, ward and calcaneal sites ($P < 0.11$, $P < 0.16$, $P < 0.13$) respectively. A negative trend was seen for iron intake with BSAP and OC excretions; a higher iron intake was associated (non-significantly) with low bone formation ($P < 0.1$, $P < 0.19$) respectively. There was a positive association (non-significant) for potassium intake and FN, FW-BMD, BUA and VOS, i.e. a higher K intake resulted in high bone mass at the femoral, trochanter and calcaneal sites ($P < 0.08$, $P < 0.10$, $P < 0.09$, $P < 0.15$) respectively. Vitamin C intake was positively associated (non significantly) with BUA and VOS, i.e. the higher vitamin C intake resulted with a higher bone mass at the calcaneal site ($P < 0.07$, $P < 0.16$) respectively. Vitamin D intake was negatively associated with BSAP and OC excretion, i.e. the higher vitamin D intake resulted in lower bone formation ($P < 0.01$, $P < 0.02$) respectively. A positive association was found between vitamin D intake and BUA; a higher intake of vitamin D resulted in high bone mass at the calcaneal sit ($P < 0.01$). There was a negative trend for protein intake with PYD and BSAP excretions, i.e. a higher protein intake resulted in lower bone turnover ($P < 0.10$, $P < 0.14$) respectively.

6.3.5. Quartile intakes of macro and micronutrient intake: effect on bone density and bone metabolism of postmenopausal women

Intakes of proteins, fibre, iron, vitamin C, vitamin D, phosphorus, calcium and potassium were divided into quartiles of intake. The data were examined to see if any differences were found in BMD bone ultrasound and bone turnover. Protein intake had no effect on bone mass or bone turnover. There was negative trend for vitamin C intake and bone turnover, i.e. a higher intake of vitamin C was associated non-significantly with lower bone resorption (DPD and PYD) excretion and bone formation (BSAP and OC) excretion. As shown in Figures (6.5, 6.6, 6.7, 6.8) those in the lower quartile of vitamin C intake (1st Qtr) had Non-significantly higher PYD, DPD, OC and BSAP excretion. Linearity between quartiles was ($P < 0.15$, $P < 0.10$, $P < 0.02$, $P < 0.07$) respectively. Differences reviewed the same after adjustment for age, weight and height.

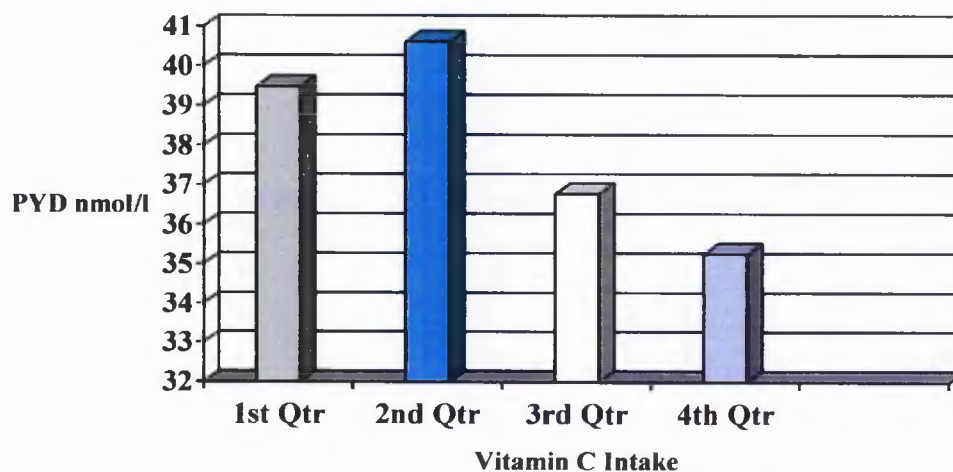


Figure 6.5: Effect of vitamin C intake by quartile on PYD excretion in postmenopausal women. Mean PYD values for Q1-Q4 were 39.47, 40.61, 36.77, and 35.26 nmol/l, respectively.

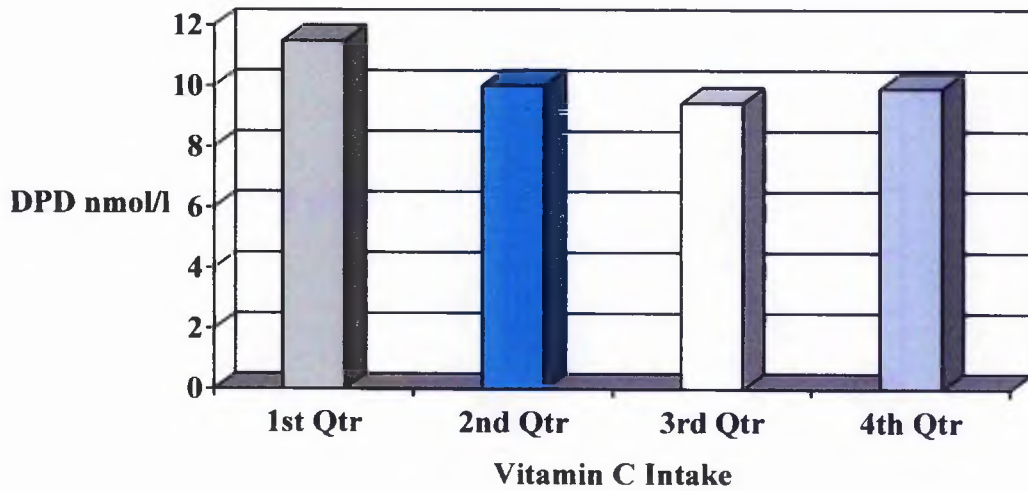


Figure 6.6: Effect of vitamin C intake by quartile on DPD excretion in postmenopausal women. Mean DPD values for Q1-Q4 were 11.49, 10.02, 9.44, and 9.92 nmol/l, respectively.

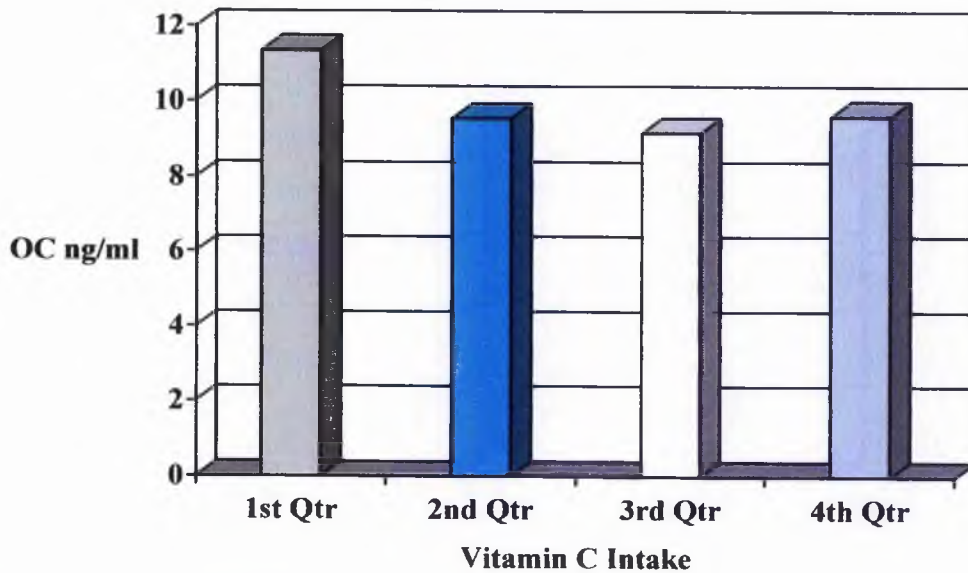


Figure 6.7: Effect of vitamin C intake by quartile on OC excretion in postmenopausal women. Mean OC values for Q1-Q4 were 11.35, 9.54, 9.17, and 9.61 ng/ml, respectively.

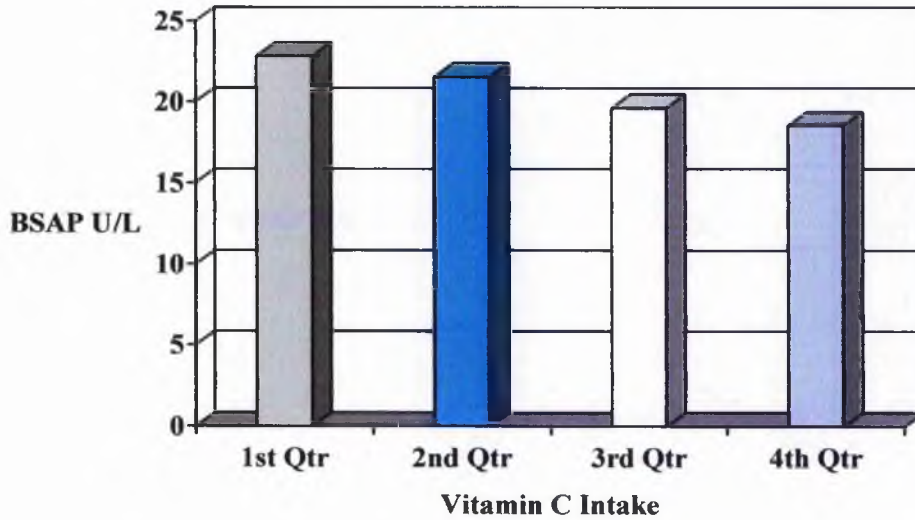


Figure 6.8: Effect of vitamin C intake by quartile on BSAP excretion in postmenopausal women. Mean BSAP values for Q1-Q4 were 22.82, 21.53, 19.62, and 18.54 U/L, respectively.

Potassium intake had no effect on bone mass but there was a negative association with bone resorption excretion; higher intake of K were associated with lower PYD and DPD excretion. As shown in Figures (6.9, 6.10) those in the lower quartile of Potassium intake (1st Qtr) had non-significantly higher PYD and DPD excretion. Linearity between quartiles was ($P < 0.07$, $P < 0.03$) respectively. Vitamin D has no effect on bone mass and bone formation, but there was a negative trend with bone resorption; i.e. a higher vitamin D intake resulted in lower DPD excretion. As shown in Figure (6.11, 6.12) lower quartile of vitamin D intake (1st Qtr) had non-significantly higher DPD excretion. Linearity between quartiles was ($P < 0.11$).

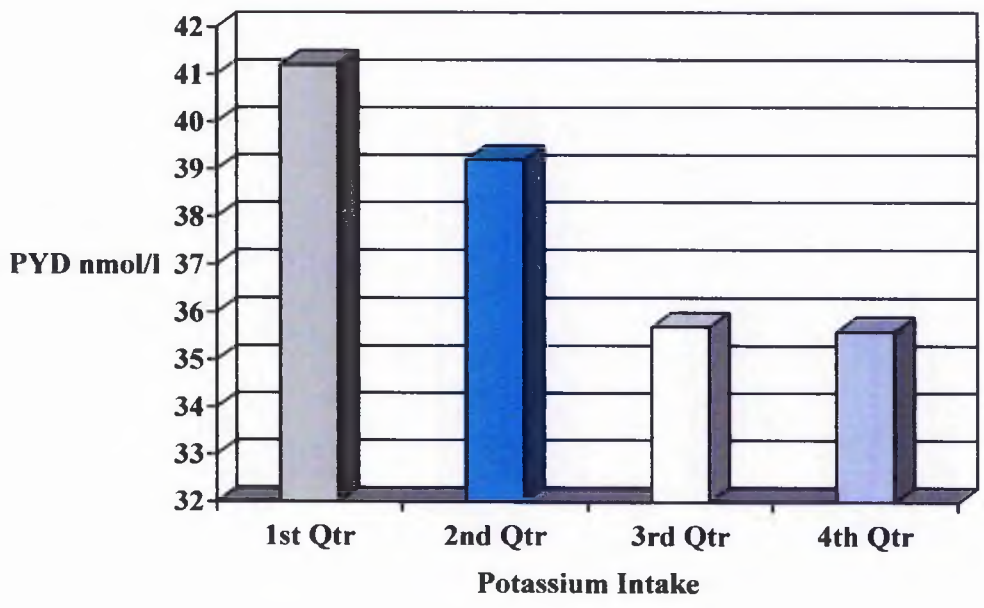


Figure 6.9: Effect of potassium intake by quartile on PYD excretion in postmenopausal women. Mean PYD values for Q1-Q4 were 41.2, 39.2, 35.7, and 35.6 nmol/l, respectively.

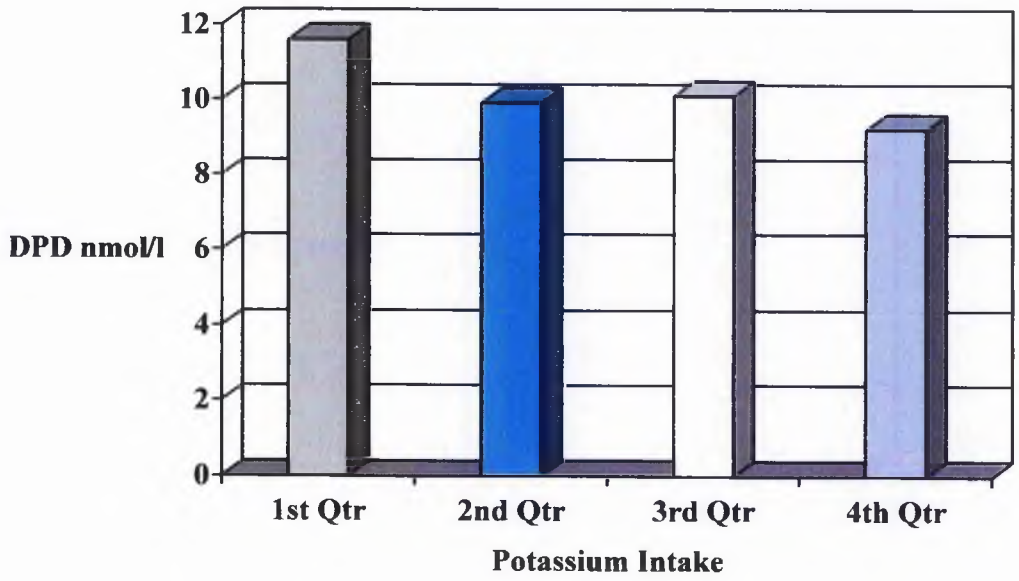


Figure 6.10: Effect of potassium intake by quartile on DPD excretion in postmenopausal women. Mean DPD values for Q1-Q4 were 11.6, 9.9, 10.1, and 9.2 nmol/l, respectively.

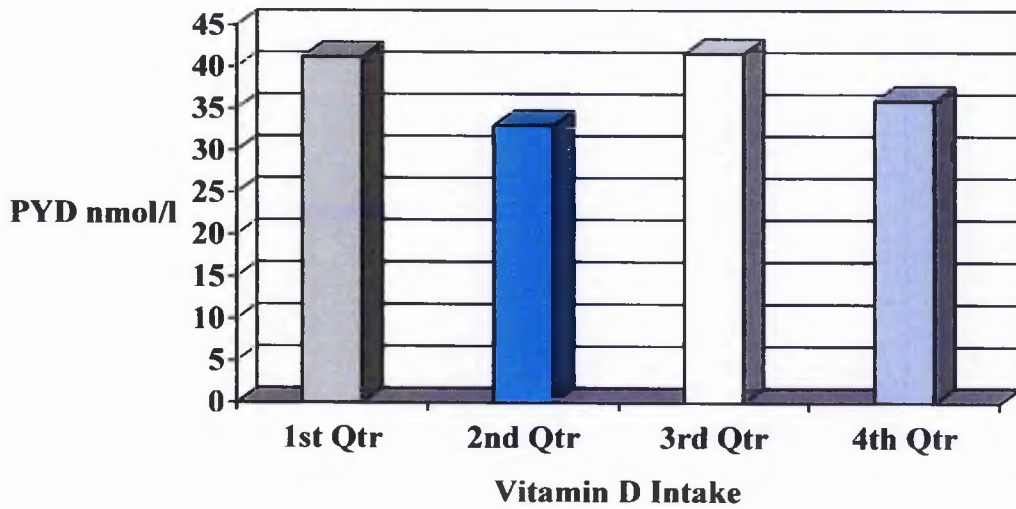


Figure 6.11: Effect of vitamin D intake by quartile on PYD excretion in postmenopausal women. Mean DPD values for Q1-Q4 were 41.2, 33.0, 41.6, and 35.99 nmol/l, respectively.

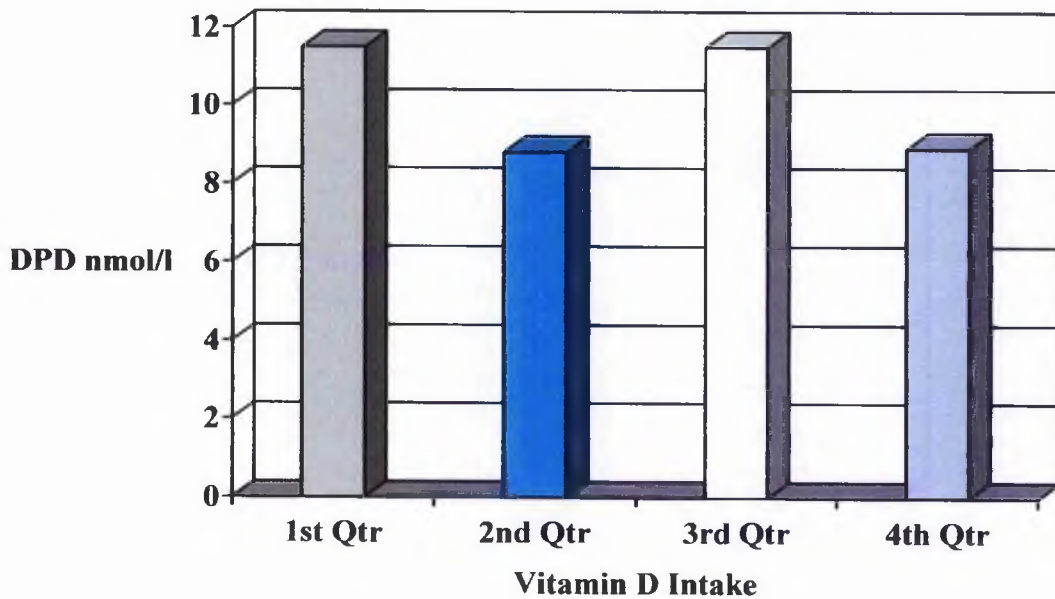


Figure 6.12: Effect of vitamin D intake by quartile on DPD excretion in postmenopausal women. Mean DPD values for Q1-Q4 were 11.4, 8.87, 11.5, and 8.9 nmol/l, respectively.

6.3.6. Quartile of macro and micronutrient intake: effect on bone density and bone metabolism of premenopausal women

Intakes of proteins, fibre, iron, vitamin C, vitamin D, phosphorus, calcium and potassium were developed into quartiles of intake. The data were examined to see if any differences were found in BMD (as assessed by DXA), bone ultrasound (as assessed by BUA) and bone turnover (as assessed by DPD, PYD, OC, BSAP). Protein intake had no effect on bone mass or bone turnover. There was a positive trend between calcium intake and VOS. i.e. a higher intake of calcium resulted in high VOS. As shown in Figure (6.13) those in the lower quartile of calcium intake (1st Qtr) had the lowest VOS concentration. Linearity between quartiles was non-significant ($P < 0.19$). A significant difference among all quartiles ($P < 0.01$) was found. There was a negative trend of Ca intake with bone resorption excretion; higher intake of Ca results in lower PYD excretion. As shown in Figure (6.14) those in the lower quartile of Calcium intake (1st Qtr) had significantly higher PYD excretion. Linearity among all quartiles was significant ($P < 0.01$). Non-significant differences were found between quartiles ($P < 0.4$).

Vitamin C intake was positively associated (non-significant) with BUA; higher intake of vitamin C resulted in higher bone mass at the calcaneal site. As shown in Figure (6.15) those in the lower quartile of vitamin C intake (1st Qtr) had (non-significantly). Lower bone mass at the calcaneal site. Linearity among all quartiles was Non-significant ($P < 0.09$). Non-significant difference was found between quartiles ($P < 0.27$) differences reviewed the same after adjustment for age, weight and height.

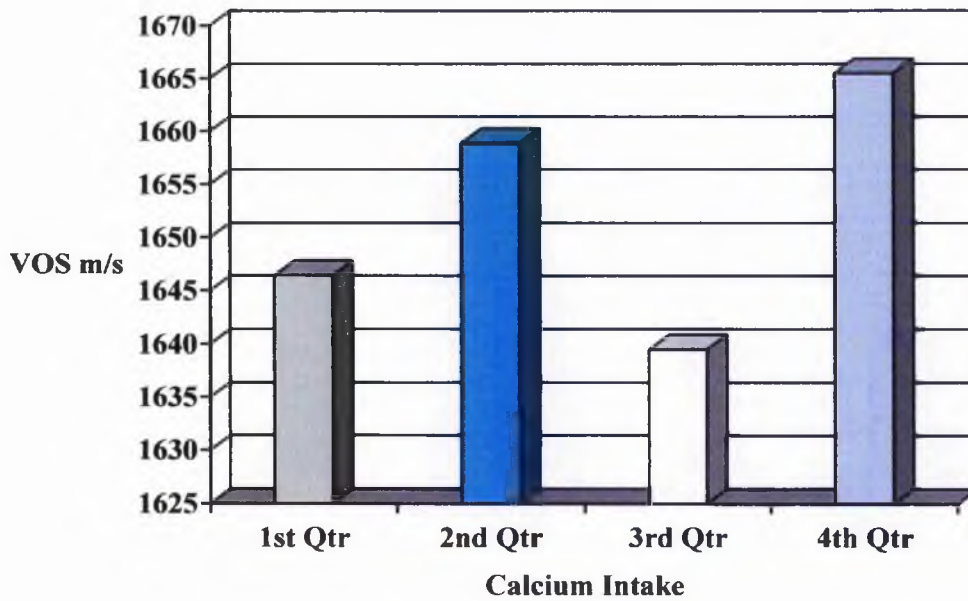


Figure 6.13: Effect of calcium intake by quartile on VOS in premenopausal women. Mean VOS values for Q1-Q4 were 1646.41, 1658.88, 1639.500, and 1665.47 m/s, respectively.

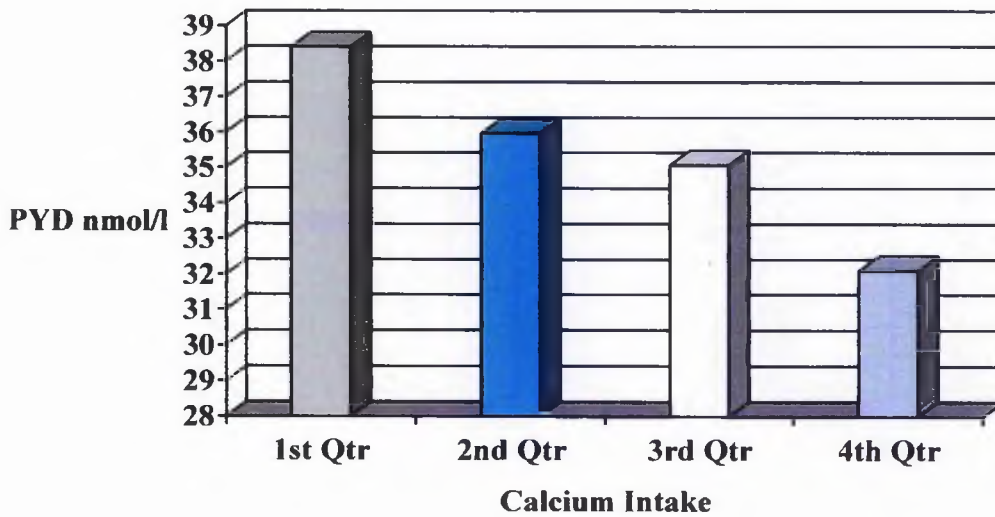


Figure 6.14: Effect of calcium intake by quartile on PYD excretion in premenopausal women. Mean PYD values for Q1-Q4 were 38.40, 35.97, 35.07, and 32.11 nmol/l, respectively.

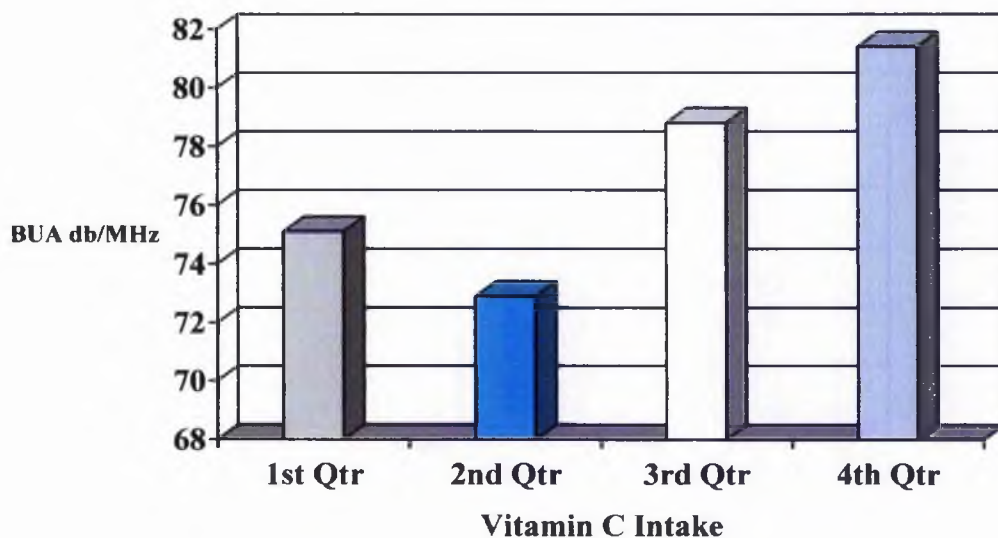


Figure 6.15: Effect of vitamin C intake by quartile on BUA (db/MHz) in premenopausal women. Mean BUA values for Q1-Q4 were 75.1, 72.9, 78.8, and 81.4 MbHz, respectively.

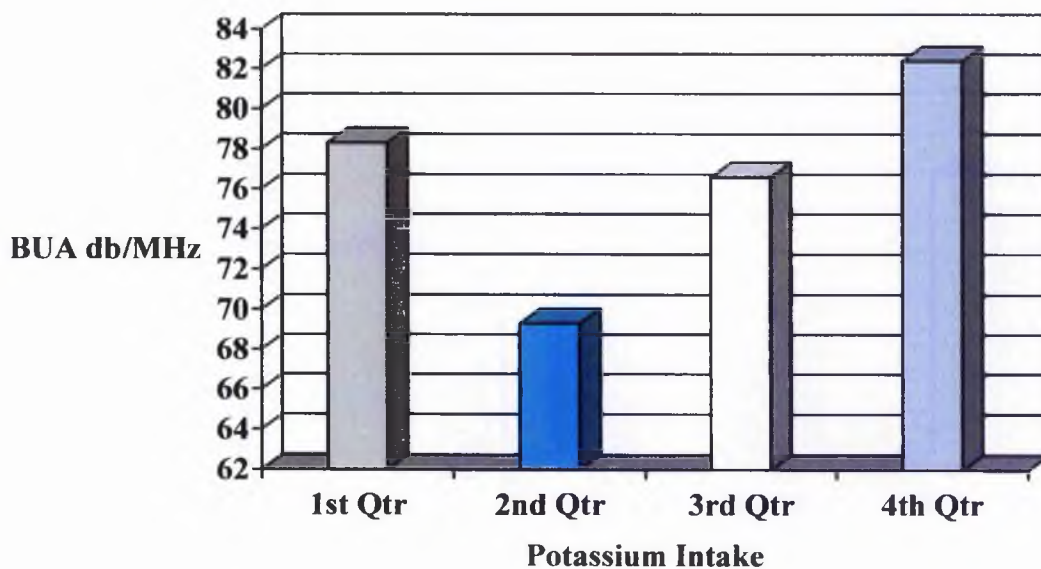


Figure 6.16: Effect of potassium intake by quartile on BUA(db/MHz) in premenopausal Women. Mean BUA values for Q1-Q4 were 78.3, 69.3, 76.6, and 82.4 MbHz, respectively.

Potassium intake was positively associated with BUA; higher intake of K resulted in higher bone mass at the calcaneal site. As shown in Figure (6.16) those in the lower quartile of K intake (1st Qtr) had (significant) lower bone mass at the calcaneal site. Linearity among all quartiles was significant ($P < 0.02$) and significant difference was found between quartiles ($P < 0.02$). A negative trend was found with phosphorus intake and BSAP excretion; that is, a higher P intake was non-significantly associated with lower bone turnover. As shown in Figure (6.17) those in the lower quartile of P intake (1st Qtr) had (non-significantly) higher BSAP excretion. Linearity among all quartiles was non-significant ($P < 0.16$). Non-Significant difference was found between quartiles ($P < 0.5$).

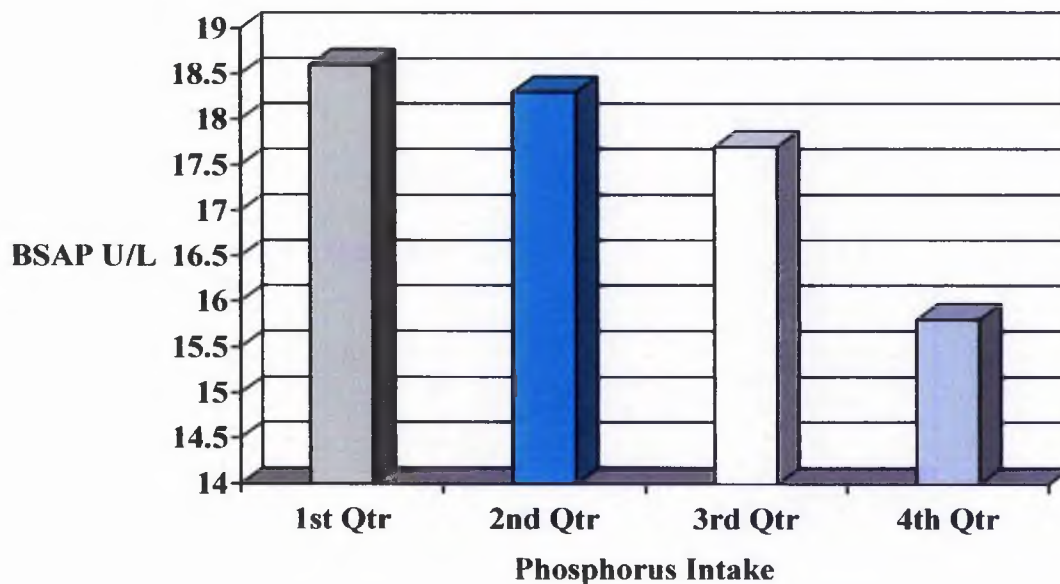


Figure 6.17: Effect of phosphorus intake by quartile on BSAP excretion in premenopausal women. Mean BSAP values for Q1-Q4 were 18.6, 18.3, 17.7, and 15.8 U/L, respectively.

Vitamin D intake was associated negatively with BSAP and OC excretion; that is the higher vitamin D intake resulted in lower bone formation. As shown in Figure (6.18, 6.19) those in the lower quartile of vitamin D intake (1st Qtr) had higher BSAP and OC excretion. Linearity among all quartiles was significant ($P < 0.05$, $P < 0.06$) respectively. Non-significant difference was found between quartiles ($P < 0.10$, $P < 0.16$) respectively. A positive association was found between vitamin D intake and BUA; a higher intake of vitamin D resulted in higher bone mass at the calcaneal sit. As shown in Figure (6.20) those in the lower quartile of vitamin D intake (1st Qtr) had (non-significantly) lower bone mass at the calcaneal site. Linearity among all quartiles was Non-significant ($P < 0.07$). Non-significant difference was found between quartiles ($P < 0.3$).

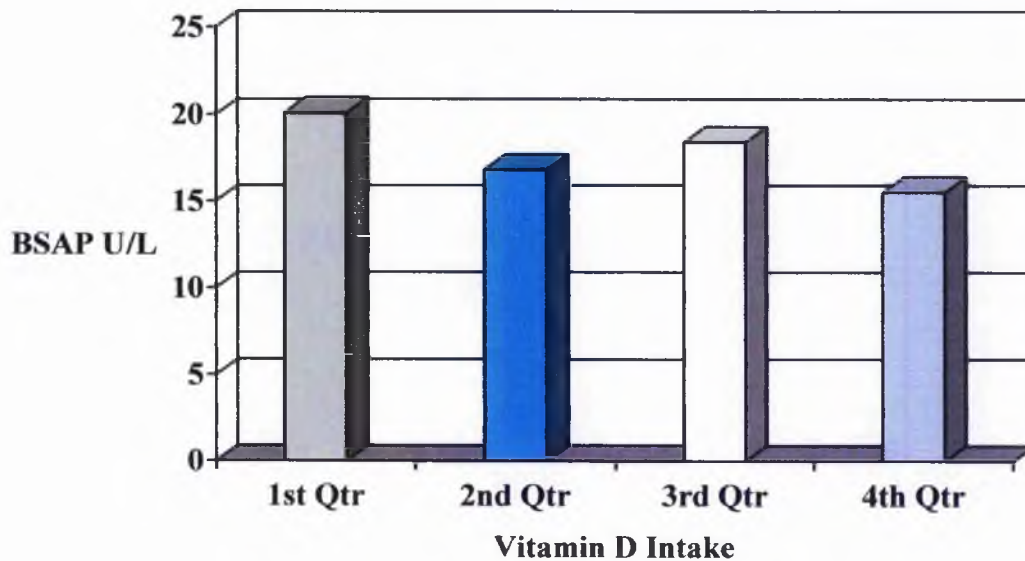


Figure 6.18: Effect of Vitamin D intake by quartile on BSAP excretion in premenopausal women. Mean BSAP values for Q1-Q4 were 20.1, 16.8, 18.4, and 15.5 U/L, respectively.

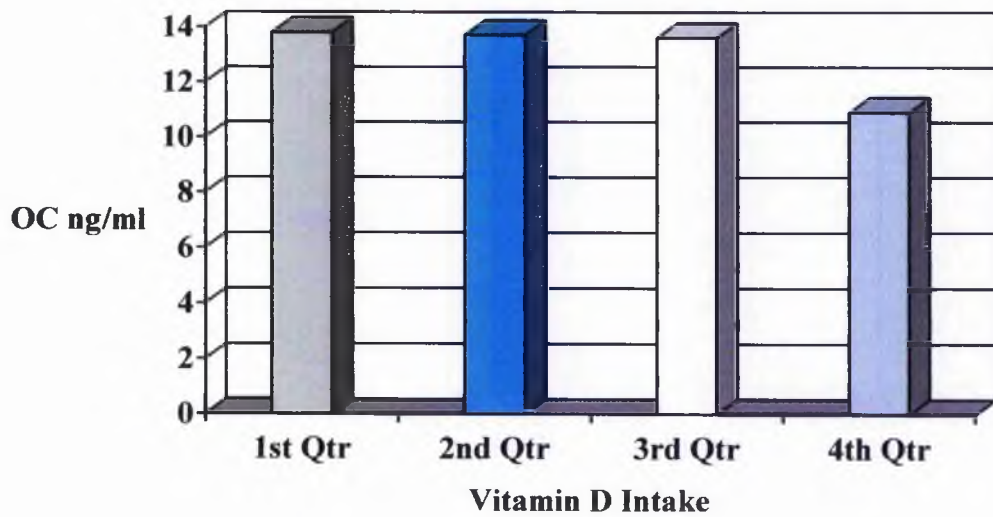


Figure 6.19: Effect of Vitamin D intake by quartile on OC excretion in premenopausal women. Mean OC values for Q1-Q4 were 13.8, 13.7, 13.6, and 10.9 ng/ml, respectively.

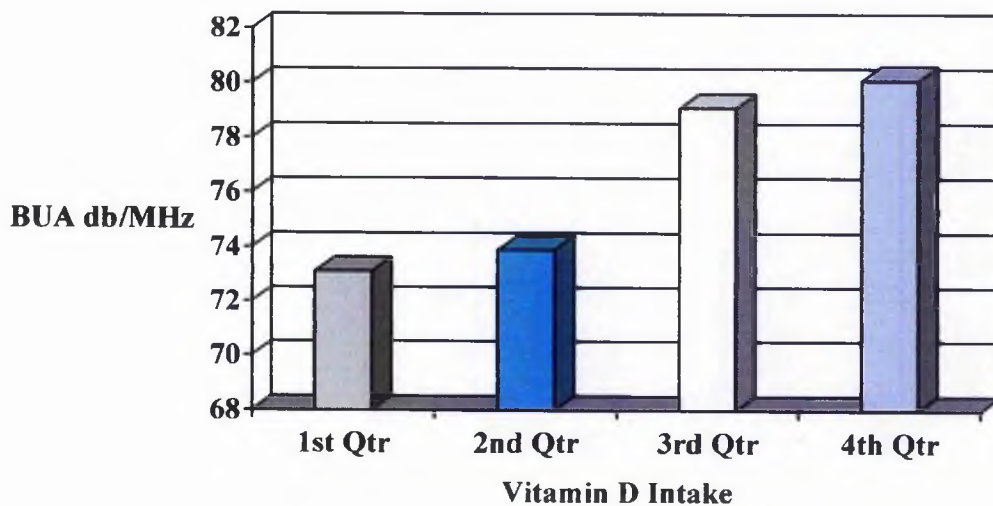


Figure 6.20: Effect of Vitamin D intake by quartile on BUA(db/MHz) in premenopausal women. Mean BUA values for Q1-Q4 were 73.1, 73.9, 79.1, and 80.1 MbHz, respectively.

6.3.7. Quartile of Fruit and vegetable intake: effect on bone density and bone metabolism of postmenopausal women

When we examined the data for postmenopausal women, there was evidence of an association between fruit and vegetable intake and bone formation markers. A significant negative association with BSAP and negative trend (non-significant) with OC ($P < 0.02$, $P < 0.2$) respectively; higher fruit and vegetable intakes were associated with BSAP and OC excretions. Data were divided into quartiles of intake. As shown in Figure (6.21, 6.22) those in the lower quartile of fruit and vegetable intake (1st Qtr) had higher BSAP and OC excretion. Linearity among all quartiles of BSAP was significant ($P < 0.04$) and for OC non-significant ($P < 0.09$). Non-Significant difference was found between BSAP and OC quartiles ($P < 0.2$, $P < 0.2$) respectively.

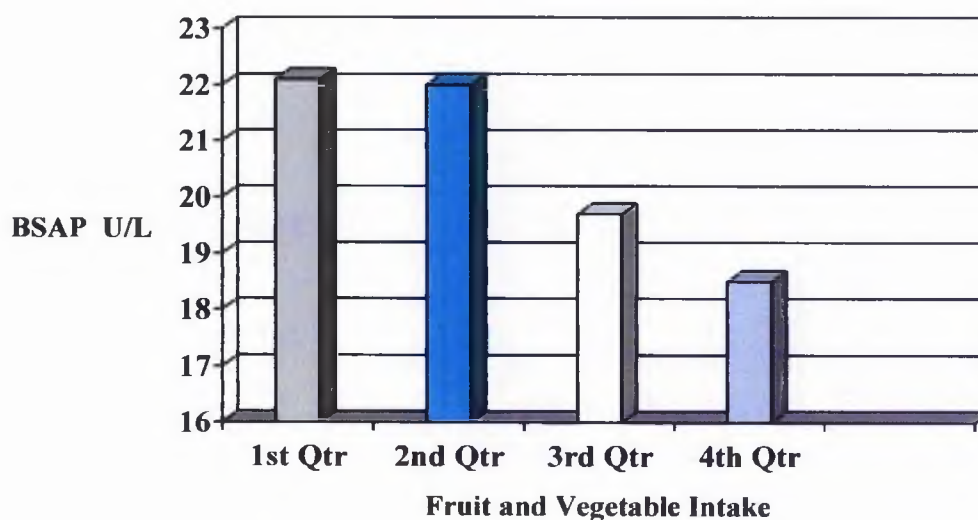


Figure 6.21: Impact of fruit and vegetable intake by quartile on BSAP excretion in postmenopausal women: Mean BSAP values for Q1-Q4 were 22.1, 22.0, 19.7, and 18.5 U/L, respectively.

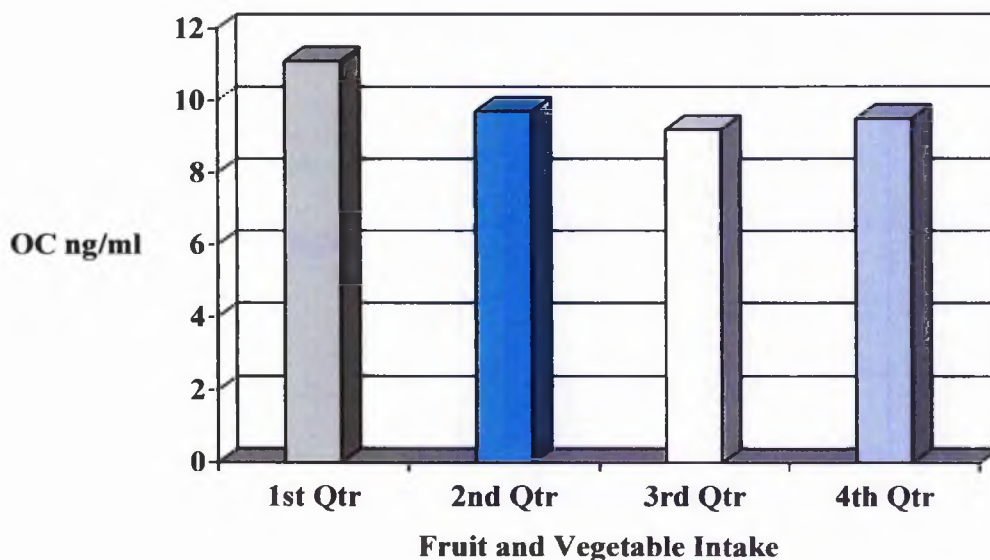


Figure 6.22: Impact of fruit and vegetable intake by quartile on OC excretion in postmenopausal women: Mean OC values for Q1-Q4 were 11.1, 9.7, 9.2, and 9.5 U/L, respectively.

6.3.8. Quartile of Fruit and vegetable intake: effect on bone density and bone metabolism of premenopausal women

Data for premenopausal women had been examined for an evidence of an association between fruit and vegetable intakes and BMD, BUA and bone turnover. There was a positive association between fruit and vegetable intakes and bone mass at the calcaneal site. Higher fruit and vegetable intakes resulted in higher the BUA. Data were developed into quartiles of intakes. As shown in Figure (6.23) those in the lower quartile of fruit and vegetable intake (1st Qtr) had lower BUA. Linearity among all quartiles of BUA was significant ($P < 0.02$). Significant difference was found between quartiles ($P < 0.01$) differences reviewed the same after adjustment for age, weight and height.

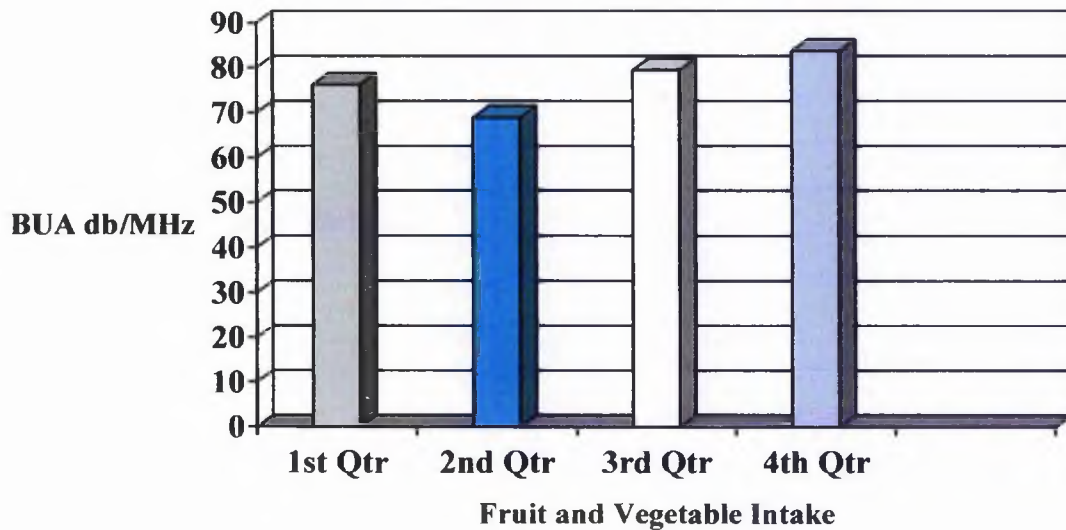


Figure 6.23: Impact of fruit and vegetable intake by quartile on BUA (db/MHz) in premenopausal women: Mean BUA values for Q1-Q4 were 76.2, 68.9, 79.5, and 83.7 MbHz, respectively.

6.3.9. Net endogenous acid production NEAP: effect on bone density and bone metabolism of postmenopausal women

Data were examined for any association existed between NEAP and bone mass/ bone metabolism in postmenopausal women. There was a negative (non significant) trend for NEAP and lumbar spine BMD ($P < 0.153$). Positive trend were found between NEAP and bone turnover. There was (non-significant) association between NEAP and DPD and BSAP and OC excretion. Lower estimates of NEAP were significantly associated with lower excretion of OC ($P < 0.05$), and a similar but (non-significant) trend was seen with DPD and BSAP ($P < 0.15$, $P < 0.09$) respectively.

6.3.10. Net endogenous acid production NEAP: effect on bone density and bone metabolism of premenopausal women

Data were examined for any association existed between NEAP and bone mass/ bone metabolism in postmenopausal women. NEAP estimate did not appear to be associated with bone mass in this population. There was a negative association between NEAP and markers of bone resorption. The lower the NEAP the higher the PYD excretion ($P < 0.05$).

6.4. Discussion

In the present study subjects were divided into three groups, non-milk group (NMG), low milk group (LMG <1 pint/d = 240-300 ml/d) and high milk group (HMG >1 pint/d = 480-500 ml/d) (Netzer, 1997) for both pre and postmenopausal women. According to this category, the quantity of milk intakes by our subjects was much lower than the quantity consumed by UK women (New *et al.*, 2000).

Evidence indicates that consumption of an adequate intake of calcium or calcium rich foods such as milk and dairy products helps to optimize peak bone mass by positively influence bone mineralization during growth and that the mechanism for its action is likely due to a suppression of bone remodeling which is achieved by age 30 or earlier (Chan *et al.*, 1995; Cadogan *et al.*, 1997; Bonjour *et al.*, 1997). In a calcium supplementation trial, calcium influenced lumbar spine BMD in late menopausal women (Dawson-Hughes *et al.*, 1990). In our study milk was not a significant predictor of absolute bone mineral density of lumbar spine or femoral neck. In our post and premenopausal women the results showed that there were no differences between none, low, and high milk consumer, concerning BMD, which is similar with results from other studies (Earnshaw *et al.*, 1997; Macdonald *et al.*, 2004).

Analysis of data obtained from three-day food diaries intakes between the three study groups strongly emphasized the significant differences between those three groups. Our results have shown a trend for lower bone ultrasound attenuation BUA with low milk consumption. These results are agreed with the evidence from recent study on adolescents, which have found that a high calcium intake in the form of dairy products had the greatest influence on BUA (Novotny *et al.*, 2004). The

results also shown that BUA t-score in high milk group is significantly greater than none and low milk groups, which are agreed with several previous studies (Tucker *et al.*, 1999; Eastell and Lambert, 2002). A comparison of the mean BUA t-scores of each groups revealed that the mean BUA t-scores for the NMG were lower than the BUA t-scores for the other two groups in both the premenopausal and postmenopausal women.

The markers of bone turnover between the three milk consumption groups have shown significant differences. Excretion of deoxypyridinoline (DPD) is greater in the non-milk group compared with low and high milk groups. indicative of a high bone resorption in this group, which is confirmed the important role of milk intakes as a good source for calcium to maintain the integrity and health of bones in postmenopausal women. In premenopausal women, excretion of PYD exhibited significant differences between the none milk and low milk groups comparing with high milk group, which they having greater bone resorption compared with high milk group due to insufficiency of milk intakes and number of servings per day. These results suggest that milk has an antiresorptive effect on bone in both postmenopausal and premenopausal women. In contrast, the bone formation markers BSAP revealed no significant differences between the three groups, which is inconsistency to the previous studies (Hasling *et al.*, 1990; Dawson-Hughes *et al.*, 1990; Prince *et al.*, 1995).

Nutrients intake such as proteins, fiber, vitamin C, vitamin D, phosphorus, calcium, and potassium were assessed by three days food diaries, but the three milk groups were assessed by food-frequency questionnaire questioner (FFQ). In postmenopausal and premenopausal women we verify the milk intake data by looking at differences in Ca consumption between the three milk groups. Our

finding regarding calcium in our population of postmenopausal and premenopausal women was interesting. Our failure to see an association between calcium and bone mass / quality / turnover in postmenopausal women, while in premenopausal women there was a positive non-significant association between calcium intake and bone quality (VOS) and a negative non-significant association with bone resorption (PYD) which is similar to other interventional study that demonstrated that calcium supplementation reduced serum OC and free pyridinoline PYD (Riggs *et al.*, 1998).

Phosphorus is the second important mineral for bone (Matkovic *et al.*, 2003). Our results showed a negative trend between phosphorus intake and lumber spine BMD in postmenopausal women. This finding is in agreement with Metz and Hoppe studies (Metz *et al.*, 1993; Hoppe *et al.*, 2000). While in premenopausal women the higher phosphorus intake was positively non-significantly associated with bone quality (VOS) and negatively with bone resorption markers (PYD). Which is similar to other studies investigating the influence of dietary phosphorus intake on bone mass support a positive association (New *et al.*, 1997; Tucker *et al.*, 1999). Grimm study supports the view that the women with adequate calcium supply, a high phosphorus intake dose not detrimental to bone health (Grimm *et al.*, 2001). Although phosphorus is an essential nutrient, there is a concern that high intakes of phosphorus might be detrimental to the bone (Calvo, 1994). For example a rise in dietary phosphorus increases serum phosphorus concentration, producing a transient fall in serum ionized Ca resulting in elevated parathyroid hormone (PTH) secretion and potentially bone resorption.

Our data result of postmenopausal women found a negative trend between potassium intake and DPD excretion demonstrated the promotion of renal calcium retention that the low potassium intake increases daily and fasting urinary

calcium excretion rate (Lemann *et al.*, 1993). Therefore, the low potassium intake effects calcium balances increased urinary DPD and hydroxyproline excretion and decrease serum osteocalcin concentration (Tucker *et al.*, 1999). While our premenopausal group of women, there was a positive non-significant link between potassium intake and BMD at the FN and FT sites and BUA at the calcaneal, which is similar to study of New *et al.* which they reported greater BMD in spine, hip and forearm in premenopausal women (New *et al.*, 2000).

Findings also showed a negative correlation between iron intakes and bone resorption marker DPD excretion among the postmenopausal women. In premenopausal women a positive non-significant association between iron intake and hip BMD and heel BUA. Negative trends were found with bone formation markers (BSAP and OC) excretions. These findings were in agreement with previous study suggesting that iron deficiency may play a role in bone fragility (Medeiros *et al.*, 1997). Another study examined the relation between bone mass and iron in adolescent girls there was a trend for a positive association between BMD and serum ferritin (Ilich-Ernst *et al.*, 1998).

Suboptimal vitamin C intakes may adversely affect the bone formation and degradation process, either by decreasing the rate of osteoblast differentiation or through the synthesis of abnormal collagen fibers which may be degraded before reaching maturity (Munday, 2003). Our results in postmenopausal women showed that lower Vitamin C intake correlate with higher excretion level of markers of bone formation (BSAP). While in our premenopausal group we found a trend for a positive association between bone mass at the calcaneal site (BUA and VOS) and vitamin C intakes. This finding is similar to Kaptoge *et al.* that suggested that low intake of vitamin C was associated with faster rate of BMD loss (Kaptoge *et al.*,

2003). Vitamin C, which is antioxidant vitamin along with others, may serve to protect the skeleton from the oxidative stress that increase free radical generation and bone resorption. In a recent study of women, high intakes of vitamin E and C significantly decreased the odds ratio for hip fracture (Ilich and Kerstetter, 2000).

The negative correlation between vitamin D intake and DPD, (postmenopausal women) BSAP, and OC excretion (premenopausal women) do however confirm the importance of vitamin D on bone health. This finding is in a agreement with a recent studies showed that Low serum vitamin D leads to secondary hyperparathyroidism, increased bone turnover, reflected by an increase in bone resorption and bone formation markers. (Gannage *et al.*, 2000; Reginster, 2005). Studies indicate that serum vitamin D makes a contribution to BMD a lumbar spine and neck of femur (Collins *et al.*, 1998; Ooms *et al.*, 1995; Chapuy *et al.*, 2002). Our results showed that BUA reflected the positive effect on bone of vitamin D intakes in premenopausal women, which is similar to those of vitamin D and calcium supplementation in a population of elderly women (Krieg *et al.*, 1999).

In our present study the results showed no trend for protein intake and lumbar spine BMD of postmenopausal and premenopausal we do not find support for the hypothesis that high protein consumed induced hypercalciuria and result in high bone resorption (Chan and Swaminathan, 1994; Lemann, 1999). Clinical intervention trials support this hypothesis and report an increase in bone resorption (Kerstetter *et al.*, 1999). Other studies report higher fractures at group consuming a high protein diet (Abelow *et al.*, 1992; Meyer *et al.*, 1997). In contrast, a cross-sectional and longitudinally studies showed a positive correlation between protein intake and bone mass in post and premenopausal women (Teegarden *et al.*, 1998; Dawson-Hughes and Harris, 2002). On the other hand numerous studies, have not

find any relationship between bone mineral mass and protein intake (Henderson *et al.*, 1995; New *et al.*, 1997).

Examination of fruit and vegetable intake in postmenopausal women has proved a correlation with bone formation markers. Higher intake of fruit and vegetable shows a significant negative association with BSAP and negative trend with OC. Several previous studies showed similar findings to our results (Tucker *et al.*, 1999; New *et al.*, 1997; Wachman and Bernstein, 1968). Data had been developed into quartiles of intakes emphasized that women at the lower quartile of fruit and vegetable intake (1st Qtr) had higher BSAP and OC excretion. In our premenopausal women the data have shown a positive correlation between fruit and vegetable intakes and bone density / bone metabolism in which higher fruit and vegetable intakes resulted in higher BUA at the calcaneal site. Data has been developed into quartiles of intakes confirmed the finding, lower quartile of fruit and vegetable intake (1st Qtr) had lower BUA. Fruit and vegetable are alkaline-producing foods (which is, the excretion of renal base exceeds excretion of acid), and long-term ingestion may have a beneficial protective effect on bone (Lemann *et al.*, 1993). Recent studies showed the greater intake of fruit and vegetable the higher the bone mineral density and the less bone loss (Hannan and Tucker, 2003; Macdonald *et al.*, 2004).

The effect of net endogenous acid production (NEAP) on bone density and bone metabolism in postmenopausal women showed a negative non significant trend with lumber spine BMD and a positive trend with bone turnover markers, which is indicate that diet with lower protein content but higher potassium content gives us lower estimates of NEAP, are significantly associated with greater BMD and lower bone resorption. These data are in agreement with a study shows that women with

the lowest estimate of NEAP were having lower pyridinoline and deoxypyridinoline secretion. These data indicate that diets that are characterized by less dietary acid are associated with a better indices of bone (Macdonald *et al.*, 2002). Another recent study by New et al showed that a link between NEAP and skeletal integrity (New *et al.*, 2004). In our premenopausal women estimate of NEAP did not show any association with bone mass and there was a negative association with bone resorption markers PYD.

CHAPTER 7

Extent of vitamin D insufficiency in Saudi Arabian women: effect on indices of bone health

7.1. Introduction

Vitamin D maintains extracellular fluid concentrations of calcium and phosphorus within normal range. Vitamin D is required to increase the efficiency of the small intestine to absorb dietary calcium which is essential for mineralization of bone. Reduction in vitamin D levels is associated with impaired calcium absorption and a compensatory increase in the level of parathyroid hormone (PTH), which stimulates bone resorption and bone loss.

7.1.1. Vitamin D status

The 25(OH) D is the principle circulating vitamin D metabolite. Its concentration in the serum is considered as an accurate assessment of vitamin D nutritional status, which reflects a dietary intake and cutaneous production (Parfitt, 1998). Serum levels of 25 (OH) D reflect both excess and deficiency states.

Serum PTH concentrations are inversely related to 25 (OH) D serum levels (Lips *et al.*, 1988). Therefore, the serum PTH concentration, in conjunction with 25(OH) D level, make valuable indicator of vitamin D status. Many studies suggested that there is a value of 25(OH) D above that there is little decrease in PTH. In assessing vitamin D status, studies have suggested that the value of 25(OH)D that considered normal range could be regarded as optimal. This has been addressed by (Malabanan *et al.*, 1998) who demonstrated that the serum 25(OH) D level minimizing PTH secretion is much higher than the “ classical” threshold of 30nmol/liter (12ng/ml).

Some cross-sectional studies relating PTH levels to 25(OH)D levels suggest that 25(OH)D concentrations as high as 100 nmol/liter may be necessary to minimize serum PTH (Reid, 1998). However, the threshold concentration of

25(OH)D that delimits deficiency from sufficiency, i.e. the lowest concentration defining the plateau level of PTH, differs between studies and ranging from 20 nmol/liter (8 ng/ml) to >100 nmol/liter (40 ng/ml) (Gloth *et al.*, 1995; Dawson-Hughes *et al.*, 1997). Recent studies have shown with increasing 25(OH) D a continuous decline in PTH and no plateau (Kudlacek *et al.*, 2003; Bates *et al.*, 2003). Other studies have been proposed a gradual scale in which vitamin D deficiency as a 25 (OH) D concentration of <25 nmol/liter (10 ng/ml), vitamin D insufficiency as a 25(OH)D concentration of <50 nmol/liter (20 ng/ml) and hypovitaminosis D is defined as a 25 (OH) D concentration of <100 nmol/liter (40ng/ml) (McKenna and Freaney, 1998).

7.1.2. Vitamin D deficiency / insufficiency

Vitamin D deficiency results in transient hypocalcaemia that causes secondary hyperparathyroidism and mild hypophosphatemia. This ultimately induces inhibition of the primary mineralization of bone matrix (Meunier, 2001). It is a major risk factor for bone loss and fracture. Vitamin D deficiency was recognized as a cause of rickets in children and osteomalacia in adults.

Vitamin D insufficiency is the preclinical phase of vitamin D deficiency that increases the risk of osteoporotic fractures (Chapuy *et al.*, 1992). It induces a secondary hyperparathyroidism without mineralization defect and accelerates bone loss.

Vitamin D sufficiency do not induces effect on calcium homeostasis (Meunier, 2001).

7.1.3. Influence of vitamin D status on bone health

Vitamin D deficiency results in a fall in ionized calcium and secondary hyperparathyroidism. The effect of high level of PTH secretion is to restore normal calcium through increasing intestinal calcium absorption, renal calcium retention and bone resorption. The consequences of these changes is inhibition of mineralization at the growth plate and impair of mineralization of performed osteoid at sites of bone remodelling on the trabecular bone surface. Secondary hyperparathyroidism causes increase in bone resorption result in thinning of the trabecular and cortical bone (Pettifor, 2003).

7.1.4. Study aim

The vitamin D nutrition status of Saudi premenopausal and postmenopausal women is unclear. The objective of this study was to determine the prevalence of vitamin D deficiency / insufficiency and to examine associated risk factors for vitamin D deficiency in those groups of women. This study assessed the serum concentrations of 25-hydroxyvitamin D as an index of vitamin D nutrition status in premenopausal women in comparison with those in postmenopausal women and investigated the influence of vitamin D status on indices of bone health.

7.2 Study Design

7.2.1. Subject selection

Serum of 25-hydroxyvitamin D [25(OH) D], 1,25-dihydroxyvitamin D [1,25(OH)₂ D], intact parathyroid hormone (PTH), and ionized calcium and phosphorus were measured in 101 premenopausal women aged 20-30 years and 112 postmenopausal women aged 45-60 years who were participating in this study. The exclusion criteria used for the study are shown in Table 2.1.

7.2.2. Biological samples collection

Morning fasting blood and second morning void urine samples were collected from every woman (section 2.5) for the measurement of the following parameters: serum calcium, phosphorus, 25 (OH)D, 1,25 (OH)D and parathyroid hormone (PTH).

7.2.3. Analytical methods for assessment of vitamin D metabolite and PTH

Serum 25-OH vitamin D was measured by two methods a competitive binding protein assay (CPBA) and high-pressure liquid chromatography (HPLC) with ultraviolet quantification (Section 2.5). The first method is based on the competition of 25-OH Vit D present in the sample with biotinylated 25-OH Vit D (tracer) for the binding pocket of vitamin D binding protein (VDBP, Gc-globulin). This method is described in (Section 2.7.1). The second methods involves the use of a tritiated recovery standard, details is described in (Section 2.7.2).

Serum 1,25-dihydroxyvitamin D was measured by a competitive enzyme immuno assay (EIA), which is based on the competition between 1,25-dihydroxyvitamin D present in the sample with labeled 1,25-dihydroxyvitamin D tracer for the binding site of the vitamin D specific antibody. This method described in (Section 2.7.3).

Measurement of Intact parathyroid hormone (PTH) was performed by a two-site enzyme-linked immunosorbent assay [ELISA] with two different polyclonal antibodies for different regions on the PTH molecule, details is described in (Section 2.7.4).

7.2.4. Analytical methods for assessment of serum chemistry

Automated clinical chemistry analyzer measured serum chemistry. Measurement of serum calcium based on colorimetric methods with endpoint details is described in (Section 2.7.5). Serum phosphorus analyze by method based on the reaction of phosphate with ammonium molybdate to form ammonium phosphomolybdate that reduced to form molybdenum blue as described in (Section 2.7.6).

7.2.5. Statistical analysis

Statistical analysis was conducted using SPSS software package (version 12; SPSS Inc, Chicago). For all variables descriptive statistics (means, medians, SDs, and ranges) were determined. In tables, data is presented as mean values with their standard deviation and percentages for each group. The paired sample t-test was used to compare means values between the two methods used to analyse vitamin D. Comparisons of means were performed by analysis of variance, adjusting

for weight, height, and physical activity. Pearson coefficient of correlation was used to study the linear correlation between 25(OH) D, PTH, 1,25(OH) D, calcium, phosphorus, and bone turnover markers (PYD, DPD, BSAP, and OC excretion) in postmenopausal and premenopausal women. Results for the effect of 25(OH) D on bone mass (BMD sites, BUA, and VOS) and bone turnover markers presented in five levels are give as mean values and their standard deviation.

7.3 Result

7.3.1. Extent of vitamin D insufficiency / deficiency in postmenopausal women

The prevalence of vitamin D deficiency was examined by using a cutoff of 12 ng/ml. In our postmenopausal women the mean level of serum 25 (OH) D was 9.71 ± 4.44 ng/ml (24.27 ± 11.1 nmol/liter) (Table 7.1). We observe that 74% of our postmenopausal women are vitamin D deficient [serum 25 (OH) D less than 12 ng/ml (30 nmol/liter)] and 26% women are adequate [serum 25 (OH) D higher than 12 ng/ml (30 nmol/liter)]. The relationship between 25(OH) D and serum PTH in this population has been analyzed a significant negative correlation ($r = -0.23$; $P < 0.02$) was found (Fig 7.1). Different levels of serum 25(OH) D were analyzed to find out the percentage of these levels (Table 7.2), most of this population concentrate at 25(OH) D levels between 5-15 ng/ml.

7.3.2. Extent of vitamin D insufficiency / deficiency in premenopausal women

As shown in (Table 7.1) the mean concentration of serum 25 (OH) D was 8.86 ± 3.6 ng/ml (22.15 nmol/liter). A cutoff of vitamin D deficiency was 12 ng/ml. We observe that 82% of our premenopausal women are vitamin D deficient [serum 25 (OH) D less than 12 ng/ml (30 nmol/liter)] and 18 % women are adequate [serum 25 (OH) D higher than 12 ng/ml (30 nmol/liter)]. A significant negative correlation between 25(OH) D and serum PTH ($r = -0.31$; $P < 0.00$) was observed (Fig 7.2). The percentage of different levels of 25(OH) D were calculated (Table 7.2), 66% of this population their 25(OH) D level runs between 5-15 ng/ml.

Table 7.1: Biochemical characteristics in postmenopausal and premenopausal women

	<u>Postmenopausal</u>			<u>Premenopausal</u>		
	Mean±SD	Range	Median	Mean±SD	Range	Median
25 (OH) D (ng /ml)	9.7±4.4	1.3–23.2	9.7	8.86±3.6	2.3-19.2	8.05
1,25 (OH) D (pg / ml)	42.5±14.8	12–87.0	41.0	37.9±12.5	7–73	37.0
PTH (pg / ml)	61.9±34.3	18–204	53.0	39.35±21.4	10–135	35.0

Table 7.2: Percentage values for different levels of 25(OH)D in postmenopausal and premenopausal women

25 (OH) D (ng /ml)	<u>Postmenopausal</u>			<u>Premenopausal</u>		
	N	valid cumulative percent	percent	N	valid cumulative percent	percent
<5	12	13.2	13.2	13	15.5	15.5
5-10	36	39.5	52.7	41	48.8	64.3
10-12	21	23.1	75.8	15	17.8	82.1
12-15	12	13.2	89.0	11	13.1	95.2
>15	10	11.0	100	4	4.8	100

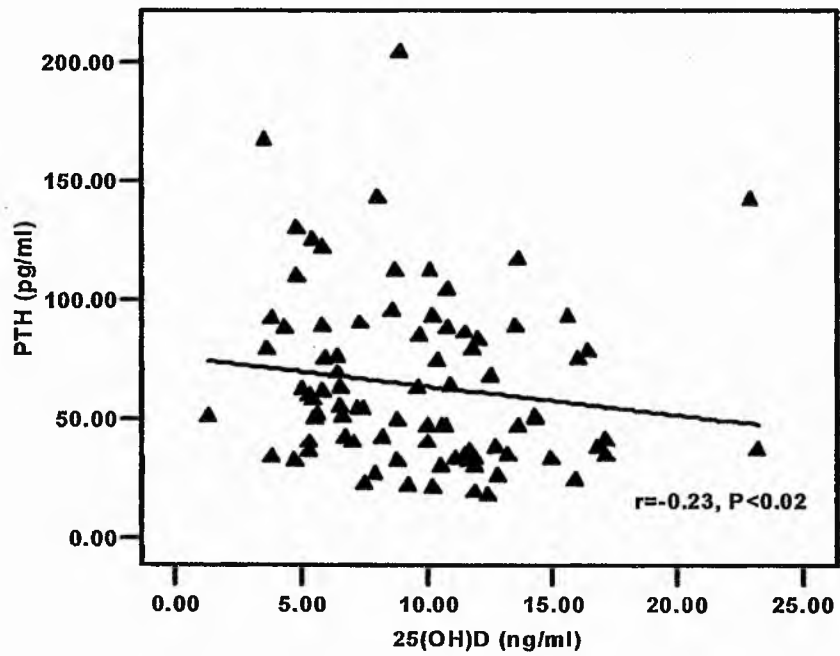


Figure7.1: Relationship between PTH and 25(OH)D in postmenopausal women

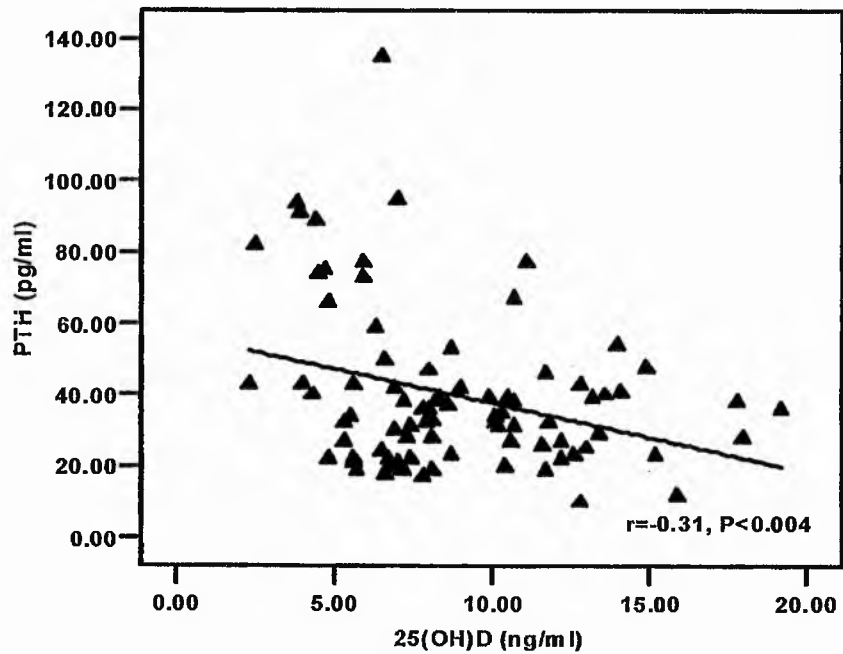


Figure7.2: Relationship between PTH and 25(OH)D in premenopausal women

7.3.3. Comparison between serum 25 (OH) D concentration by two different methods

The serum 25 (OH)D level were analyzed by two different methods, competitive protein binding assay (CPBA) and high performance liquid chromatography (HPLC). There was no significant difference between the two methods (Table 7.3).

Table 7.3: Comparisons between CPBA and HPLC methods in assessing serum 25 (OH) D

	<u>Postmenopausal</u>				<u>Premenopausal</u>			
	Mean±SD	Median	Range	P value	Mean±SD	Median	Range	P value
25 (OH) D (ng /ml) CPBA	8.5±7.3	7.5	(0.5-39.9)	P<0.11	8.6±7.2	6.6	(1.0-43.0)	P<0.7
25 (OH) D (ng /ml) HPLC	9.7±4.4	9.7	(1.3-23.2)	P<0.11	8.8±3.6	8.0	(2.3-19.2)	P<0.7

7.3.4. Relationship between serum PTH, 25(OH)D and 1,25(OH)₂ D in postmenopausal women

In analyzing the relationship between vitamin D status and serum PTH in our population, showed that in postmenopausal group 25(OH) D was significantly inversely correlated to PTH ($r = -0.23$; $P < 0.02$), while 1,25(OH)₂D was significantly positively associated with PTH ($r = 0.21$; $P < 0.03$). The relationship between PTH and 25(OH)D showed an increase of PTH when 25(OH)D fell below 8 ng/ml (20 nmol/l)(Fig. 7.2).

7.3.5. Relationship between serum PTH, 25(OH)D and 1,25(OH)D in premenopausal women

We analyzed the relationship between vitamin D status and serum PTH in our group of premenopausal women. A significant negative correlation was found between serum intact PTH and serum 25(OH)D values ($r = - 0.31$; $P < 0.004$). Serum PTH held almost a stable level at about 30pg/ml as long as serum 25(OH)D values were above than 7 ng/ml (17.5 nmol/l) (Fig 7.3). Serum 1,25(OH)D were not related to PTH in this group of premenopausal .

7.3.6. 25(OH)D and PTH in relation to biochemical parameters in postmenopausal women

There was a positive trend between 25(OH)D and calcium ($r = 0.19$; $P < 0.06$) as well as with phosphorus ($r = 0.18$; $P < 0.07$). Serum calcium was significantly negatively correlated to PTH ($r = - 0.22$; $P < 0.02$). There was a significant negative association between PTH and phosphorus ($r = - 0.24$; $P < 0.01$).

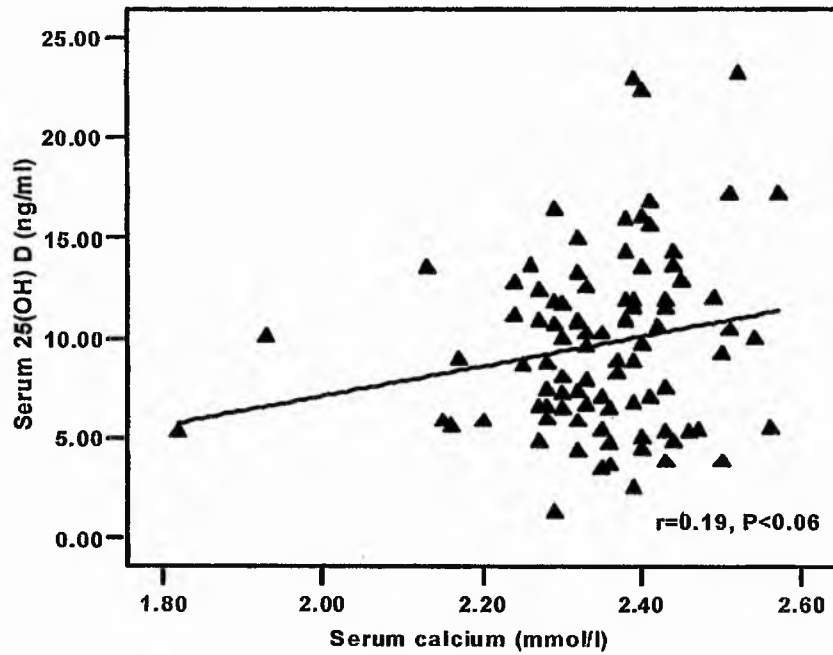


Figure 7.3: Relationship between serum 25(OH)D and serum calcium in postmenopausal women

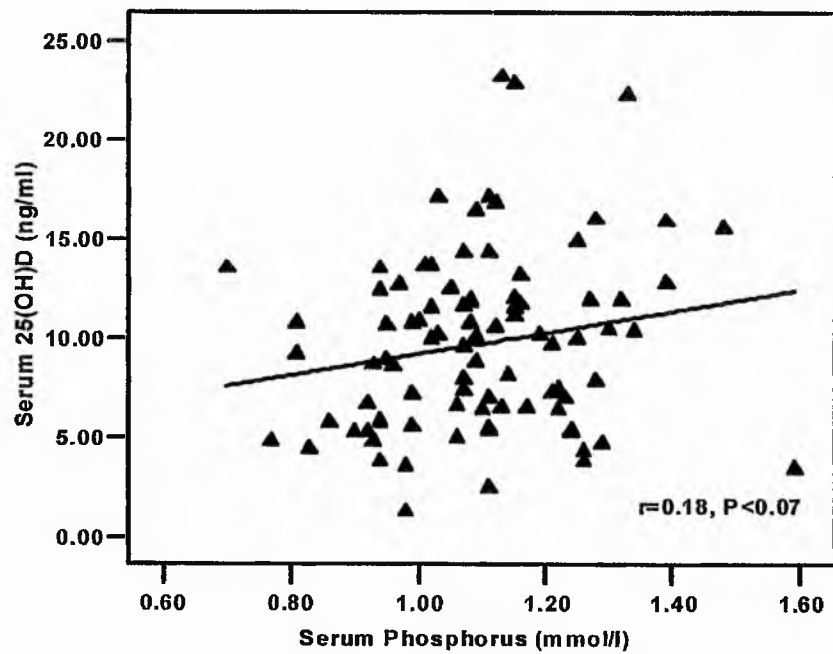


Figure 7.4: Relationship between serum 25(OH) D and serum phosphorus in postmenopausal women

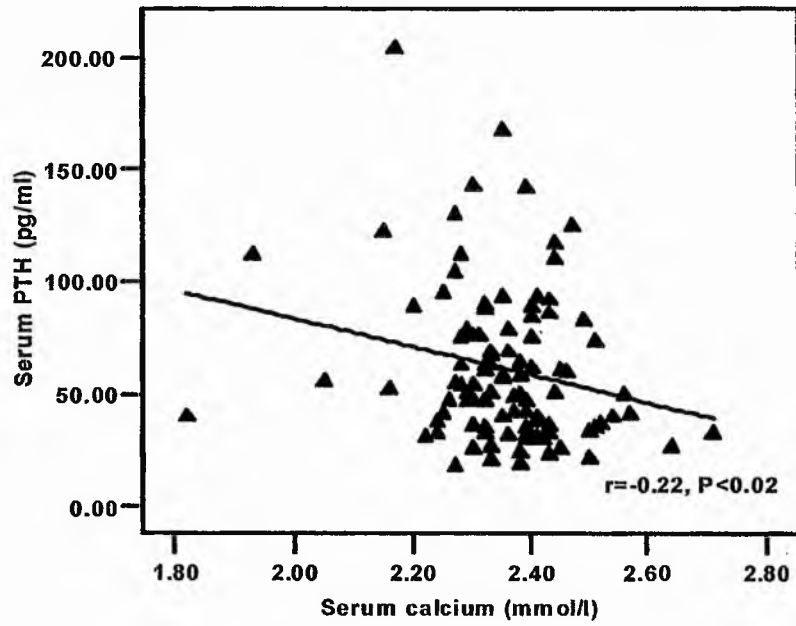


Figure7.5: Relationship between serum PTH and serum calcium in postmenopausal women

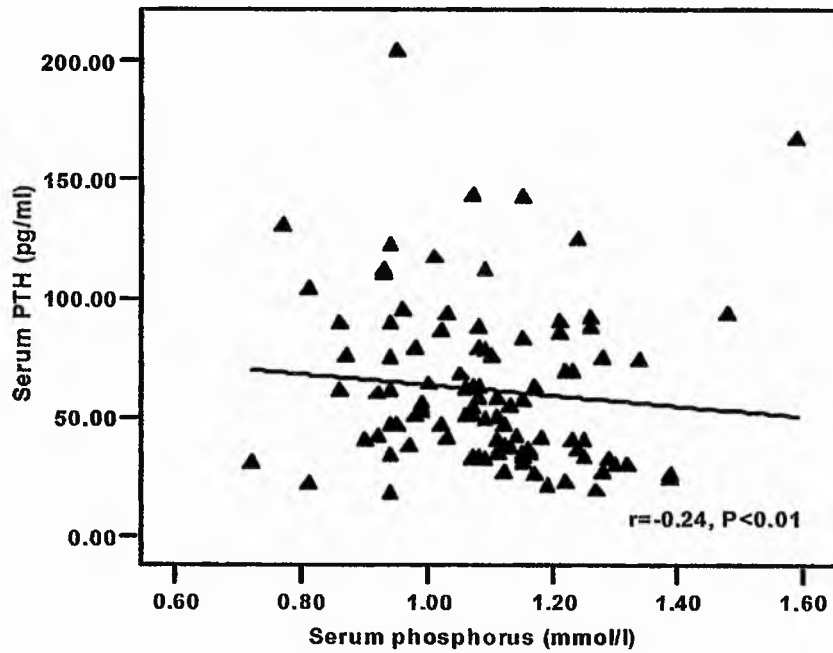


Figure7.6: Relationship between serum PTH and serum phosphorus in postmenopausal women

7.3.7. 25(OH)D and PTH in relation to biochemical parameters in premenopausal women

Data were analyzed for premenopausal women. None of the biochemical parameters were significantly related to 25(OH)D and PTH. Calcium intake (mg/d) was significantly negatively correlated to PTH ($r = -0.24$; $P < 0.05$).

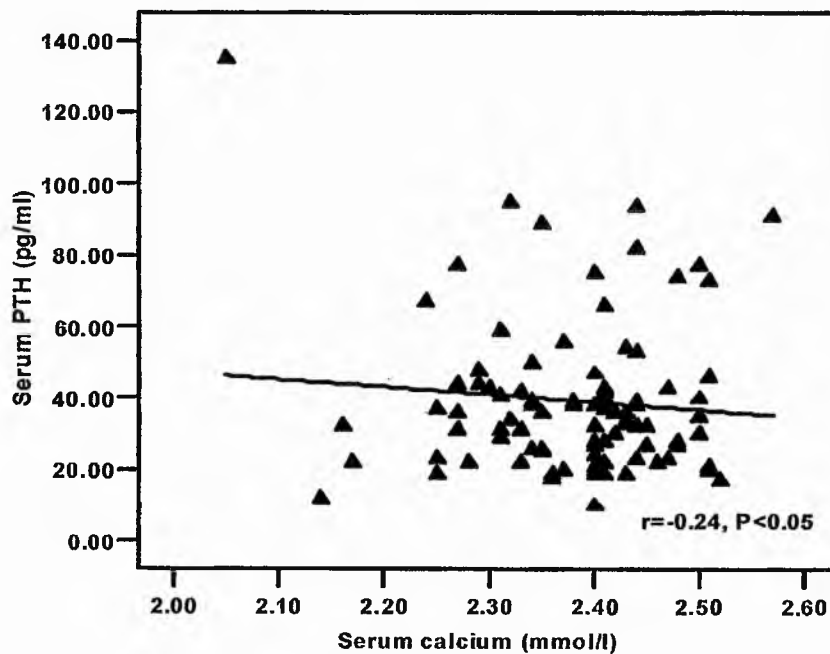


Figure 7.7: Relationship between serum PTH and serum calcium in premenopausal women

7.3.8. Relationship between 25(OH)D and PTH with bone markers in postmenopausal women

The data were examined to see if any association existed between 25(OH)D and PTH with bone resorption (PYD and DPD) / formation (BSAP and OC). We observed that non of the bone markers were associated with 25(OH)D in this group. With PTH we observed a significant positive association with bone formation markers BSAP ($r = 0.21$; $P < 0.03$) and OC ($r = 0.20$; $P < 0.04$).

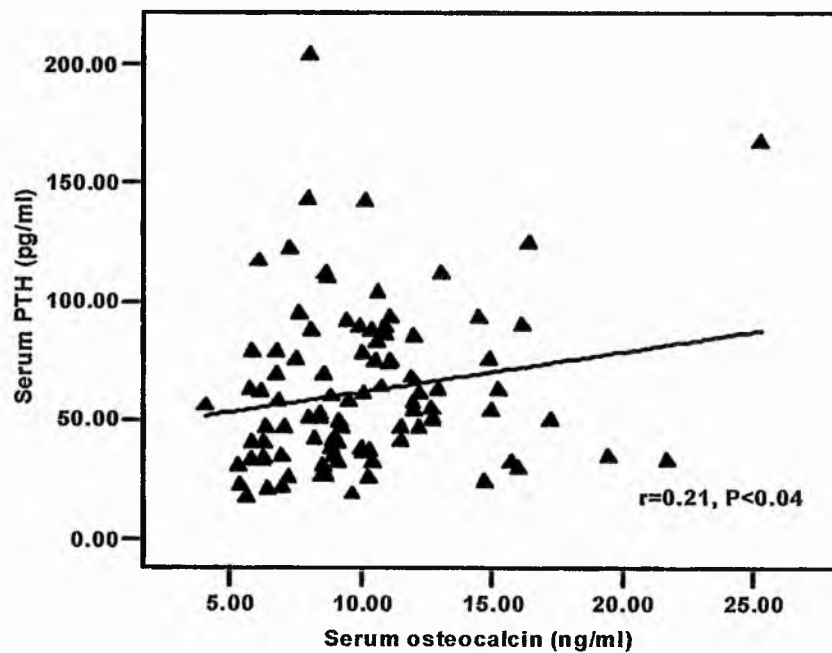


Figure7.8: Relationship between serum PTH and OC in postmenopausal women

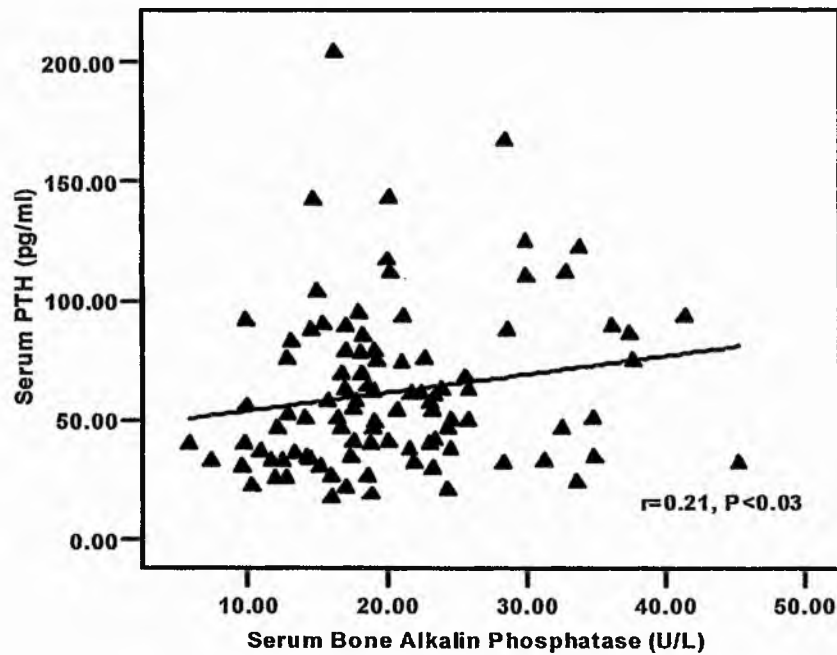


Figure 7.9: Relationship between serum PTH and BSAP in postmenopausal women

7.3.9. Relationship between 25(OH)D, PTH and bone markers in premenopausal women

There was a significant positive association between 25(OH)D and bone resorption marker PYD ($r = 0.24$; $P < 0.03$) and positive trend with DPD ($r = 0.19$; $P < 0.08$). While with PTH there wasn't any association related to bone markers.

In this group of premenopausal women we observed significant positive association between serum calcium and bone resorption marker DPD ($r = 0.27$; $P < 0.008$), bone formation marker OC ($r = 0.25$; $P < 0.01$). There was a positive significant correlation between serum calcium and serum phosphorus ($r = 0.26$; $P < 0.007$)

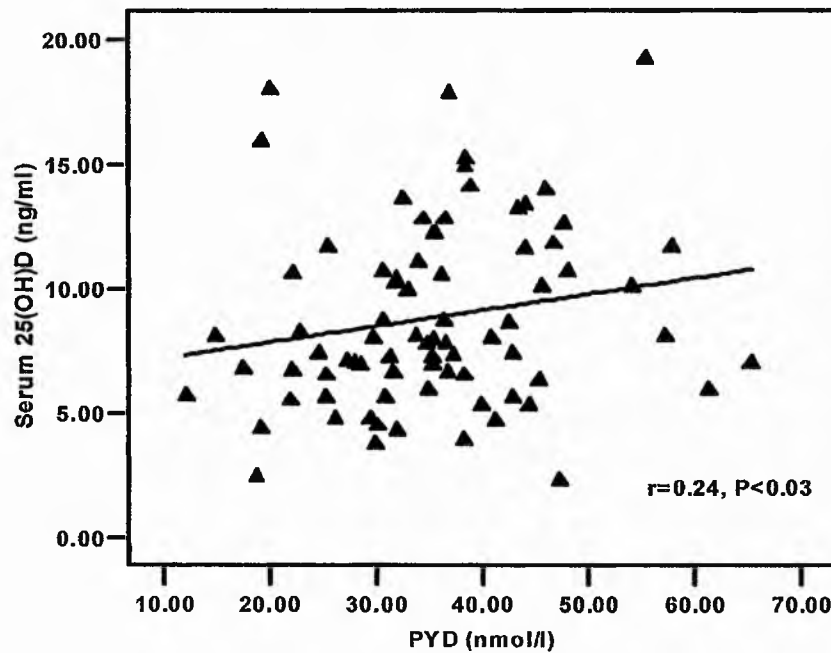


Figure 7.10: Relationship between serum 25(OH) D and urine PYD in premenopausal women

7.3.10. Effect of 25(OH) D levels on indices of bone health in postmenopausal women

The mean values of serum 25(OH) D were divided into five different levels and the mean values of bone density at sites (LS, FN, FW, and FT), BUA, and VOS for postmenopausal women are shown in Table 7.4. These data were examined for differences in bone density, BUA, and VOS at different levels of vitamin D. There were significant differences in lumbar spine; femoral neck; femoral ward; femoral trochanter BMD at different vitamin D levels ($P<0.02$; $P<0.00$; $P<0.00$; $P<0.00$) respectively. No differences were found at the BUA and VOS ($P<0.22$; 0.12).

7.3.11: Effect of 25(OH) D levels on indices of bone health in premenopausal women

The data were also examined in premenopausal women to see if there are any differences in bone density at sites (LS, FN, FW, and FT), BUA, and VOS at different 25(OH)D levels. There were no significant differences were found in the lumbar spine, femoral neck, femoral ward, and femoral trochanter ($P < 0.9$; $P < 0.2$; $P < 0.27$; 0.7) respectively. There was only trend in the BUA and none was founding the VOS ($P < 0.14$; $P < 0.8$) respectively. The mean values of bone density at sites (LS, FN, FW, and FT), BUA, and VOS for premenopausal women at different 25(OH) D levels are shown in Table 7.5.

Table 7.4: Effect of 25(OH) D level on bone indices of postmenopausal women

	25(OH)D <5 (ng/ml) n=12	25(OH)D 5-10 (ng/ml) n=36	25(OH)D 10-12 (ng/ml) n=21	25(OH)D 12-15 (ng/ml) n=12	25(OH)D >15 (ng/ml) n=10	P value
Age (y)	48.83±4.13	49.38±4.24	49.85±5.6	47.75±4.5	54.90±4.65	P< 0.007
Weight (kg)	90.95±15.2	77.24±13.08	71.53±9.26	73.61±16.7	68.83±9.9	P< 0.000
Height (cm)	157.95±8.3	157.63±5.4	156.24±5.3	154.58±4.3	155.5±6.8	P< 0.4
BMI (kg/m²)	36.38±5.15	31.1±5.17	29.30±3.53	30.80±6.8	28.62±5.0	P< 0.002
LS BMD (g/cm²)	1.16±0.16	1.11±0.16	1.07±0.16	1.0±0.13	0.98±0.17	¹ P<0.02 ² P<0.27
FN BMD (g/cm²)	1.0±0.14	0.96±0.17	0.87±0.12	0.89±0.13	0.81±0.11	¹ P<0.00 ² P<0.1
FWBMD (g/cm²)	0.90±0.16	0.82±0.20	0.78±0.15	0.75±0.17	0.65±0.13	¹ P<0.00 ² P<0.1
FTBMD (g/cm²)	0.86±0.13	0.84±0.16	0.75±0.11	0.76±0.17	0.67±0.1	¹ P<0.00 ² P<0.1
LS t-score	-0.32±1.3	-0.68±1.36	-1.06±1.32	-1.62±1.09	-1.83±1.4	¹ P<0.02 ² P<0.3
TF t-score	0.50±1.11	0.10±1.47	-0.51±1.09	-0.41±1.44	-1.29±0.73	¹ P<0.00 ² P<0.2
BUA (db/MHz)	70.0±19.12	73.47±15.7	71.15±11.0	63.33±15.9	63.30±18.7	¹ P<0.22 ² P<0.29
VOS (m/s)	1614.5±25	1608.9±27	1623.0±18	1607.9±28	1598.7±39	¹ P<0.125 ² P<0.08

1. ANOVA 2. ANCOVA

Table 7.5: Effect of 25(OH) D level on bone indices of premenopausal women

	25(OH)D <5 (ng/ml) n=13	25(OH)D 5-10 (ng/ml) n=41	25(OH)D 10-12 (ng/ml) n=15	25(OH)D 12-15 (ng/ml) n=11	25(OH)D >15 (ng/ml) n=4	P value
Age (y)	24.1±4.03	23.19±3.5	23.0±3.20	20.41±0.66	23.0±3.93	P< 0.08
Weight (kg)	59.85±12.5	62.4±14.83	61.8±12.35	61.96±22.9	54.74±10.3	P< 0.8
Height (cm)	158.6±6.58	158.73±5.0	158.9±6.80	158.83±7.0	159.0±5.91	P< 1.0
BMI (kg/m ²)	23.83±4.83	24.68±5.45	24.41±4.20	24.62±9.67	21.56±3.38	P< 0.8
LS BMD (g/cm ²)	1.15±0.15	1.29±0.11	1.13±0.1	1.11±0.12	1.15±0.08	¹ P<0.9 ² P<0.6
FN BMD (g/cm ²)	0.90±0.14	0.97±0.11	0.93±0.10	1.0±0.12	1.0±0.107	¹ P<0.2 ² P<0.07
FW BMD (g/cm ²)	0.88±0.15	0.94±0.13	0.88±0.14	0.97±0.168	1.0±0.14	¹ P<0.27 ² P<0.17
FT BMD (g/cm ²)	0.75±0.24	0.79±0.22	0.75±0.11	0.78±0.30	0.75±0.39	¹ P<0.7 ² P<0.7
LS t-score	-0.39±1.27	-0.56±0.92	-0.58±0.77	-0.65±1.0	-0.36±0.67	¹ P<0.9 ² P<0.7
TF t-score	-0.37±1.12	-0.027±1.0	-0.34±1.05	0.033±1.24	0.02±0.95	¹ P<0.7 ² P<0.59
BUA (db/MHz)	80.54±15.3	77.85±12.0	75.53±11.68	84.75±12.9	67.80±21.1	¹ P<0.14 ² P<0.15
VOS (m/s)	1655.2±29	1651.6±32	1647. 1±27	1660.4±27	1654.2±24	¹ P<0.83 ² P<0.89

1. ANOVA 2. ANCOVA

7.3.12. Effect of 25(OH) D levels on PTH and bone turnover in postmenopausal women

The data were examined to see any differences between mean values of PTH, 1,25(OH)₂D, and all biochemical markers of bone turnover (PYD, DPD, BSAP, and OC) between different 25(OH) D levels of postmenopausal women are shown in Table 7.6. Data exhibited non-significant differences in mean values of PTH, 1,25(OH)₂ D, PYD, DPD, BSAP, and OC between 25(OH) D levels (P<0.2; P<0.8; P<0.7; P<0.1; P<0.2; P<0.1) respectively.

7.3.13. Effect of 25(OH) D levels on PTH and bone turnover in premenopausal women

The data were examined to see any differences between mean values of PTH, 1,25(OH)₂D, and all biochemical markers of bone turnover (PYD, DPD, BSAP, and OC) and different 25(OH) D levels in premenopausal women (Table 7.7). There was significant difference in PTH among 25(OH) D different levels (P<0.00). The mean serum PTH was higher at 25(OH) D level <5 ng/ml and lowest at 25(OH) D level >15 ng/ml. Osteocalcin was significantly different between different levels of 25(OH) D (P<0.04). Other bone turnover markers (PYD, DPD, and BSAP) exhibited non-significant differences in their mean values between different 25(OH) D levels (P<0.2; 0.4; 0.4) respectively.

Table 7.7: Effect of 25(OH) D levels on PTH and bone turnover of postmenopausal women

	25(OH)D <5 (ng/ml) n=13	25(OH)D 5-10 (ng/ml) n=41	25(OH)D 10-12 (ng/ml) n=15	25(OH)D 12-15 (ng/ml) n=11	25(OH)D >15 (ng/ml) n=4	P value
PTH (pg/ml)	84.5±42.9	66.27±37.5	57.3±29.84	52.0±29.25	62.5±38.07	P< 0.2
1,25(OH)D (pg/ml)	39.2±16.27	43.4±17.7	44.4±12.2	44.6±11.26	45.0±10.8	P< 0.8
PYD (nmol/l)	42.3±12.36	38.96±15.4	36.14±8.39	38.0±14.83	39.33±13.2	P< 0.7
DPD (nmol/l)	12.21±5.14	9.54±2.99	10.12±3.77	10.26±3.64	11.77±3.22	P< 0.1
BSAP (U/L)	22.48±7.95	20.08±6.72	20.28±8.61	19.4±6.85	26.06±10.3	P< 0.2
OC (ng/ml)	9.61±5.35	9.90±2.90	9.5±2.96	9.39±3.82	12.56±3.05	P< 0.1

Table 7.8: Effect of 25(OH) D levels on PTH and bone turnover of premenopausal women

	25(OH)D <5 (ng/ml) n=13	25(OH)D 5-10 (ng/ml) n=41	25(OH)D 10-12 (ng/ml) n=15	25(OH)D 12-15 (ng/ml) n=11	25(OH)D >15 (ng/ml) n=4	P value
PTH (pg/ml)	65.36±24.5	37.56±23.0	36.93±15.9	33.41±12.7	27.4±10.5	P< 0.00
1,25(OH)₂D (pg/ml)	33.9±12.4	39.24±13.0	35.73±11.7	38.58±10.4	40.6±13.16	P< 0.6
PYD (nmol/l)	31.2±9.05	34.2±11.22	39.11±11.0	39.33±5.13	34.0±15.0	P< 0.2
DPD (nmol/l)	9.44±3.14	10.35±5.0	11.69±2.5	12.65±4.27	10.32±5.55	P< 0.4
BSAP (U/L)	18.57±4.44	18.92±6.0	17.08±4.8	17.6±4.83	14.43±4.8	P< 0.4
OC (ng/ml)	13.8±3.55	11.88±3.62	12.81±3.27	12.06±2.67	11.74±4.24	P<0.04

7.4. Discussion

This study is a cross-sectional investigation and hence the study is limited by design in that only associations and not relationships can be described. The results are trying to reflect what is happening in the population and is based on volunteers which is new to Saudi society and more so to women due to their movements restriction and their dependent on male companion for transportation. The most important limitation of this study it reliance on self-reporting, which are potentially biased by non-responders. The study is also limited by demographic bias toward the rich (post 61%, pre 59%) and educated (post 76%, 100%) members of the society. This is not a true representation of the Saudi women population. This will eliminate the more passive and subjugated women who may have worse results. Study exclusion criteria were very stringent and this limited the number of cases to be studied in addition to financial limitations. Several other limitations should be noted such as response biases; women tend to hide important information of which affect the results of the study (i.e. as using vitamin D and calcium supplementation which affect vitamin D measurements). According, the generalization of our finding to the Saudi population women should be treated with some caution. With respect to vitamin D status our result were consistent with those of previous work in the Middle Eastern population, which increase confidence in our findings.

This study showed that the mean serum levels of 25(OH)D of women population are much lower in Saudi Arabia 9.285 ± 4.0 ng/ml (23 ± 10.0 nmol/l) comparing to the Westerner countries e.g. France 24.0 ± 12 ng/ml (60.0 ± 30 nmol/l) (Chapuy *et al.*, 1997), USA 33.0 ± 0.6 ng/ml (82.5 ± 1.5 nmol/l) (Nesby-O' Dell *et al.*, 2002) but similar to other Saudi and Arab studies. In the study by Ghannam in Riyadh city (capital of Saudi Kingdom) mean serum levels of 25(OH)D in women

were 9.8 ± 6.9 ng/ml (24.5 ± 17.2 nmol/l) (Ghannam *et al.*, 1999). At Gannage-Yared study in Beirut (Lebanon) mean serum levels of 25(OH) D in (men and women) were 9.71 ± 7.07 ng/ml (24.27 ± 19.25 nmol/l). It has been well documented that vitamin D deficiency causes impairment of skeletal mineralization, resulted in rickets and osteomalacia in children or osteomalacia and osteoporosis in adults. Women with vitamin D deficiency are at high risk for bone loss, bone fractures and disability. The high prevalence of vitamin D deficiency is being increasingly recognized globally. However, less severe degrees of vitamin D insufficiency contribute to bone loss without impairing bone mineralization. Specifically, vitamin D inadequacy produces calcium malabsorption with resultant secondary hyperparathyroidism producing by time bone loss and osteoporosis (Parfitt, 1990)

Vitamin D deficiency is highly prevalent in our studied population of women. The threshold of vitamin D insufficiency, which is defined by serum 25(OH) D concentration, varies widely between studies ranging from 4 – 20 ng/ml (10 – 50 nmol/l) (Chapuy *et al.*, 1997; Lips *et al.*, 1988). We used the “classical” threshold of 12 ng/ml value (30 nmol/l) that is chosen by (Chapuy *et al.*, 1997). In this study it is notable that (74% and 82%) of our population of postmenopausal and premenopausal women respectively is below the threshold of vitamin D insufficiency <12ng/ml. This observation is similar with a study done in Lebanon (Gannage-Yared *et al.*, 2000) were 72.8% of their population (men and women) is below <12 ng/ml but in women alone 83.9% were below <12 ng/ml. Severe vitamin D deficiency below <5 ng/ml were found in our postmenopausal and premenopausal women (13.2% and 15.5%) respectively.

Although vitamin D deficiency was common in our population, 25(OH)D level did not correlate with any of the BMD measurements, this observation is

similar to the study done in Saudi Arabia (Ghannam *et al.*, 1999). The possible explanation for the observed lack of correlation is may be the distribution of 25(OH)D level in this population was far below the threshold needed for maintaining normal bone density. In several epidemiological studies serum 25(OH)D was not a risk factor for hip fractures (Lips, 2004).

Secondary hyperparathyroidism is a well-known consequence of vitamin D deficiency (Parfitt *et al.*, 1982; Chapuy *et al.*, 1997). Our results show a significant negative correlation between PTH and 25(OH)D in both group of postmenopausal and premenopausal ($P < 0.02$, $P < 0.004$) respectively. In our study it was observed that there was a gradual continuous increase in serum PTH levels as the 25(OH)D levels continually decline. In most studies serum PTH levels were start to increase when serum 25(OH)D equal or falls below 31 ng/ml (78 nmol/l) (Chapuy *et al.*, 1997). Most of our population falls in between 5-12 ng/ml (12.5-30 nmol/l), which is below the threshold of serum 25(OH)D levels inducing an increase in serum PTH secretion. Low levels of serum 25(OH)D have been associated with high levels of serum PTH, low bone density and hip fractures.

The increase in serum PTH levels that follows a fall in serum 25(OH)D, may be due to a loss of the calcemic effect of vitamin D on bone (Jones *et al.*, 1998). Vitamin D is a key modulator of intestinal calcium and phosphorus absorption (Chapuy *et al.*, 2002). Our data show a positive trend between serum calcium and serum phosphorus with serum 25(OH)D ($P < 0.06$, $P < 0.07$).

Dietary calcium intake influences serum PTH and serum PTH may influence the turnover of vitamin D metabolites. Low calcium intake causes an elevation of serum PTH and serum 1,25(OH)₂D thereby lowering the half life of serum 25(OH)D and inducing vitamin D deficiency (Lips, 2004). Calcium intake

was (791, 747 mg/day) for postmenopausal and premenopausal respectively. In a study in postmenopausal women, it shown that increasing calcium intake from 800 to 2,400 mg/day caused a decrease of serum PTH of 30% (McKane *et al.*, 1996).

Vitamin D intake was very low in Saudi postmenopausal and premenopausal women (112, 116 IU/d), which is due to a relatively low consumption of milk, low consumption of fish, and lack of fortified food except milk (highly fortified in dried and low in fresh). Recent studies reveal that current dietary recommendation for vitamin D, (200 - 600 IU/d) are inadequate to maintain circulating levels of 25(OH)D at or higher than 32 ng/ml (80 nmol/l) consequently, expert opinion suggest that substantially more than (1000 IU) may be required (Hollis, 2005).

A marked variability in serum 25 (OH) D measurement is reported which is confounds the diagnosis of vitamin D insufficiency/deficiency and hamper comparison between results from different populations (Lips *et al.*, 1999). During the past 30 years, different methods have been developed for determining vitamin D status in humans. These techniques have involved competitive protein binding assays (CBPA), high-performance liquid chromatography (HPLC) with UV detection and radioimmunoassay (RIA). The HPLC method give slightly lower results than RIA and CBPA methods. The competitive binding assay and high-performance liquid chromatography (HPLC) with UV detection methods were used in this study. The difficulty in vitamin D measurements is the standardization of assays. Different laboratories, using different methods, yield different results for the same specimens by as much as two fold (Binkley *et al.*, 2004).

CHAPTER 8

General Discussion

The present research could be considered as the first academic investigation for PhD degree in the Kingdom of Saudi Arabia in one of the most common diseases affecting millions of people worldwide osteoporosis. Unfortunately, in the past little attention has been given to bone diseases in Saudi Arabia (Khan and Khoja, 2003). Only a few studies have been published from several cities in the Kingdom such as the capital Riyadh (central region; Ghannam *et al.*, 1999; El-Desouki, 1995), Eastern region (Al-Nuaim *et al.*, 1995) and Jeddah city from western region (Ardawi *et al.*, 2005), which has a population number of approximately five million. Therefore, unfortunately we have a lack of information and precise statistics about the prevalence of the disease in the Kingdom especially in the southern region, which is known as an area of crowded population and low income. Recently, Ardawi *et al.* (2005) published the first large – scale report on reference value on the BMD of the lumbar spine and the neck femur in randomly selected healthy Saudis of both sexes of various age groups (20 – 79 years) in Jeddah city only.

As far as my confirmed knowledge, this project is the first study concerned the association between bone disease, nutrition intake and lifestyle factors in the Kingdom of Saudi Arabia. The data and finding results presented in this Thesis were dealing with most common factors affecting bone health including nutritional habits, the quality and quantity of food consumed, physical activities and non-dietary lifestyle (e.g. sunlight exposure) beside anthropometric measurements. The results obtained in this study has been mainly compared with several studies for similar female age groups in the Western countries and the reason of that is the lack of similar studies in this country except of course, Ardawi *et al.* (2005) report as mentioned above.

This PhD study involved a total of 212 females, 112 postmenopausal and 100 premenopausal aged 45-60 years and 20-30 years respectively. The findings obtained showed significant differences between the two groups, postmenopausal and premenopausal women depend for a number of aspects selected. Although, these finding are very important they still remain a limitation for several reasons including:

- a) The scarce of studies in bone disease as cross the country and in general Middle East countries which discourage to make a comparison study between different regions of the Kingdom to find out the accurate reasons and the accurate prevalence of the disease.
- b) Absence of references value for Arab countries (except recently, Lebanese reference, Malouf *et al.*, 2000) forces us to compare our results with USA and European references value, which limit the accuracy of this comparison.
- c) The nutritional habits are definitely profoundly differences between this country and UK / USA habits which, confirmed the limitation of comparison and this can be attributed to inexistence of food composition tables for Middle Eastern countries limit our ability to examine clearly diet diseases relationships and comparisons between UK / USA habits.
- d) Lifestyle factors, in particularly style of clothing and dwelling location are also profoundly different between Saudi females and UK/ USA females. Therefore, the alternate comparison between the results obtained in this study and other results obtained from several studies carried out in USA or European countries are not precisely accurate which believed that recommendations of strategies nutritional experts would be

unrealistic for population of Saudi Arabia or other Arab countries. In this study one thing made clear that the work has focused on intensively on women in post- and premenopausal ages.

The study focused on indices of bone health and the factors affecting skeletal activity as determined by bone mineral density (BMD, g/cm^2) from the anterior – posterior lumbar spine and three sites of the left femur and broadband ultrasound attenuation (BUA) at the calcaneal site in both groups. As shown in the results, bone health indices indicated a high prevalence of low bone mass in both age groups. The percentage of women classified as osteopenic and osteoporotic according to WHO criteria (WHO, 1994) a total of 52 % of postmenopausal and 37 % of premenopausal women were osteopenic at the lumbar spine, and similar results were found for the femoral neck 32 % of postmenopausal and 23 % of premenopausal was osteopenic. For the calcaneous although were 62 % postmenopausal and 36 % premenopausal, who were osteopenic and for osteoporotic changes were 13% and 2 % respectively. Our findings are similar to Ardawi et al (2005) which they found the prevalence of osteoporosis in Saudi females ranged between 30.5 – 49.6 % (except in the age groups 50 – 79 years), and according to Ardawi report the mean values for the lumbar spine BMD in both sexes (men and women) of Saudis were lower than those reported previously for USA and northern European population (Mazess and Barden, 1999; Mazess *et al.*, 1990; Karlsson *et al.*, 1993; Kroger *et al.*, 1992; Laitinen *et al.*, 1991; Lilley *et al.*, 1994; Truscott *et al.*, 1996; Kroger and Laitinen, 1992; Wetzel *et al.*, 1996; Burger *et al.*, 1994) and Kuwaitis (Gougherty and Al-Marzouk, 2001) whereas similar for Lebanese females (except lower in the age groups 60 – 79 years).

A similar finding to Ardawi reports has observed in our study that there was a significant positive association between body weight and BMD at all five skeletal sites, the lumbar spine, the femoral neck, the femoral Ward, the femoral Trochanter and the calcaneal. Our study shows that the body weight had greater effect on BMD than the height. Our result also indicate that there was a positive correlation between the physical activity and BMD; higher physical activity levels associated with greater bone density. This is in agreeable with several previous studies (Boot *et al.*, 1997; Slemenda *et al.*, 1991). Nguyen *et al* (1998) reported that the effect of physical activity on bone loss was independent of age and baseline BMD, but was dependent on baseline weight and weight change. They emphasize that physical activity level had an effect only in those women who lost weight and in those with stable weight, but not in women who's increased.

It is important to note that in this study obesity was common amongst women, which is likely to be reflective of poor physical activity levels and increased energy intake. This is a concern given the complications associated with obesity such as diabetes and coronary heart disease. Public health strategies to improve body weight ratios are required as a matter of urgency. Although obesity is protective to osteoporosis, as a key health outcome, further efforts to reduce the prevalence of obesity are urgently required.

In this study, sunlight exposure was very low which is reflected in the poor vitamin D status. Sunlight plays an important role in protecting against vitamin D deficiency. Data would suggest that short periods (30minutes per day) of time exposed to sunlight without wearing sun protection such as sun-screen and protective clothing is suffice to maintain optimum vitamin D levels.

Vitamin D status and calcium intake indicate the condition of healthy bone. The deficiency or insufficiency of vitamin D induced frankly to the mineralization of bone matrix. Severe hypovitaminosis leads to rickets in children and its equivalent in adult osteomalacia. Mild to moderate hypovitaminosis D causes secondary hyperparathyroidism and increased the risk of fracture, particularly femoral neck fracture (Yared and Halaby, 2001). The results presented in this study showed that the vitamin D deficiency less than 12 ng/ml was prevalent in our postmenopausal women in 74 % with only 26 % higher than 12 ng/ml. Whereas in premenopausal the deficiency was 82 % less than 12 ng/ml and only 18 % are higher than > 12 ng / ml.

It is obvious that the circulating 25OHD level was much lower in Saudi females than in Western populations. There were no significant differences between the mean, normal Saudi males and females value (Woodhouse and Norton, 1982). The deficiency of vitamin D is from poor dietary intake and reduced synthesis of vitamin D by the skin, although, the country is a sunny climate all the year. Mishal (2001) reported that there were no significant differences between valid women and western style clothing women in his study to Jordanian females, therefore and similar to the above findings, in our study the effect of clothing showed a tiny effect between valid and non valid women, but it is not that much of influence and definitely not the mean factor for vitamin D deficiency.

The data presented in this study for food consumption revealed that the consumption of food groups 3 and 4 (meat products and diary products) are quite similar to the western countries as well as for postmenopausal and premenopausal women. In the few last years, the world trend is increased to develop efficient and precocious strategies in the prevention of osteoporosis by encouraging people in the

early stages to intake regularly and in adequate amounts the important necessary minerals and vitamins.

In this group of women, milk consumption was low and was associated with higher bone resorption. Furthermore, a high intake of fruit and vegetables were negatively associated with bone formation markers and in premenopausal women was associated with higher bone mass at the calcaneal site. Nutrients including vitamin C and potassium were also associated with improvements in bone indices. It is important to note that the nutrient data should be treated with some caution since a full nutrient composition database was not available for the nutrient intake analysis.

Areas for future research

On this sight and according to the above cases, more work is urgently needed in this domain. Future research ideas include

- a) More research is needed to determine the prevalence of osteopenia and osteoporosis in every city across the country for both sexes in all age stages.
- b) More research is needed to study the influences of habitual nutrition on bone health.
- c) More research is needed to examine the influence of nutrients including calcium and vitamin D as well as other nutrients with specific reference to improving the vitamin D status of the population.
- d) The long-term relationship between measures of bone mass in adulthood and bone health in old age.
- e) The long-term study for childhood nutritional intake and the effect of those nutrition on peak bone in old age.

- f) The food consumption table and measurement of food portion size.
- g) Large scale cross-section study to determine the most fractures bone in old age and young age.

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APPENDICES

Appendix I- Chapter 2

Measures the level of vitamin D, Calcium and Bone density in Saudi Women

Scientific research will be done by King Abdul Aziz University
in Jeddah & Surrey University in UK

Research Aim

To find out the level and the cause of Osteoporosis diseases in Saudi Women.

Dear Lady

Do you know what is Osteoporosis disease?

Osteoporosis is a disease of progressive bone loss associated with an increased risk of fractures. It literally means "**Porous bone**" with no symptoms or discomfort, until a fracture occurs.

Osteoporosis often causes a loss of height and dowager's hump (a severely rounded upper back)

There are one in 3 women over 55 years will sustain bone fractures due to Osteoporosis.

Many of these are painful fractures of the Hip, spine, wrist, arm and leg.
That often occurs as a result of a fall.

Dear Madam

If you wish to participate at this Scientific research about Osteoporosis
It will be a great benefit to all women in our community.

What we ask from you Madam

- Please write your name and your telephone number.
(All names and telephone No. will be secure and very restricted)

and we will call on you for appointment to collect samples of blood and urine and to measure your bone density.

- We will ask of you Madam:
 - Fasted blood sample.
 - Single urine sample 2nd morning from 8-11 am.
- We will need to measure your bone density by special machine called DXA it will take a few minutes from your time.

I agree to participate in this research

Name:

Telephone No.:

Remarks:

Signature :

The cost of the analysis and x-ray around 2500 S.R. University will absorb all expenses. Thank you for your participations and cooperation, which make us achieve our goal that will help our community.

Diet and Lifestyle Questionnaire

Dear participant in this scientific research about Osteoporosis:

Thank you for agreeing to participate with us to know the causes of this disease in our community.

Please fill in with me this questionnaire about your personal history, physical activity, life style, medical information and food intake.

Section One	Personal
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First: What is your age?

Second: What is your occupation?

Third: What is your Husband occupation?

Forth: Where do you live?

Vila	Apartment	Cottage

Fifth: What is your level of education?

Primary school	Secondary school	University degree	Higher education

Section Two**Physical Activity and Life Style****First:** Do you smoke?

(if your answers yes what kind did you smoke?)

Yes	No

Cigarettes	Haply bubbly Moassel	Haply bubbly Jurrak	More specify
Daily use	Daily use	Daily use	Daily use

Second: Please tell us how do you cover yourself when you go out?

All veiled	Part veiled	Not veiled

Third: How long do you spend out side in the sunlight per day?

Less than 15 mints.	15 mints.	30 mints.	1 Hrs.	1-2 Hrs.	3-4 Hrs.	5-6 Hrs.

Forth: How long do you practice any of the following activities on a weekly basis?

Activity	Hours
Walking	1-2-3
Jogging	1-2-3
Cycling	1-2-3
Swimming	1-2-3
Aerobics	1-2-3
Other's Please specify	1-2-3

Section Three**Medical Information****First:** How old were you when your periods started?

Years

Second: Is there a gap of 25-35 days between the start of each periods?

Yes	No	Not sure

Third: Have your periods ever stopped for longer than months for reason other than pregnancy?

Yes	No

six

Forth: How many times have been pregnant
(Including miscarriage, terminations Stillbirths)
Fifth: How many pregnancies have resulted in the birth of live child?
Sixth: Have you ever broken or fractured any bone?

Yes	No	Not sure

If your answer YES please give us more detailed in the next schedule

Name of broken bone	Your age when it's broke	How did it happen? Fall, Car accident, Sport incident

Seventh: Have any of your female relative ever-suffered fractured or broken hips?

Relatives	Yes	No	Do not know
Mother			
Grandmother			
Aunt			
Sister			
Cousin			
Others			

Section Four

Dietary Intake

First: Intake of milk.

- 1) How many glasses of milk do you drink during the day including milk used in tea or coffee, chocolate, cereals, sweet drinks etc.?
(Please check one box only)

Rare	1 glass	2 glass	3-4 glass	5-6 glass	7 < glass

- 2) What kind of milk do you use?

Full fat milk	Semi skimmed milk	Skimmed milk	Others (e.g. condensed)	Others kinds

3) Is this milk

Fresh	
Long life	
Dried	

usually?

Second: Intake of milk products

How many times a day / week do you eat the following milk products?

	A day	A week	Fortnight	Rare
1) Yogurt	1-2-3	1-2-3-4-5-6-7		
2) Cream	1-2-3	1-2-3-4-5-6-7		
3) Ice-cream (all kinds)	1-2-3	1-2-3-4-5-6-7		
4) Hard cheese	1-2-3	1-2-3-4-5-6-7		
5) Soft cheese	1-2-3	1-2-3-4-5-6-7		
6) Milk Based Pudding, e.g. Custard	1-2-3	1-2-3-4-5-6-7		

Third: Intake of eggs

1) How many eggs do eat per day / week?
(Number of eggs does you usually eaten each week)
(Boiled, poached, fried, and scrambled)

A day	0	1	2	3	4	5	6	7	8	9	10	<
A Week	0	1	2	3	4	5	6	7	8	9	10	<

(if you do not eat eggs please check 0)

2) How many eggs in baked dishes do you eat each day /week?
(e.g. flans, quiches...etc.)(In sweet e.g. soufflés, egg custard, crème caramel)

A day	0	1	2	3	4	5	6	7	8	9	10	<
A Week	0	1	2	3	4	5	6	7	8	9	10	<

Forth: Intake of fatty fish

How many times a day / week do you usually eat a portion of the following?
Fatty fish (salmon, tuna, sardines)

A day	A week	Fortnight	Rare
1-2-3	1-2-3-4-5-6-7		

Fifth: Intake of cereals

How many times a day / week do you eat these different types of cereals?

1) Porridge

2) Bran flakes

3) All Bran, Wheat Bran

4) Corn flakes, Nutri-grain, Special K

5) Unsweetened muesli, Weetabix,

Shredded wheat

6) Coco-pops, crunchy nut cornflakes, frosties, ricicles, sugar puffs. Etc.

A day	A week	Fortnight	Rare
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		

Sixth: Intake of butter, margarine and oil

1) What kind of fat do you most often use?

	Type		Brand Name
1	Butter		
2	Soft margarine		
3	Vegetables oil		

2) How do you normally use it?

Thickly Heavy	Average Medium	Thinly Light
------------------	-------------------	-----------------

3) How many times a day / week do you use it?

1) Butter

(Please give brand name and type)

(.....)

A day	A week	Fortnight	Rare
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		

2) Soft margarine

(Please give brand name and type)

(.....)

3) Vegetables oil

(Please give brand name and type)

(.....)

Seventh: Intake of hot and cold beverages

A) How many cups of coffee do you have a day		
1	Turkish coffee	Cups
2	Nescafe	Cups
3	Decaffeinated coffee	Cups
B) How many cups of tea do you have a day		
1	Red tea	Cups
2	Green tea	Cups
3	Mint tea	Cups

C) How often a day/week do you drink natural fruit juice?

A day	A week	Fortnight	Rare
1-2-3	1-2-3-4-5-6-7		
1-2-3	1-2-3-4-5-6-7		

D) How often a day/week do you drink cola drinks and other caffeine containing drinks?

E) Are these drink usually?

Diet	Normal

Eights: Intake of vitamin and mineral

1) Are you taking any vitamin or mineral?

YES	NO

If yes please give us the details

Type	Brand	Frequency

Ninth: Intake of salt

1) How much salt is added to your food on cooking?

2) How much salt do you add to your food on the plate?

A lot	A little	None

Tenth:

1) Are you on a special diet?

1	No	
2	Sliming diet you decided for yourself	
3	Sliming diet prescribed by your doctor	
4	Cholesterol lowering diet	
5	Vegetarian (but does eat milk & milk products)	
6	Vegetarian (does not eat any animal products at all)	

2) How long have you been on your diet?

Years	Months

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