بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Evaluation of 25-OH Vitamin D, Total Calcium and Phosphorus Levels in Post-Menopausal Sudanese Women with Osteoporosis

تقويم مستويات فيتامين (د) والكالسيوم والفوسفور لدي النساء السودانيات بعد سن اليأس المصابات بهشاشة العظام

A dissertation Submitted in partial Fulfillment for the requirements of

M.Sc. Degree in Medical Laboratory Science (Clinical Chemistry)

By

Abdalrahman Ismail Hamad Mohammed

B.Sc.in Medical Laboratory Sciences - Clinical Chemistry

(Omdurman Islamic University-2015)

Supervisor:

Associate Professor. Abdelgadir Ali Elmugadam

Medical laboratory Science - Clinical Chemistry

October- 2018

الآية

قال تعالي:

وَضَرَبَ لَنَا مَثَلًا وَنَسِيَ خَلْقَهُۖ قَالَ مَن يُحْيِي الْعِظَامَ وَهِيَ رَمِيمٌ (78) قُلْ يُحْيِيهَا الَّذِي أَنشَأَهَا أَوَّلَ مَرَّةٍۖ وَهُوَ بِكُلِّ خَلْقٍ عَلِيمٌ (79)

سورة يس

DEDICATION

First and Foremost, I would like to dedicate this research

To:

To my parents and my family who loves and encourage me all the time.

To my teachers who helping and supporting me.

To my favorite friends who are always there for me.

Moreover, I am grateful for Sudan University for Science and Technology for giving me the chance to be a part of the project.

In addition, special dedication to my supervisor Associate Professor. Abdelgadir Ali Elmugadam for giving me the Opportunity to work with him.

Moreover, special dedication to Dr. Ghada Abdalrahman Elfadil and Dr. Mohammed Karrar Abdalla for helping me to finish this work.

ACKNOWLEDGEMENTS

First of all, I would like to thank my God for blessing my life and lighting my way.

Greatest thanks for my Supervisor Associate Professor. Abdelgadir Ali Elmugadam for supporting me all the time.

Special thank for Dr. Ghada Abdalrahman Elfadil and Dr. Mohammed Karrar Abdalla who always support and give me the hope and light in the dark street.

In addition, I thank all the staff of alrayan medical center specially Dr. Mawahib ibn idriss aledrissy for facilitating collection of the samples.

Then I give the nicest words for workers at research laboratory in Sudan University for science and technology for sample analysis.

Abstract

Osteoporosis is disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture incidence. The osteoporosis is becoming public health problem in Sudan and neighboring countries and worldwide. As the environmental risk factors for osteoporosis in women are similar, the study aim to evaluate 25-OH vitamin D, total calcium and phosphorus levels among Sudanese post-menopausal osteoporotic patients compared to non-osteoporotic healthy women as controls. A case control study was conducted in Khartoum state from March to October (2018), total of 50 osteoporotic patients and 50 non-osteoporotic healthy controls matched for sex and presence of other diseases were enrolled in this study. Serum 25-OH Vitamin D were measured using ELISA technique, while total calcium measured via methylthymol blue method and phosphorus were measured using molybdate method. Serum 25-OH Vitamin D level and calcium level were significantly decreased while serum phosphorus level was significantly increased ($p<0.001$) in case group compared with control healthy women. We also found insignificant correlation between 25-OH vitamin D level and calcium, phosphorus levels in osteoporotic women and insignificant correlation between serum calcium and phosphorus level.

The data of the study suggest that Sudanese postmenopausal osteoporotic women had lower 25-OH vitamin D and calcium level and higher phosphorus level. Meanwhile, no associations were observed between age and 25-OH vitamin D and study variables.

المستخلص

مرض هشاشة العظام هو مرض يتميز بانخفاض الكتلة العظمية وتدهور الأنسجة الدقيقة في العظام مما يؤدي إلى زيادة هشاشة العظام وزيادة في حدوث الكسور. أصبح مرض هشاشة العظام مشكلة صحية عامة في السودان والدول المجاورة والعالم. وبما أن عوامل الخطر البيئي لترقق العظام لدى النساء متشابهة، تهدف هذه الدراسة إلى تقييم مستويات فيتامين (د) والكالسيوم والفوسفور في النساء السودانيات بعد سن اليأس المصابات بهشاشة العظام مع النساء الأصحاء غير المصابات بهشاشة العظام كأداة مقارنة. وقد أجريت دراسة الحالة في ولاية الخرطوم من مارس الي اكتوبر 2018. تم تسجيل مجموعه 50 من مرضى هشاشة العظام و50 غير مصابين بمرض هشاشة العظام في هذه الدراسة. تم مطابقة الحالات علي حسب الجنس ووجود الأمراض الأخرى. تم قياس مستوي فيتامين (د) باستخدام طريقة مقايسة الامتصاص المناعي المرتبط بالإنزيمات، وتم قياس مستوي الكالسيوم باستخدام الميثيل زايول الأزرق، وتم قياس مستوي الفوسفور باستخدام طريقه الموليبدات.

انخفض مستوى فيتامين (د) ومستوى الكالسيوم بشكل ملحوظ بينما ارتفع مستوى الفوسفور بشكل كبير في حالة المرضي مقارنة مع النساء السليمة. وجدنا أيضا علاقة طفيفة بين فيتامين (د) والكالسيوم (<0.001) ومستويات الفوسفور في النساء المصابات بهشاشة العظام والعلاقة غير ذات دلالة بين مستوى الكالسيوم في الدم ومستوى الفوسفور.

تشير بيانات الدراسة الي ان النساء السودانيات المصابات بمرض هشاشة العظام بعد سن اليأس لديهن نسبة اقل من فيتامين د والكالسيوم ومستوي الفوسفور اعلي. في الوقت نفسه لم يلاحظ أي ارتباط بين فيتامين د ومتغيرات الدراسة.

List of Contents

List of tables

List of figures

List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ALP	Serum Alkaline phosphatase
ATP	Adenosine Triphosphate
BMC	Bone Mineral Contents
BMD	Bone Mineral Density
BSP	Bone Sialoprotein
CTSK	Cathepsin K
CTX-1	Amino-Terminal Crosslinked Telopeptide of type 1 Collagen
DDK1	Dickkopf-1
DPD	Deoxypyridinoline
DXA	Dual Energy x-ray Absorptiometry
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immune Sorbent Assay
ER	Estrogen Receptor
GMCSF	Granulocyte/Macrophage Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HYP	Hydroxyproline
IOF	International Osteoporosis Foundation
MMP	Matrix Metalloproteinases
NTX-1	Amino-Terminal Crosslinked Telopeptide of type 1 Collagen
OPG	Osteoprotegerin
pDXA	Peripheral Dual Energy X-ray Absorptiometry
PH	Hydrogen Number
PICP	Precollegen type I C-terminal Propeptide

PINP	Procollagen Type I N-terminal Propertied
Pqct	Peripheral Quantitative Computed Tomography
PTH	Parathyroid Hormone
PYD	Pyridinoline
QCT	Quantitative Computed Tomography
QUS	Quantitative Ultrasound
RA	Radiographic Absorptiometry
RANK	Receptor Activator for Nuclear Factor κB
RANKL	Receptor Activator for Nuclear Factor κB Ligand
RPM	Round Per Minute
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
SXA	Single Energy X-ray Absorptiometry
TMB	Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TRAP 5b	Tartrate-resistant acid Phosphatase 5b
UV	Ultra Violet
VDBP	Vitamin D-binding Protein
VDR	Vitamin D Receptor
WHO	World Health Organization

Chapter One

Introduction

1. Introduction, Rationale and Objectives

Osteoporosis is described by the World Health Organization (WHO) as a "progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Society *et al*., 2017).

Osteoporosis and low bone mass are currently estimated to be a major public health threat for almost 44 million US men and women aged 50 and older, or 55% of the population in that age range (Nieves, 2005). Because of increased bone loss after the menopause in women, and age-related bone loss in both women and men, the prevalence of osteoporosis increases markedly with age from 2% at 50 years to more than 25% at 80 years in women (Ferdous *et al*., 2016).

Bone remodeling (turnover) is a lifelong process in which osteoclasts remove (resorb) old bone and osteoblasts form new bone in a coordinated and continuous fashion. However, incomplete replacement of old bone with new bone occurs with certain diseases and with aging. Net loss with each cycle, in conjunction with a higher rate of bone turnover, reduces bone density. Higher bone turnover also increases fracture risk independent of bone density (Mary *et al.*, 2010).

Worldwide variation in the incidence and prevalence of osteoporosis is difficult to determine because of problems with definition and diagnosis. The WHO definition of osteoporosis is a bone mineral content (BMC) or bone mineral density (BMD), measured by techniques such as dual-energy X-ray absorptiometry, that is more than -2.5 SD below the young adult mean for the population (Prentice, 2004).

Many environmental factors have been identified as risk factors of osteoporosis, including exercise and calcium intake. In addition, twin and family studies have shown that approximately 50–85% of heritability for BMD in the general population

may be attributed to genetic factors. Genetic factors may also play a role in the development of osteoporosis (Zhao *et al.*, 2016).

Osteoporosis is evaluated by a bone mineral density (BMD) test that measures the amount of mineral per square centimeter and can be performed by a number of radiological densitometry procedures, most often dual energy x-ray absorptiometry (DXA). The most common areas evaluated are the lumbar spine or proximal hip and distal radius. With osteoporosis, bone mineral density is decreased due to the breakdown of bone without compensatory, subsequent remodeling (Oleson and Morina, 2017).

Osteoporosis has generally been divided into two categories: primary and secondary osteoporosis. Primary osteoporosis is age related, affects 95 % of women and about 80 % of men, and is related to estrogen loss in women and a testosterone deficiency in men; other factors include low calcium and vitamin D intake as well as hyperparathyroidism. In contrast, secondary osteoporosis stems from other conditions including hormonal imbalances, diseases, and medications that predispose to bone loss. It may arise at any age and affects both men and women (Oleson and Morina, 2017).

Vitamin D is a topic of great interest for the scientific community, The commonly known function of vitamin D has been associated with skeletal tissue, in which vitamin D influences mineralization, bone turnover rate, and occurrence of fractures, contributing to the prevention and treatment of osteoporosis (Cândido and Bressan, 2014).

Vitamin D its metabolically active form $1,25(OH)_2$ D3 ,is a steroid hormone obtained after hepatic (C-25 position), and not exclusively, kidney (C-1) hydroxylation. Its precursors can be acquired from the diet as well as sun exposure the latter being due to non-enzymatic reactions by exposing the skin to ultraviolet radiation ,The active

vitamin D is capable of binding to an intracellular transcription receptor called vitamin D receptor [VDR] (Cândido and Bressan, 2014).

The 1,25-$(OH)_2$ D3 molecule is a hormone that regulates calcium and phosphorous metabolism. This being so, its primary function is to maintain serum calcium and phosphorus levels in a normal state that is capable of providing the conditions for the majority of metabolic functions, including bone mineralization. Because it is involved in growth of the skeleton, vitamin D is essential during childhood and adolescence (Bueno and Czepielewski, 2008).

The skeleton consists of cortical bone (70–80%) and trabecular bone (20–30%). In the normal axial skeleton, about 25% of the anatomic bone volume is specific bone tissue and 75% bone marrow and fat , but this varies widely between different parts of the skeleton (Ahota, 2000).

Bone tissue is a complex, metabolically active organ of which the bone mineral is composed essentially of calcium and phosphate salts. These salts account for about two-thirds of the total dry weight of bone and most of total body calcium and phosphate (Ahota, 2000).

Calcium is an element that is a fundamental part of the body and its importance is related to the functions it performs in bone mineralization, primarily related to bone health, which include formation and maintenance of the structure and rigidity of the skeleton (Bueno and Czepielewski, 2008).

Calcium is the most abundant stored nutrient in the human body. More than 99% (1.2-1.4 kg) is stored in the bones and teeth. Less than 1% is found in extracellular serum calcium (Beto and Beto ,2015) Level of calcium in the blood is regulated by parathyroid hormone, 1,25- dihydroxycholecalciferol (calcitriol) and calcitonin (Fialová and Vejražka, 2017).

Phosphorus is an important structural component of cells (nucleic acids, phospholipids) as well as bone tissue (hydroxyapatite), participates in energy

conservation in the form of macroergic phosphates (ATP, creatine phosphate), plays an important role in regulation of enzyme activity (phosphorylation and dephosphorylation of enzymes), and contributes to buffering of blood and urine in the form of hydrogen and dihydrogen phosphates, Phosphorus found in bones (80 %) of total in form of hydroxyapatite and in muscles and visceral organs (10–20%) of total and in extracellular fluid (1%) (Fialová and Vejražka, 2017).

1.2 Rationale

Osteoporotic fractures are increasing worldwide as the population ages, with substantial human, economic and social costs. More than 2 million fractures each year in the US are attributed to low bone mass, including 300,000 hip fractures and 550,000 vertebral fractures.

Osteoporotic fractures are a major cause of morbidity and disability in the elderly and, in the case of hip fractures, can lead to premature death. In addition, they impose a considerable economic burden on health services, costing many billions of dollars each year.

Osteoporotic fractures can lead to chronic pain, lack of independence, institutionalization or even death. Direct annual costs of osteoporosis were estimated at $19 billion for 2005, with an increase to $25.3 billion by 2025.

Osteoporosis strikes not only women, as 20% of those affected are men , All ethnic groups are affected, including whites, Native American, Hispanics and Asians, although African-American individuals are at lower risk.

Osteoporosis and low bone mass are currently estimated to be a major public health threat for almost 44 million US men and women aged 50 and older, or 55% of the population in that age range. In fact, 1 in 2 women and 1 in 4 men over the age of 50 will fracture at some point in their lifetime.

Osteoporosis is set to arise alarmingly in the Middle East and Africa with Rates of fragility fracture incidences expected to quadruple in several countries as the

population ages, says a new report by the International Osteoporosis Foundation (IOF).

Lack of research into osteoporosis in our country is aggravating the situation due to lack of funding for research in general and for this type of research in particular , along with the lack of human capacity trained in conducting good quality research are important reasons for the scarcity of research.

This study designed to investigate the association of 25-OH vitamin D and total calcium and phosphorus levels among Sudanese post-menopausal woman with osteoporosis despite the large numbers of research on role of vitamin D in control of other Diseases, there is relatively few studies on the association of vitamin D and osteoporosis.

1.3 Objectives

1.3.1 General objective

To evaluate serum levels of 25-OH vitamin D, total calcium and phosphorus among Sudanese post-menopausal woman with osteoporosis.

1.3.2 Specific objectives

1. To estimate serum 25-OH vitamin D, total calcium and phosphorus levels in Sudanese post-menopausal woman with osteoporosis compared to healthy woman.

2. To determine association between 25-OH Vitamin D, total calcium and phosphorus in study groups.

3. To correlate between serum (25-OH vitamin D, total calcium and phosphorus) levels in case group.

Chapter Two

Literature Review

2. Literature Review

2.1 Bone Definition

Bone provides mechanical support and protection to soft organs, enables movement, hosts hematopoietic tissue, and serves as storage of calcium, phosphate, and magnesium ions. From one third it consists of protein matrix, and from two thirds of the bone mineral (Fialová and Vejražka, 2017).

The bone protein matrix contains mostly type I collagen (90 %) together with other proteins such as osteocalcin , osteonectin, osteopontin, etc. (10 %) (Fialová and Vejražka, 2017).

The bone mineral is composed from small crystals of hydroxyapatite **Ca**10(**PO4**)6(**OH**)2. Other compounds, such as calcium carbonate, calcium fluoride and magnesium phosphate, are present as well (Fialová and Vejražka, 2017).

The metabolic activity of bone tissue is provided by bone cells. Osteoblasts form osteoid (the bone protein matrix), into which mineral salts are deposited. Other cell types include osteoclasts, whose main function is bone resorption, and osteocytes, which complement activity of osteoclasts by providing fine tuning of bone resorption (Fialová and Vejražka, 2017).

2.2 Bone remodeling

Bone remodeling is the process by which old bone is replaced by new bone. The normal bone remodeling process consists of five phases: the resting phase, activation, resorption, reversal, and formation (Gallagher and Harsha, 2013).

In the activation phase of remodeling, osteoclasts are recruited to the surface of the bone (Gallagher and Harsha, 2013).

In the resorption phase, osteoclasts generate an acidic microenvironment between the cell and the surface of the bone dissolving or resorbing the mineral content of the bone (Gallagher and Harsha, 2013).

In the reversal phase osteoclasts undergo apoptosis and osteoblasts are recruited to the bone surface (Gallagher and Harsha, 2013).

In the formation phase, osteoblasts then deposit collagen; this is mineralized to form new bone (Gallagher and Harsha, 2013).

In normal process of bone turn over the rate of bone resorption and bone formation is equal in which by acidification osteoclasts remove bone and by secreting osteoid into the resorption cavity osteoblasts build bone. The rate of bone turnover is increased due to elongation of the life span of osteoclasts and reduction in the lifespan of osteoblasts (Shakoor and Putra, 2015).

2.3 Role of Hormones in bone formation

2.3.1 Vitamin D

Vitamin D refers to a group of fat-soluble secosteroids and is important for intestinal absorption of calcium, phosphate, magnesium and zinc. It is well known that the vitamin D deficiency leads to poor bone development and health. Vitamin D maintains healthy calcium and phosphate levels by aiding the absorption of calcium from the intestine in the body as well as by influencing kidney function. Calcium homeostasis maintained by vitamin D improves the strength of human bones by increasing bone density and thereby preventing bone disease such as osteoporosis and rickets (Balwant and Prashant, 2017).

Skin is the main source of vitamin D, Amount of Vitamin D synthesized depends upon skin’s exposure to sunlight. Most important vitamin D forms are: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Both forms are ingested from the

diet and supplements. These forms are biologically inactive and require enzymatic conversion in the liver and kidney (Balwant and Prashant, 2017) Vitamin D is converted in the liver to calcifediol (prohormone) and ergocalciferol (vitamin- D2). Vitamin D2 is converted to 25-hydroxyergocalciferol [25-hydroxyvitamin D2- abbreviated as 25(OH)2 D2]. Some of the calcifediol which has entered in kidney is converted to calcitriol (1, 25-dihydroxycholecalciferol, abbreviated as 1, 25 $(OH)_2$ D3 (hormone), is biologically activated form (Balwant and Prashant, 2017).

Calcitriol plays major role in calcium and phosphate homeostasis and also affects immune and neuromuscular functions. Calcitriol is released into the blood circulation. It binds to vitamin D-binding protein (VDBP), which is a career protein in plasma and is transported to various tissues/organs. In addition to skin, liver and kidney, calcitriol is also synthesized by immune system cells like monocyte /macrophages (Balwant and Prashant, 2017).

Vitamin D in its active form ($1,25(OH)_2$ D3) is able to increase circulating levels of calcium and phosphorus to normal levels through three pathways. The first pathway, and most well established, is by stimulating the absorption of calcium and phosphate in the intestine, particularly in the duodenum and jejunum. This occurs due to the opening of calcium channels and by the formation of calcium-binding protein, independent of PTH. Vitamin D is able to increase the absorption rate of calcium in the intestine. The second pathway, dependent on PTH, occurs through mobilization of calcium and phosphorus from bone. In this process, there is increased expression of the receptor activator for nuclear factor κB ligand (RANKL) protein in the osteoblasts, capable of binding to the pre-osteoclast RANK and promoting osteoclastogenesis and bone resorption. Vitamin D in the osteoblasts is also capable of highly stimulating the synthesis of osteocalcin and moderately osteopontin , two structural proteins present in the organic matrix related to bone remodeling that have a hormonal function in peripheral tissues. In osteoclasts, vitamin D exerts a direct

function by stimulating osteoclastogenesis, although the indirect action via osteoblasts is the most recognised. The third pathway is also dependent on the PTH and involves the increase in renal retention of calcium due to increased tubular reabsorption or a decrease of filtered load. The renal function of vitamin D is well known and many proteins involved in the process have been identified (Cândido and Bressan, 2014).

In vitamin D deficiency, there is a decrease in circulating levels of calcium and increased PTH levels. PTH acts by increasing P450C1 hydroxylase activity in the kidney, which consequently increases vitamin D serum levels, and is a potent agent in bone resorption. In this new phase, the circulating levels of vitamin D and calcium are normal, but the bone reserves become compromised. If vitamin D deficiency occurs for a prolonged period, substrates for synthesis of the active form of the vitamin may be reduced and the resulting bone loss can lead to osteoporosis (Cândido and Bressan, 2014).

2.3.2 Parathyroid hormone

Parathyroid hormone is an 84-amino acid polypeptide, which is secreted by the parathyroid glands in response to decreases in calcium concentration. Its main actions are to increase renal tubular calcium reabsorption, to stimulate renal calcitriol, or 1,25 dihydroxyvitamin D, production thereby indirectly increasing intestinal calcium absorption, and to regulate bone remodeling. Its ligand is the PTH - 1 receptor, a G protein-coupled receptor expressed primarily in kidney and bone, PTH results in an increase in the number of bone-forming cells by promoting osteoblast growth and de- creasing osteoblast cell death or apoptosis and also PTH also stimulates osteoclastogenesis (Lin and Lin, 2011).

Both PTH and PTH analogues bind on G-protein-dependent receptors on osteoblasts and renal tubular cells to conduct signal transduction that activates protein kinase C and phospholipase C. These signal transduction pathways increase the number of

activated osteoblasts, suppress apoptosis in osteoblasts, attract bone-lining cells, and ultimately increase bone strength, bone mass and bone diameter, as well as stabilize bone structure (Lin and Lin, 2011).

Parathyroid hormone stimulates both osteoclast-mediated bone resorption and osteoblast-mediated bone formation. This increased bone turnover is evidenced by marked increases in biochemical markers of both bone formation and resorption (Augustine and Horwitz, 2013).

2.3.3 Sex hormones

Sex hormones are important regulators of bone remodeling and in post-menopausal females estrogen deficiency makes the major contribution to the cause of osteoporosis.

2.3.3.1 Estrogen:

Estrogen is the major hormonal regulator of bone metabolism in women and men. Therefore, there is considerable interest in unraveling the pathways by which estrogen exerts its protective effects on bone Although the major consequence of the loss of estrogen is an increase in bone resorption. estrogen deficiency is associated with a gap between bone resorption and formation indicating that estrogen is also important for maintaining bone formation at the cellular level. Direct estrogen effects on osteocytes, osteoclasts, and osteoblasts lead to inhibition of bone remodeling, decreased bone resorption, and maintenance of bone formation, respectively. Estrogen also modulates osteoblast/ osteocyte and T-cell regulation of osteoclasts. Unraveling these pleiotropic effects of estrogen may lead to new approaches to prevent and treat osteoporosis (Khosla *et al.*, 2012).

At the tissue level, estrogen reduces bone turnover. It would appear that osteocytes may regulate the activation of bone remodeling via connections with bone lining cells. It is likely that the antiremodeling effects of estrogen are mediated via the

osteocyte. Osteocytes are cells embedded within the bone matrix, derived from the osteoblast that helps to bone remodeling (Tulay, 2015).

It was reported that osteoblasts, osteocytes and osteoclasts express functional estrogen receptors (ERs). These receptors are also expressed in bone marrow stromal cells (SCs), the precursors of osteoblasts. Estrogen signals through two receptors ER alpha and beta, Bone cells contain both receptors, but their distributions within bone are not homogeneous. In humans, ER alpha is the predominant in cortical bone, but ER beta is predominant in trabecular bone (Tulay, 2015).

Estrogen suppresses both directly and indirectly bone resorption. The dominant acute effect of estrogen is blocking the new osteoclast formation. In addition, estrogen also modulates RANK (Receptor activator of nuclear factor κB) signaling in osteoclastic cells and induces apoptosis of osteoclasts (Tulay, 2015).

Estrogen deficiency induces bone loss and increases in production of cytokines interleukin-1, interleukin-6, tumor necrosis factor (TNF-a) and granulocyte/macrophage colony stimulating factor (GMCSF) these cytokines appears to associated with increased stimulation of bone resorption which then leads to increased bone loss and a reduction in BMD (Tulay, 2015).

Finally, estrogen important for the maintenance of bone formation. Human studies show that acute estrogen deficiency is associated with a fall in bone formation markers ,Chronically estrogen deficient increased both bone-resorption and bone-formation markers (Tulay, 2015).

2.3.3.2 Androgen

Sex steroids (estrogen and testosterone) are other important factors for bone metabolism in both women and men. In vitro and in vivo studies indicate estrogens and androgens act via different cellular mechanisms. The bone-sparing effect of estrogen is antiresorption by inhibition of osteoclast activity. The skeletal effect of androgens may be partly mediated by local aromatization to estrogen. However,

there are also data that support a direct androgen action on bone. The presence of specific androgen receptors in cultured osteoblasts has been reported. Androgens were shown to stimulate proliferation and differentiation of osteoblasts and to inhibit apoptosis (Huong *et al.*, 2014).

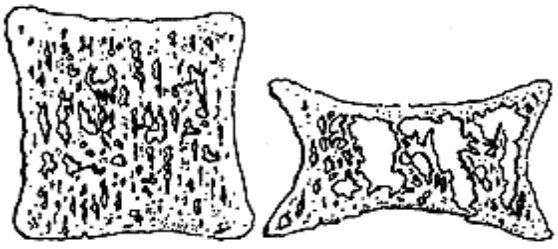
Men with gonadal insufficiency have lower BMD than healthy subjects of the same age and testosterone replacement therapy has been suggested to reverse this condition (Huong *et al* ., 2014).

2.4 Osteoporosis can be defined as

Osteoporosis is a progressive systemic skeletal disease characterized by reduced bone mass/density and microarchitectural deterioration of bone tissue, It is a "silent disease" as there are no symptoms prior to a fracture (Ferdous *et al* .,2016).

According to the World Health Organization osteoporosis is considered a silent epidemic of the century, causing great economic and social impacts (Moreira *et al*., 2017).

Literal meaning of osteoporosis is "porous bone". It is a multifactorial skeletal disorder which leads to low bone mineral density and is the major cause of fracture incidences, disabilities, reduced mobility and poor quality of life (hirani *et al.*, 2014).



(A) (B)

Figure (1): Normal Bone (A) **Vs** Osteoporotic Bone (B) (Huda ,2008)

2.4.1 Etiology of osteoporosis

Bone strength reflects the integration of two main features bone density and bone quality. Many factors contribute to the risk of osteoporotic fractures, all of which should be taken into account in the assessment of fracture risk in patients (Lane, 2006).

The final clinical outcome of the osteoporotic process is a fracture, which can occur as a result of minimal trauma or even spontaneously. At present low bone mass is regarded as the main contributor to bone fragility, but possible qualitative changes in the bone matrix must also be considered. Two factors which determine the level of bone mass at any age are the obtained peak bone mass and duration and rate of bone loss. Peak bone mass is achieved during the first three decades of life. Genetic and nutritional factors as well as mechanical stress on the skeleton obviously play crucial roles in determining peak bone mass. Two phases of bone loss age-related and menopause related dictate the final bone mass at old age (Huda, 2008).

Postmenopausal osteoporosis is a particular example of unbalanced bone resorption leading to net bone loss. An increasing number of systemic and local factors have been found to participate in the regulation of bone remodeling (Huda, 2008).

2.4.2 Osteoporosis Symptoms

Osteoporosis occurs "silently" and progressively and there are often no symptoms until the first fracture occurs. The most common osteoporotic fractures occur at the hip, spine and wrist. A hip fracture often results in disability and higher mortality vertebral fractures may have serious consequences, such as loss of height, intense back pain and deformity. In this context it is important to bear in mind that many fractures characterised as osteoporotic are in fact caused by falling, a known strong risk factor for fracture (Hallström, 2013).

2.4.3 Classification of Systemic Osteoporosis

It is conventional to classify osteoporosis as either primary or secondary osteoporosis. Primary osteoporosis includes involutional (postmenopausal and age-

related) forms and so-called idiopathic osteoporosis of premenopausal women and young or middle-aged men. Secondary osteoporosis arises in response to an identifiable catalyst, i.e. it is secondary to an underlying condition or therapeutic treatment (e.g. resulting from long-term corticosteroid therapy, rheumatoid disease, chronic liver disease or intestinal malabsorption) (Theobald, 2005).

2.4.3.1 Primary osteoporosis

Primary osteoporosis is the most common type of osteoporosis. It is more common in women than men and can also be divided into two subgroups:

2.4.3.1.1 Involutional Osteoporosis Type I

It is also known as postmenopausal osteoporosis, caused by the deficiency of estrogen, mainly affecting the trabecular bone , therefore women are more susceptible to osteoporosis than men (Sözen, Özışık and Başaran, 2017).

2.4.3.1.2 Involutional Osteoporosis Type II

It is also called senile osteoporosis, and it is related to bone mass lost due to the aging of cortical and trabecular bones (Sözen, Özışık and Başaran, 2017).

2.4.3.2 Secondary osteoporosis

Secondary osteoporosis results from many causes that contribute significantly to accelerated bone loss, these causes includes: Lifestyle changes (Vitamin D insufficiency, High salt intake alcohol abuse, Low calcium intake), Genetic diseases (Cystic fibrosis, Glycogen storage diseases Gaucher's disease), Endocrine disorders (Central obesity , Cushing's syndrome , Diabetes mellitus "types 1 and 2" , Hyperparathyroidism , Thyrotoxicosis), Others (AIDS/HIV , Congestive heart failure , End-stage renal disease , Systemic lupus , Other rheumatic and autoimmune diseases) (Sözen, Özışık and Başaran, 2017).

2.4.3.3 Juvenile Idiopathic osteoporosis

Juvenile Idiopathic osteoporosis is very rare condition of primary bone demineralization, the exact prevalence is unknown, and diagnosis based on clinical

presentation, skeletal X-ray, BMD and exclusion of other common causes of osteoporosis in this age, The exact pathogenesis of this condition is unknown but there is very low bone formation rate and decreased bone volume have been described (Gemma and Maria, 2015).

2.4.5 Risk factors of osteoporosis

The risk factors of osteoporosis are divided into controllable and uncontrollable risk factors:

2.4.5.1 Uncontrollable Risk factor

2.4.5.1.1 Gender

i.e. Being female ,Women are five times more likely to develop osteoporosis than men (Vanessa *et al* .,2018) Gender differences can affect the prevalence and severity of osteoporosis ,Beginning at age 40 both sexes lose axial bone mass at relatively slow rates, but women lose bone mass more rapidly because of the onset of menopause in the late 40s or early 50s, contributing to increased risk of fracture to the axial skeleton. For men, who do not experience the sudden loss of gonadal sex steroid secretion, the reduction of reproductive hormones is more gradual, and bone loss occurs at a slower rate (Oleson and Morina, 2017).

2.4.5.1.2 Advancing age

Age is one of the non-modifiable risk factors of osteoporosis. Elderly are at high risk of getting osteoporosis and osteoporotic fracture. Age was reported to be the main factor that contributes to osteoporosis, especially with the age of 70 years and above. The BMD reduces with age resulting in thinning of the bone. Low BMD was associated with higher incidence of osteoporotic fracture in elderly more than 50 years old, Around 90% of patients suffering from hip fracture were above 50 years old. The high incidence was believed to be related to deterioration of the bone mineral density with advancing age (Ahmad *et al*., 2015).

2.4.5.1.3 Race (ethnicity)

Caucasian and Asian women are at highest risk while African and Hispanic women are at low risk (Vanessa *et al*., 2018).

2.4.5.1.4 Family history of osteoporosis

Osteoporosis can be categorized as a genetic disease. Therefore, genetic make-up can be one of the non- modifiable risk factors for osteoporosis and osteoporotic fracture. This is because BMD is highly correlated with heredity ,Genetic factors accounted for 50% of the variance in BMD across the populations (Ahmad *et al*., 2015).

2.4.5.1.5 Menopause

Menopause is a phase in a woman's life, not a disease. Between the ages of 45-55, a woman's ovaries slow down and in time stop producing an egg every month. A woman's body also begins to make female hormones (estrogen and progesterone) in smaller amounts. When Menopause occurring before the age of 45 years is regarded as premature (early onset) menopause. Women who undergo an early menopause, potentially start to lose bone at a significantly earlier age than women who undergo menopause in their fifties. This puts them at a greater risk of developing osteoporosis at an earlier age, than women who undergo menopause at midlife (Huda, 2008).

2.4.5.1.6 Amenorrhea

This condition typically affects athletes women who do endurance activities or ballet dancers with low body weight and intense exercising. Studies show that women with amenorrhea 20 to 30 percent less bone mineral content than those with regular cycles. The condition is associated with faster bone resorption seen with estrogen deficiency and low body weight (Huda, 2008).

2.4.5.2 Controllable Risk factor

2.4.5.2.1 Nutrition

The nutrients that have received the bulk of attention are vitamin D and calcium, two pivotal contributors to bone mineralization , there is certainly evidence that fracture

risk is increased with vitamin D levels below 50 nmol/l .Interestingly, randomized intervention studies have shown that neither vitamin D supplementation nor calcium supplementation on its own reduce the risk of osteoporotic fractures (Abrahamsen *et al*., 2014).

2.4.5.2.2 Smoking

Smoking has been identified to be one of the risk factor for osteoporosis since 20 years ago Some studies have shown that there is a strong relationship between tobacco use and low bone density. Smoking is also an established risk factor for osteoporotic fracture. Current and former smokers have higher risk of low BMD. Therefore, smoking has been recognized to cause poor bone health. Female smokers lost around 5 to 10 % of bone tissue more than female non-smokers when they reach menopause (Ahmad *et al*., 2015).

2.4.5.2.3 Lack of exercise

Physical exercise, simple exercise and activities such as weight bearing and resistance training improved bone strength, encouraged bone growth and preserved bone mass. Regular exercise may also increase bone mineral density (BMD). Lack of exercise or sedentary lifestyle is a risk factor for osteoporosis (Ahmad *et al* .,2015),recent studies found Bedridden people lose bone faster than people who exercise regularly (Vanessa *et al.*, 2018).

2.4.5.2.4 Medications

Several medications have been shown to contribute to the development of osteoporosis Glucocorticoids (corticosteroids), the leading secondary cause of osteoporosis, decrease bone formation by downregulating osteoblasts and prolonging their life span. In addition, an inhibitory effect on sex hormones influences bone formation. When used chronically in high doses, glucocorticoids restrict intestinal vitamin D-dependent calcium absorption, increase calcium

excretion, and can cause osteomalacia, a softening of bone generally caused by vitamin D deficiency (Oleson and Morina, 2017).

2.4.5.2.5 Lack of exposure to sunlight

Vitamin D is produced through the exposure of skin to ultraviolet (UV) radiation in sunlight. However the amount of sun exposure required will vary based on season, location, area of skin exposed and skin type (Nowson *et al*., 2012).

2.5 Menopause

The term "menopause" comes from two Greek words that mean "month" and "to end". In other words, it translates as "the end of the monthlies" (Huda, 2008) Menopause is defined as the permanent cessation of menstruation resulting from the loss of ovarian follicular activity (Helena, 2014) It is the result of irreversible changes in the hormonal and reproductive functions of the ovaries, Hormonal fluctuations affect more than a woman's reproductive system .During menopause a woman's body slowly produces less of the hormones estrogen and progesterone. This often happens between ages 45 and 55, A woman has reached menopause when she has not had a period for 12 months in a row (Suri and Suri, 2016).

2.5.1 Stages of menopause

2.5.1.1 Perimenopause or (menopause transition)

Perimenopause can begin 8 to 10 years before menopause, when the ovaries gradually produce less estrogen. It usually starts in women at 40 but can start in the 30 as well, Perimenopause lasts up until menopause, the point when the ovaries stop releasing eggs in the last 1-2 years of Perimenopause, the drop in estrogen accelerates. At this stage, many women can experience menopause symptoms. Women are still having menstrual cycles during this time and can get pregnant (Cleveland, 2017). Perimenopause was assessed according to the WHO definition

as experiencing noticeable changes in the length and duration or amount of flow in the menstrual cycle (Helena, 2014).

2.5.1.2 Menopause

Menopause is point when a woman no longer has menstrual periods. At this stage, the ovaries have stopped releasing eggs and producing most of their estrogen. Menopause is diagnosed when a women has gone without a period for 12 consecutive months (Cleveland, 2017).

2.5.1.3 Post menopause

These are years after menopause, During this stage menopausal symptoms such as hot flashes can ease for many women , But as a result of a lower level of estrogen. Postmenopausal women are at increased risk for a number of health conditions such as osteoporosis and heart diseases. Medication such as hormone therapy and/or healthy life styles changes may reduce the risk of some of these conditions (Cleveland, 2017).

2.5.2 Symptoms of menopause

Menopause symptoms begin gradually while the ovaries are still functioning and a woman is still having menstrual periods. Some of symptoms are common such as:

Changes in period : time between periods or flow may be different (Questions, 2013).

Hot flashes ("hot flushes"): getting warm in the face, neck and chest with and without sweating (Questions, 2013).

Night sweats that may lead to problems sleeping and feeling tired, stressed or tense (Questions, 2013).

Vaginal changes : the vagina may become dry and thin, and sex may be painful (Questions, 2013).

Thinning of bones: which may lead to loss of height and bone breaks (osteoporosis) (Questions, 2013).

2.5.3 Causes of menopause

The menopause happens when the ovaries stop responding to certain hormones from the brain, and so eggs stop maturing regularly. There is a drop in the levels of estrogen and progesterone (the two female sex hormones produced by the ovaries). It is this fall in hormone levels that causes symptoms of menopause (Huda, 2008).

2.6 Bone mineral density

A bone mineral density test (BMD), a non-invasive and painless test, is the best way to determine bone health. BMD tests can identify osteoporosis, determine the risk for fractures and monitor the response to an osteoporosis treatment. Different BMD tests may measure the hip, spine, wrist, finger, shinbone or heel. The National Osteoporosis Foundation recommends BMD testing for the following individuals:

All women aged 65 and older regardless of risk factors.

Younger postmenopausal women with one or more risk factors.

Postmenopausal women who present with fractures (to confirm the diagnosis and determine disease severity).

Estrogen deficient women at clinical risk for osteoporosis.

Individuals with vertebral abnormalities.

Individuals receiving, or planning to receive, long-term glucocorticoids (steroid) therapy.

Individuals with primary hyperparathyroidism.

Individuals being monitored to assess the response or efficacy of an approved osteoporosis drug therapy.

2.6.1 Common bone mineral density tests

Dual Energy X-ray Absorptiometry (DEXA).

Peripheral Dual Energy X-ray Absorptiometry (pDXA).

Single Energy X-ray Absorptiometry (SXA)

Peripheral Quantitative Computed Tomography (pQCT).

Radiographic Absorptiometry (RA).

Quantitative Computed Tomography (QCT).

Quantitative Ultrasound (QUS) (Mythili, 2007).

2.7 Bone markers

2.7.1 Markers of bone formation

Bone formation markers are products of active osteoblasts expressed during different phases of their development and are considered to reflect different aspects of osteoblast function and bone formation. All markers of bone formation are measured in serum or plasma (Shetty *et al*., 2016).

Bone formation markers are categorized as:

By-products of collagen synthesis: Propeptides of type 1 collagen: (C-terminal: P1CP, N-terminal: P1NP) (Shetty *et al*., 2016).

Osteoblast enzymes: Alkaline phosphatase (ALP) (total and bone-specific) (Shetty *et al*., 2016).

Matrix proteins: Osteocalcin (OC) (Shetty *et al*., 2016).

2.7.1.1 Procollagen type I Propeptides

Procollagen Type I N-terminal propertied (PINP) and precollegen type I C-terminal propeptide (PICP) are peptides derived from posttranslational cleavage of type I procollagen molecules by proteases at N- and C-terminal, respectively. PINP and PICP originate predominantly from proliferating osteoblasts and fibroblasts with small contributions from skin, tendon, dentin, and cartilage.PINP is preferred for clinical use as a marker of bone formation to PICP as PICP, unlike PINP, is cleared by the mannose receptor, which in turn can be regulated by growth hormone and

thyroid hormones, thus complicating the interpretation in patients with pituitary or thyroid dysfunction. P1NP exists in serum as trimeric or monomeric form. Immunoassays detect either trimeric (automated IDS ISYSS assays) or both forms which are otherwise called as total P1NP assays (automated Roche Elecsys assay) (Shetty *et al*., 2016).

P1NP is proposed as a reference bone formation marker by IOF in view of its predictable response to treatment and the reliability of P1NP assays as evidenced by low intra-individual variability, smaller circadian variation, stability at room temperature, and a good assay precision (Shetty *et al*., 2016).

2.7.1.2 Serum alkaline phosphatase (Total and bone specific alkaline phosphatase)

ALP is membrane-bound tetrameric enzyme present in the plasma membrane of the osteoblasts. It plays an important role in osteoid formation and mineralization by enzymatic degradation of the inhibitor of mineralization, pyrophosphate at an alkaline pH (Shetty *et al*., 2016) Several isoforms of ALP have been identified in liver, intestine, placenta, and bone. Unlike the hepatic isoenzyme which is heat stable, that of bone origin is thermolabile (Shetty *et al*., 2016).

2.7.1.3 Osteocalcin

is a hydroxyapatite-binding protein exclusively synthesized by osteoblast and it constitutes 15% of the noncollagenous bone matrix, Being a late marker of osteoblastic activity, it has been used as a bone formation marker but is limited by its short half-life, unstable intact molecule. Osteocalcin has been found to be a useful biomarker in steroid-induced osteoporosis (Shetty *et al*., 2016).

2.7.2 Markers of bone Resorption

These markers which are formed during the bone resorption phase of bone remodeling include by products of osteoclasts activity released during bone

resorption (Shetty *et al*., 2016) The bone resorption markers are categorized as follows:

Collagen degradation products:

Telopeptides of type 1 collagen (C-terminal: CTX-1 and CTX-matrix metalloproteinases [MMP], N-terminal: NTX-1), Hydroxyproline, Pyridinium crosslinks (pyridinoline [PYD], deoxypyridinoline [DPD]).

Noncollagenous proteins: Bone sialoprotein (BSP).

Osteoclastic enzymes: Tartrate-resistant acid phosphatase, Cathepsin K.

Osteocyte activity markers: Receptor activator of nuclear factor kappa-B ligand (RANKL), Osteoprotegerin (OPG), Dickkopf-related protein 1, Sclerostin (Shetty *et al*., 2016).

2.7.2.1 Amino-terminal Crosslinked Telopeptide of type 1 collagen (CTX-1)

Telopeptides of type 1 collagen are extensively investigated and used bone resorption biomarkers included carboxy terminal crosslinked (CTX-1) and amino terminal crosslinked (NTX-1). CTX-1 and NTX-1 are both are released during collagen degradation. ELISA is used to measured CTX-1 with a monoclonal antibody against an octapeptide sequence (EKAHD-β-GGR) in the α-1 (I) chain of the β-isoform. Recent study has shown that CTX-1 is a specific and sensitive biomarker of bone resorption that can rapidly indicate the response to bisphosphonate therapy for postmenopausal osteoporosis. However, serum CTX-1 is influenced by food intake and blood withdrawal must take place in the fasting state because food intake substantially decreases the levels of CTX-1 (Kuo and Chen, 2017).

2.7.2.2 Amino-terminal Crosslinked Telopeptide of type 1 collagen (NTX-1)

NTX-1 is stable in urine at room temperature for up to 24 h and is usually quantified by ELISA with urine sample. The urinary NTX-1 has been used as a bone resorption biomarker to assess the risk of fracture in postmenopausal women . The urinary NTX-1 is selected as the preferred biomarker compared with serum CTX-1 for practical application because it is not affected by food intake and it prevents blood withdrawal (Kuo and Chen, 2017).

2.7.2.3 Hydroxyproline (HYP)

HYP is an amino acid derived from the post-translational hydroxylation of proline. HYP provides about 12–14% of the total amino acid content of mature collagen. During the degradation of bone collagen, about 90% of the HYP is released and then the HYP is primarily metabolized in the liver. The level of HYP has significantly increased in urine with postmenopausal osteoporosis women in comparison with the postmenopausal non osteoporosis women. The increase of urinary HYP indicates that the degradation of collagen type I from the bone matrix is raised in osteoporotic women. Although the HYP is principally used as a resorption biomarker (Kuo and Chen, 2017).

2.7.2.4 Pyridinoline (PYD)

Collagen crosslink of PYD is produced during the extracellular maturation of fibrillar collagens and released into the circulation from degradation of mature collagens. Previous study shows that the PYD exhibits long term chemical stability in both the free and conjugated forms by HPLC analyses. However, PYD is found in cartilage, bone, ligaments and blood vessels. Therefore, PYD is a non specific bone resorption biomarker in comparison with DPD (Kuo and Chen, 2017).

2.7.2.5 Deoxypyridinoline (DPD)

DPD is a molecule to mechanically stabilize collagen by crosslinking between individual collagen peptides. During the process of bone resorption, the crosslinked collagens are proteolytically broken down and then the DPD is released into the

circulation and excreted by urine. Most of DPD are found in the bone and dentin. Therefore, DPD is used as a specific biomarker for bone resorption. In previous work, the DPD has been pretreated with preanalytical hydrolysis and extraction before HPLC analysis because DPD is excreted in the urine in free (40%) and peptide-bound (60%) forms. To improve the accuracy, the peptide bound form is transferred into free form for the HPLC measurement. The drawbacks for HPLC measurement of DPD are complicated procedure and variable recovery .Recently, the automated chemiluminescence immunoassay and enzyme immunoassay have been developed for the direct detection of urinary free DPD. The experimental results of chemiluminescence immunoassay and enzyme immunoassay methods have shown the correlation with HPLC measurement of urinary free DPD. The measurements of free DPD in urine by the immunoassay approaches have provided the possibility for the clinical application in the monitoring of patients with bone pathology and metabolic bone disease (Kuo and Chen, 2017).

2.7.2.6 Bone Sialoprotein (BSP)

BSP is a phosphorylated glycoprotein with an apparent molecular weight of 60–80 kDa. BSP is an element of mineralized tissues such as bone, dentin, cementum and calcified cartilage. BSP is an important component of the bone extracellular matrix and has been demonstrated to for the formation of approximately 8% of all non-collagenous proteins found in bone and cementum. BSP is generated by osteoblasts, odontoblasts and osteoclasts. Therefore, BSP is considered as an important factor for cell-matrix adhesion processes and stimulation of osteoclast-mediated bone resorption. Many studies have developed immunoassays for the measurement of BSP in serum. For example, radioimmunoassay,BSP has shown great potential as a bone resorption biomarker for osteoporotic assessment (Kuo and Chen, 2017).

2.7.2.7 Tartrate-resistant acid Phosphatase 5b (TRAP 5b)

TRAP 5b is normally secreted by osteoclasts during bone resorption .Thus ,TRAP 5b is used as a reference for osteoclast activity and numbers. In the circulation, the hydrolyzed TRAP 5b is metabolized in the liver and then excreted in the urine. TRAP 5b can be specifically detected in serum by immunoassays. Previous report shows that serum TRAP 5b has been used to identify limited or extensive bone metastasis in breast cancer patients. Furthermore serum TRAP 5b has been applied to monitor the efficiency of alendronate treatment. The bone resorption biomarker of TRAP 5b is extensively studied and revealed good specificity and high sensitivity in comparison with other bone biomarkers (Kuo and Chen, 2017).

2.7.2.8 Cathepsin K (CTSK)

Cathepsins are members of the cysteine protease family with 11 isoforms CTSK is mainly expressed at the ruffled border of actively resorbing osteoclasts. Osteoclasts secrete CTSK into bone resorption defect for degradation of bone matrix proteins included type 1 collagen, osteopontin and osteonectin. Therefore, CTSK is an important factor in process of bone resorption. The level of CTSK has revealed significantly different between controls and patients with osteoporosis. The result indicates that serum level of CTSK could serve as a potential biomarker for fracture prediction and bone mineral density (Kuo and Chen, 2017).

2.7.2.9 Receptor activator of NF-κB ligand (RANKL)

During the process of bone remodeling, osteoblasts produce RANKL and OPG to regulate the differentiation and maturation of osteoclasts. Serum levels of RANKL from humans have been observed for assessments of the states in metabolic bone diseases. Although the serum RANKL has been studied for fracture risk prediction and evaluation of the response from osteoporosis treatment, many works still need to be investigated for the clinical application of RANKL (Kuo and Chen, 2017).

2.7.2.10 Osteoprotegerin (OPG)

OPG is generally considered to be a secreted soluble receptor and is produced by many different tissues and cell types including osteoblasts. The role of OPG is used as a decoy receptor for RANKL and inhibitor of osteoclastogenesis. Studies in mice have revealed that the OPG knockout mouse develops severe osteoporosis, whereas the overexpression of OPG in transgenic mouse models and OPG treatment of normal mice leads to osteopetrosis. OPG can be measured in serum, plasma EDTA, citrate and heparin samples. There are commercially available sandwich ELISA assays for analyzing OPG by using a monoclonal capture and polyclonal detection antibodies. However, the clinical use of serum OPG as a biomarker for evaluation of bone disease activity still needs additional demonstration (Kuo and Chen, 2017).

2.7.2.11 Dickkopf-1 (DDK-1)

DKK-1 and sclerostin are the inhibitors of Wnt signaling and are applied as bone remodeling biomarkers. DKK-1 is produced by osteoblasts and is secreted into circulation. The serum levels of DDK-1 reflect the inhibition of bone formation, DKK-1 has shown the correlation with the BMD of the femoral neck and of the total hip. Further long-term studies are necessary to identify the clinical application of the regulator DKK-1 as a biomarker for assessment of osteoporosis (Kuo and Chen, 2017).

2.7.2.12 Sclerostin

In the presence of sclerostin, the Wnt pathway is downregulated and consequently osteoblastic differentiation is inhibited. Sclerostin is produced by osteocytes. Sclerostin is secreted into circulation, and serum levels reflect inhibition of bone formation. However, the clinical trial is further needed for the use of sclerostin as a biomarker of bone turnover (Kuo and Chen, 2017).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Materials

3.1.1 Study design

Cross sectional case control study to evaluate 25-OH vitamin D, total calcium and phosphorus levels among Sudanese postmenopausal Women with Osteoporosis.

3.1.2 Study area and period

The study carried out in Khartoum state, in the period (March to October 2018).

3.1.3 Sample population

The study involved 50 osteoporotic patients as a test group and 50 healthy subjects as a control group, were enrolled in this study, both groups were sex matched.

3.1.4 Inclusion criteria

The target populations Sudanese post-menopausal with Osteoporosis.

3.1.5 Exclusion criteria

People with other types of bone diseases.

3.1.6 Ethical considerations

This study approved by the Ethical committee of Sudan university of Science and technology and agreement of general hospitals, private centers and all participants known with aim of study.

3.2 Methods

3.2.1 Sample Collection

About 5 ml of venous blood was obtained using disposable needle and syringes, each sample was centrifuged at 4000 (rpm) for 5 min and the plasma was separated and stored at -20c until analysis.

3.2.2 Vitamin D estimation principle

The ELISA Kit is designed for the in vitro determination of 25-OH vitamin D in human serum or plasma samples, in the first analysis step the calibrators and patient samples are diluted with biotin-labelled 25-OH vitamin D and added to microplate wells coated with monoclonal anti 25-OH vitamin D antibodies. During the incubation, an unknown amount of 25-OH vitamin D in the sample patients and a known amount of biotin-labelled 25-OH vitamin D compete for the antibody binding sites in the microplate wells plate. Unbound 25-OH vitamin D is removed by washing. For the detection of bound biotin-labelled 25-OH vitamin D, a second incubation is performed using peroxidase-labelled streptavidin. In a third incubation using the peroxidase substrate tetramethylbenzidine (TMB) the bound peroxidase promotes a colour reaction. The colour intensity is inversely proportional to the 25-OH vitamin D concentration in the sample. Result for the samples can be calculated directly using standard curve (Appendix (2)).

3.2.3 Calcium estimation principle

Calcium in the sample reacts with methylthymol blue in alkaline medium forming a coloured complex that can be measured by spectrophotometry, hydroxyquinoline is included in the reagent to avoid magnesium interference (Appendix (3)).

3.2.4 Phosphorus estimation principle

Inorganic phosphorus in the sample reacts with molybdate in acid medium forming a phosphomolybdate complex that can be measured by spectrophotometry (Appendix (4)).

3.2.5 Quality control

The results of the EUROIMMUN 25-OH vitamin D ELISA correlate excellently with target values in the vitamin D external Quality Assessment Scheme (DEQAS), which were assigned by the National institute of Standard and Technology (NIST) Reference Measurement Procedure (RMP).

3.3 Statistical analysis

Statistical analysis was performed using SPSS (Statistical package for the Social sciences) software (version 20). The mean and standard deviation (SD) for variables of the test and control group was obtained independent t-test was utilized for comparison between quantitative variables of the two groups and p-value less than 0.05 was considered Significant.

Chapter Four

Results

4. Results

In our study, 50 individuals with Osteoporosis participated and 50 healthy individuals as control match with case in sex and presence of diseases, the mean of age was 64.46±14.12 and 51.62±13.12 of both group case and control respectively (Table 1), study observed percentage of cases have decreased Serum 25-OH Vitamin D level less than control. The mean serum level of 25-OH Vitamin D was 41.9± 23.6 and 43.0±16.0 ng/ml of both case and control respectively and observed percentage of cases have decreased total calcium level less than control. The mean serum level of total calcium was 7.31 ± 2.28 and 9.03 ±1.20 mg/dl of both case and control respectively, and observed percentage of cases have increased phosphorus level more than control. The mean serum level of phosphorus was 4.47±1.13 and 4.00± 1.09 mg/dl of both case and control respectively (Table 2). The study also showed insignificant correlation between age and 25-OH vitamin D (R= -0.11, p.value= 0.938) in case group (Figure 2), and also showed insignificant correlation with total calcium (R= 0.206, p.value = 0.151) (Figure 3), and insignificant correlation with phosphorus (R= 0.118, p.value = 0.414) in cases group (Figure 4).

Our study also showed insignificant correlation (R= 0.181, p. value 0.209) between 25-OH Vitamin D and total calcium levels in cases group (Figure 5) and also showed insignificant correlation (R= 0.074, p. value 0.610) between 25-OH Vitamin D and phosphorus levels in cases group (Figure 6).Study showed insignificant correlation (R= - 0.059, p. value 0.683) between total calcium and phosphorus levels in cases group (Figure 7).

Table 4.1 showed Demographic characteristics Age (years), weight (Kg), height (cm) and BMI (Kg/m^2) of cases and control were highly significant in Osteoporotic patients comparing with healthy population, result expressed as (M±SD), minimum and maximum.

Table 4.1 Shows Demographic characteristics of cases and control

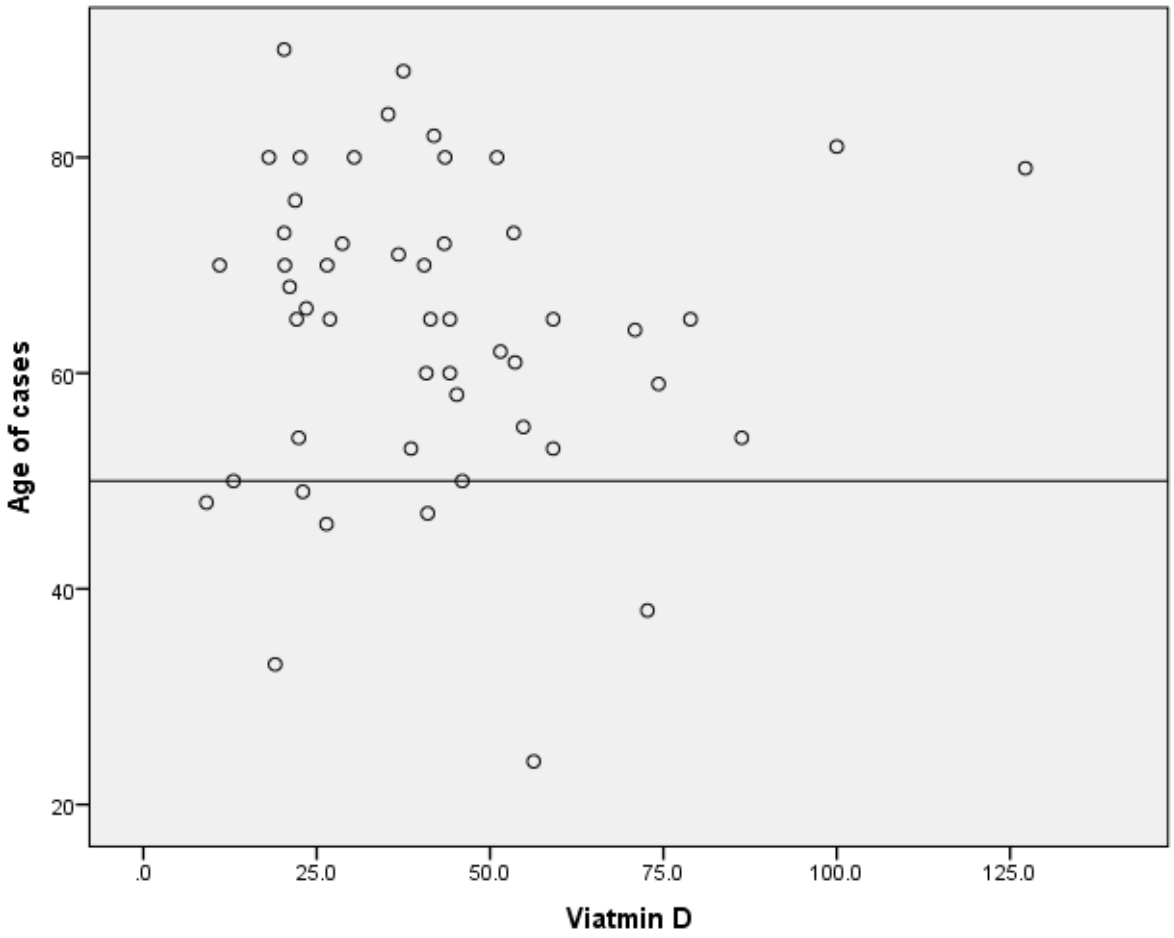
characteristics	**Mean ± Standard deviation Case and Control**		**Minimum Case and Control**		**Maximum Case and Control**	
Age (years)	**64.46±14.12**	**51.62±13.12**	**24**	**26**	**90**	**81**
Height (cm)	**155±9.8**	**159±7.3**	**126**	**146**	**178**	**179**
Weight (Kg)	**71.7±14.3**	**70.8±16.7**	**42.5**	**36**	**93.7**	**108**
BMI (Kg/m^2)	**29.7 ± 5.93**	**27.8 ± 6.19**	**19.3**	**15.1**	**45.3**	**41.1**

Table 4.2 Shows mean of parameters level in study group classified as case and control groups. Result expressed as (M±SD).

Variable	Case	Control	p. value
25-OH Vitamin D (ng/ml)	41.9 ± 23.6	43.0 ± 16.0	0.000
Calcium (mg/dl)	7.31 ± 2.28	9.03 ± 1.20	0.000
Phosphorus (mg/dl)	4.47 ± 1.13	4.00 ± 1.09	0.000

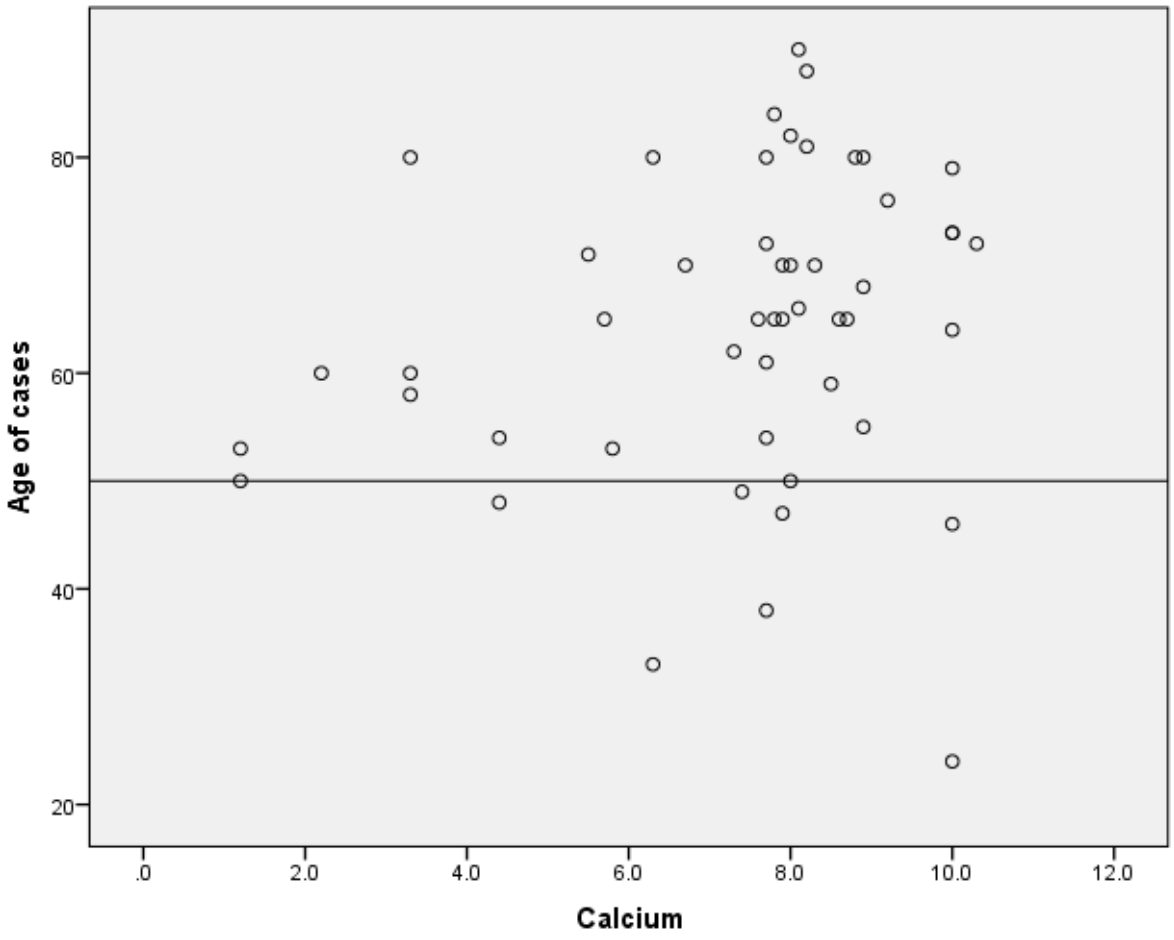
Table 4.3 shows frequencies of cases and controls according to 25-OH Vitamin D levels.

25-OH Vitamin D Concentration	**Number of cases N= (50)**	**Frequencies of cases % (100%)**	**Number of control N= (50)**	**Frequencies of control % (100%)**	**ng/ml**
Very severe vitamin D deficiency	**0**	**0**	**0**	**0**	**< 5**
severe vitamin D deficiency	**1**	**2**	**1**	**2**	**5 - 10**
Vitamin D deficiency	**4**	**8**	**2**	**4**	**10 - 20**
Suboptimal vitamin D provision	**14**	**28**	**9**	**18**	**20 - 30**
Optimal vitamin D level	**16**	**32**	**19**	**38**	**30 - 50**
Upper normal	**8**	**16**	**16**	**32**	**50 - 70**
Over dose , but not toxic	**7**	**14**	**3**	**6**	**70 - 150**
Vitamin D intoxication	**0**	**0**	**0**	**0**	**> 150**



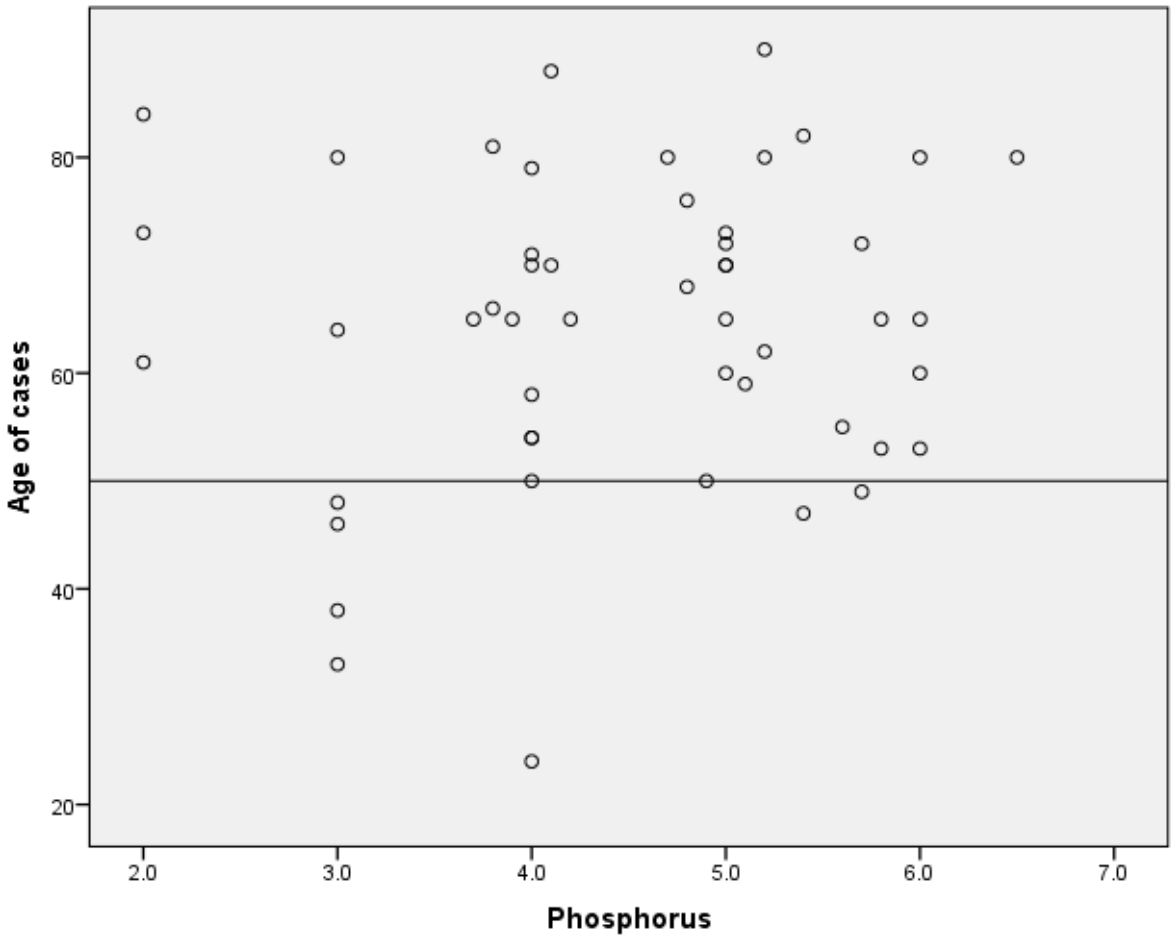
R= -0.11 p.value = 0.938

Figure (2) A scatter plot shows a correlation between age and 25-OH vitamin D levels in case group.



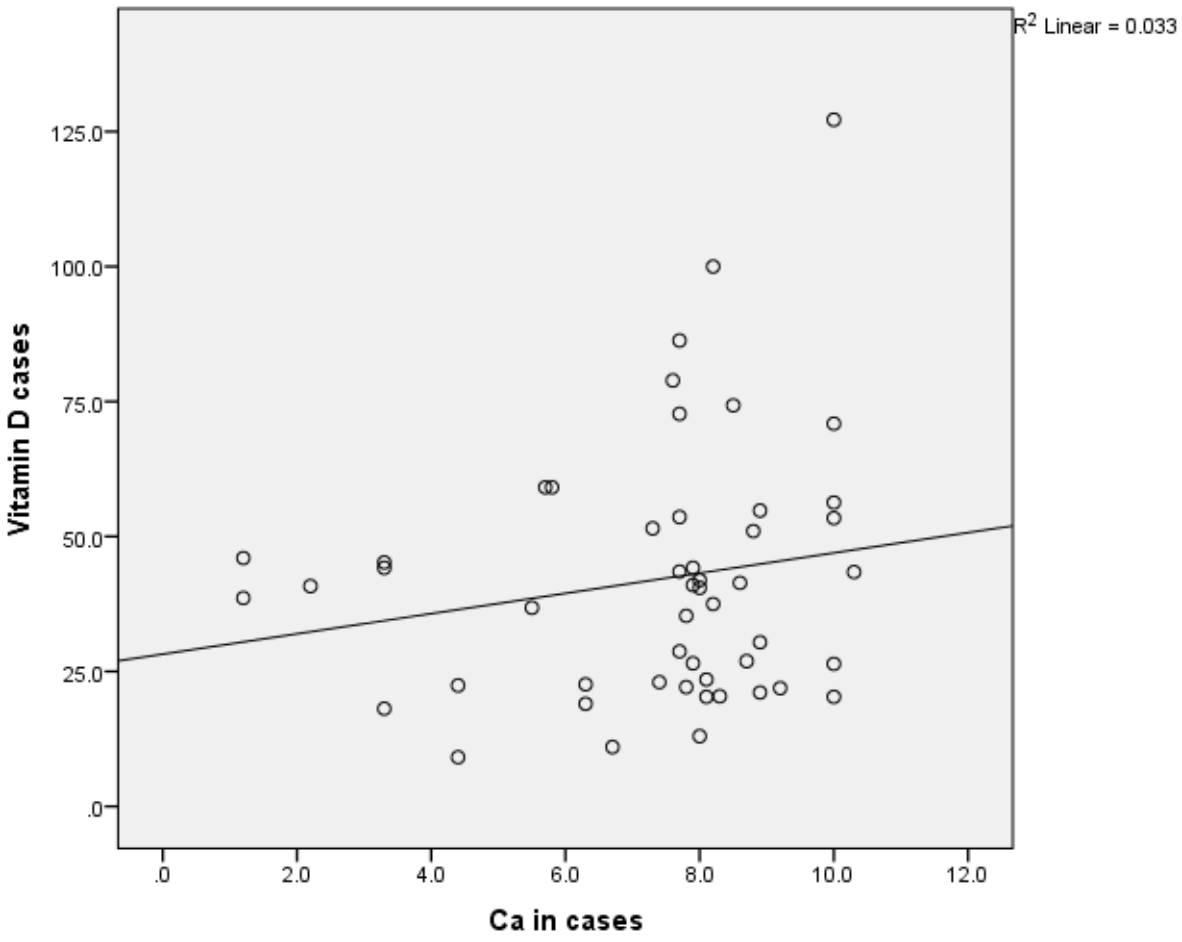
R= 0.206 p.value = 0.151

Figure (3) A scatter plot shows a correlation between age and total calcium levels in case group.



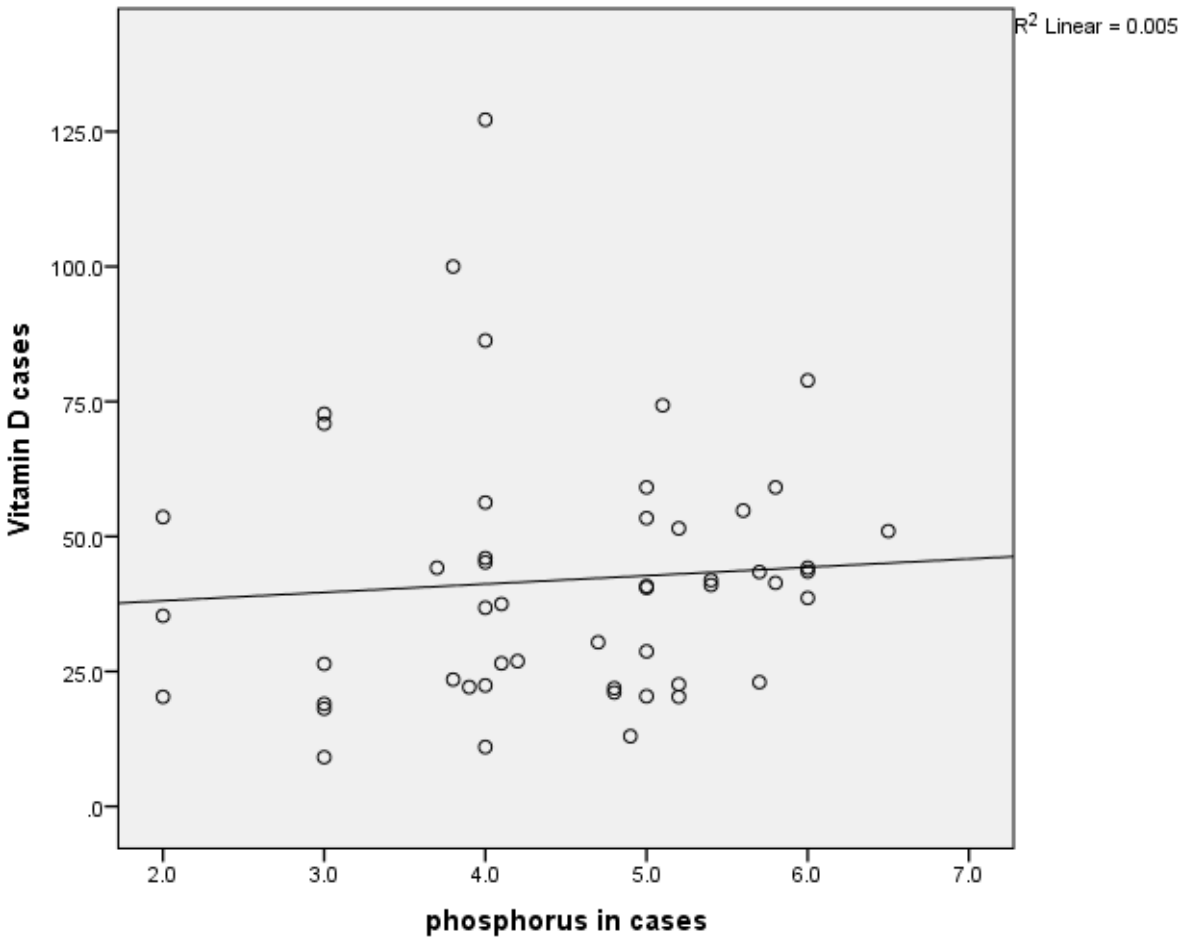
R= 0.118 p.value = 0.414

Figure (4) A scatter plot shows a correlation between age and phosphorus levels in case group.



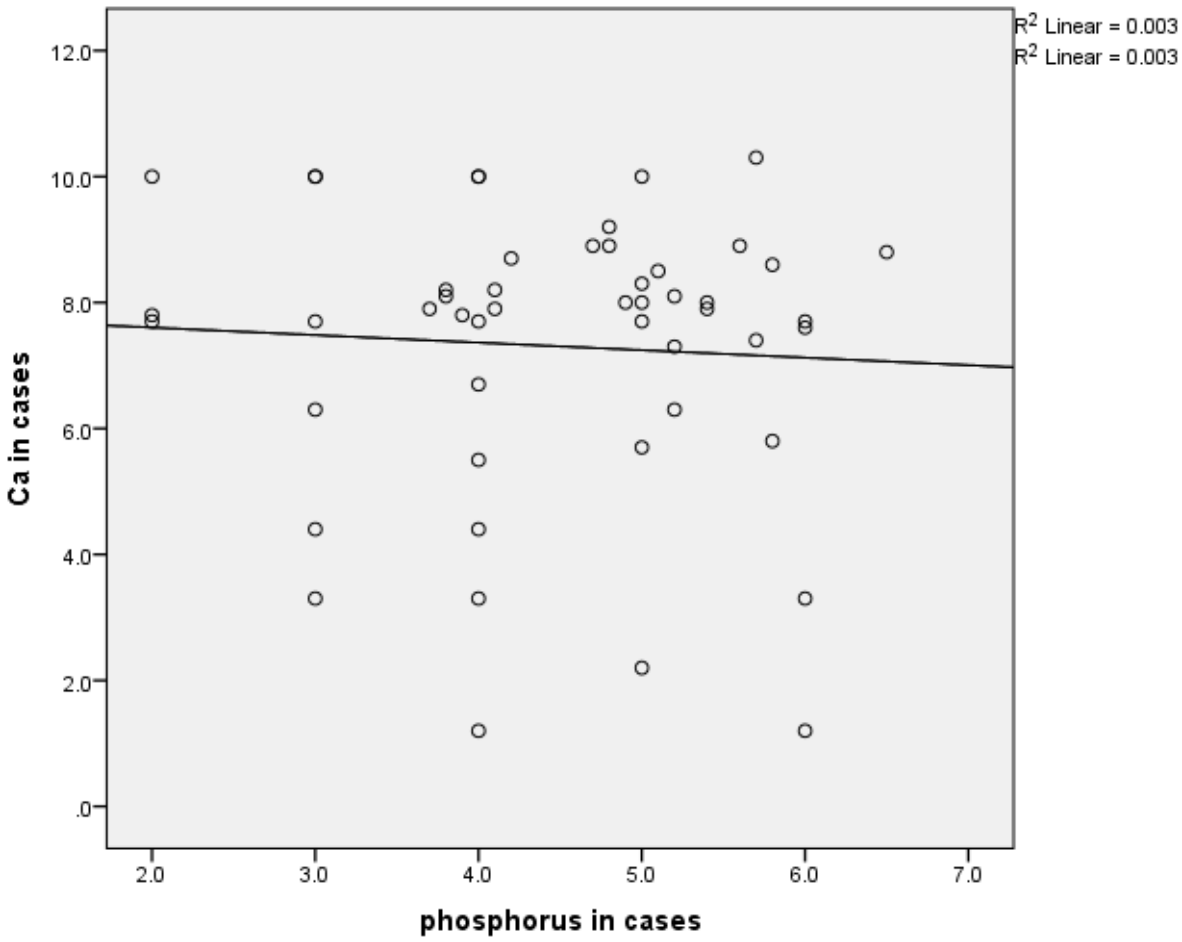
R= 0.181 p. value 0.209

Figure (5) A scatter plot shows a correlation between 25-OH Vitamin D and total calcium levels in case group.



R= 0.074 p. value 0.610

Figure (6) A scatter plot shows a correlation between 25-OH Vitamin D and phosphorus levels in case group.



R= - 0.059 p. value 0.683

Figure (7) A scatter plot shows a correlation between total calcium and phosphorus levels in case group.

Chapter Five

Discussion, Conclusion and Recommendations

5.1 Discussion

Osteoporosis effects both bone quantity and quality with thinning in cortical bone and cavity expansion due to bone absorption at the endosteum surface as well as reduced bone density. It leads to enhanced bone fragility and an increase in fracture risk, the goal of this study was to evaluate the relationship between 25-OH vitamin D, total calcium and phosphorus and osteoporosis in Sudanese post-menopausal women, since there were positive relationship between these parameters and bone strength (Wkh *et al.*, 2016).

In the current study BMI of osteoporosis group were significantly higher than control group (p.value 0.000), the finding was relevant to finding of Act and Ali (2018) who found BMI significantly increase in postmenopausal women with osteoporosis compared to healthy women and also consistent to finding of another study done by Indumati *et al* (2013), these results are also similar to the study of shakoor (2015) who observed the same finding, and this may be due to increases of age decreases the metabolic rate and fat formation and deposition in adipose tissues also increased which leads to weight gain.

In the present study, highly significant decrease of 25-OH vitamin D level in case group when compared with control group with a significant 'p' value of <0.001. This study agreed with the works of Alkhenizan *et al* (2017) who demonstrated the lower level of 25-OH vitamin D among osteoporosis patients, and also agreed with another study done by Beg *et al* (2014), The decrease of serum vitamin D levels may be due to less outdoor activities of the women also with decrease exposure to sun light and due to our habits in wearing long dress this will prevent the vitamin D in the skin to induce and convert to the active form which is the major storage form of vitamin D consecutively it is important for calcium and phosphorus absorption, when it is level decrease lead to increase parathyroid hormone which lead to decrease calcium absorption then effect on bone health.

The data in this study showed highly significant decrease (p.value 0.000) in total calcium level in case population when compared to healthy population, observed result agreed with study done by East *et al* (2015) who conducted a study in India and found that older postmenopausal women with osteoporosis had significant lower serum total calcium level, this may be duo to Ageing and loss of estrogen which leads to a significant increase in osteoclastic activity. In addition decrease in calcium intake or impaired absorption of calcium from the gut lowers the serum level of calcium. Decrease could be due to decline in either the active calcium transport or

diffusion component of the calcium absorption system, probably a result of loss of direct effect of estrogen on calcium transport in gastro intestinal tract. Deficiency of calcium and malabsorption due to hormonal imbalance may lead to disorder of bone mainly osteoporosis.

In the current study serum phosphorus showed significant increase (p.value 0.000) in case group when compared with control group. The value in the present study are in consistent with the study of Act and Ali (2018) and also consistent with work of East *et al* (2015). Phosphorus and calcium are regulated mainly by two hormones PTH and active form of vitamin D, there for any interference with action of PTH can lead to lowering serum calcium and increase of the serum phosphorus.

The study also observed insignificant correlation between age and 25-OH vitamin D (p. value 0.938), and total calcium (p. value 0.151), and insignificant correlation with phosphorus (p. value 0.414), this finding disagree with finding of Shakoor (2015) who demonstrate the significant correlation between age and these parameters.

The study also demonstrate insignificant correlation between 25-OH vitamin D and total calcium (p.value 0.209) and phosphorus (p. value 0.610). the result was similar to finding of Narang *et al* (2004) who found no correlation between 25-OH vitamin D level and total calcium and phosphorus levels.

The present study also had observed insignificant correlation (p. value 0.683) between serum total calcium and phosphorus level, this finding was similar to finding of Mahdavi and Khabazi (2018) who found no correlation between total calcium and phosphorus levels.

5.2 Conclusion

Our study conclude that 25-OH vitamin D and serum total calcium decrease with age in Sudanese post-menopausal women with Osteoporosis. Body mass index and serum phosphorus increases with age in Sudanese post-menopausal women with Osteoporosis, Meanwhile no associations were observed between age and study variables or association between 25-OH vitamin D and total calcium or phosphorus.

5.3 Recommendations

As Osteoporosis are very common among postmenopausal women in Sudan so following, recommendations and strategies can be useful to reduce the incidences:

It is recommended that every woman above 30 should take Calcium and vitamin D supplements and fortified foods.

In addition, screening should be done after menopause to prevent from consequences.

Create awareness about osteoporosis and its consequences and encourage people for taking steps for prevention and treatment before reaching severe conditions.

Exercise, walking daily for 30 minutes and weight bearing exercises on a treadmill are helpful in prevention and treatment.

Preclinical assessment, clinical trials for osteoporosis should be taken into account according to WHO guidelines.

References

- **Afsheen H, Arifa F, Muniza, Neelum, Sania R, Umer H, Zoya K (2014).**Osteoporosis in Post-Menopausal Women, *I-manager's Journal on Nursing*, Volume (2), Issue (4) ; pages (7-8)

- **Aline L. Bueno, Mauro A. Czepielewski (2008).**"The Importance for Growth of Dietary Intake of Calcium and Vitamin D" *Jornal de Pediatria*. Volume (84) Issue (5):pages: (386–394).

- **Balwant Chauhan and Prashant Sakharkar (2017).** 'Role of vitamin D Receptor (VDR) Gene. *World Journal of pharmacy and pharmaceutical Sciences.* Volume 6, Issue 7; pages (1083-1095).

- **Beg M, N Akhtar, MF Alam, I Rizvi, J Ahmad, A Gupta (2014)** 'Vitamin D status and serum osteocalcin levels in postmenopausal osteoporosis : Effect of bisphosphonate therapy, *Journal, Indian Academy of Clinical Medicine* , Volume (15), Issue (4) ;pages (172–176).

- **Bo Abrahamsen ,Dorthe B, Katrine H and Peter S (2014).**" A Review of Lifestyle , Smoking and Other Modifiable Risk Factors for Osteoporotic Fractures." *BoneKEy Reports* 3 (July). Nature Publishing Group, Volume (3), Issue (7); pages (1–7).

- **Christina V. Oleson and Amanda B. Morina (2017). "**Causes and Risk Factors of Osteoporosis" *Springer International Publishing Switzerland*, Osteoporosis Rehabilitation book, chapter (2); pages (5-14).

- **Cleveland clinic foundation (2017)**."perimenopause menopause and menopause" *American College of Rheumatology,*Volume (69), Issue (8); pages (1521-1537).

- **East I, G district, A pradesh (2015)** 'A Study of Bone Markers (Serum Calcium ,Serum Phosphorus And Serum Alkaline Phosphatase) In Post Menopausal Women', *Journal of Dental and Medical Sciences (IOSR-JDMS)* Volume (14), Issue (6):pages (1–3).

- **Ferdous HS, Afsana F, Qurishi NK, Rouf RSB (2016). "**Osteoporosis (Review)".*Birdem Medical Journal,* Volume (5), Issue (1): pages (30-36).

- **Flávia G, Cândido and J Bressan (2014). "**Vitamin D :Link between Osteoporosis , Obesity , and Diabetes " *International Journal of Molecular Sciences*, Volume (15), Issue (1): pages(6569–6591).

- **Food and Drug Administration (FDA) (2013)** "Common Questions" *Menopause & Hormones Department of Health and Human Services,* Volume (6), Issue (4): pages (1-3).

- **Gemma marcucci , Maria luisa Brandi (2015)**."Rare causes of osteoporosis"(Mini-review) *Clinical cases in mineral and bone metabolism*, Volume (12), Issue (2) ,pages :(151–156).

- **Hannah Theobald (2005)** "Dietary Calcium and Health," *British Nutrition Foundation journal*, London, UK , Volume (30), Issue (5) ;pages (237–277).

- **Helena Hallström (2013)**."*Coffee Consumption in Relation to Osteoporosis and Fractures Observational Studies in Men and Women"* Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. Acta Universitatis Upsaliensis Uppsala.

- **Helena Rubinstein (2014).**"The Meanings of Menopause : Identifying the Bio-Psycho-Social Predictors of the Propensity for Treatment at Menopause." thesis submitted for the degree of Doctor of Philosophy. Lucy Cavendish College , The University of Cambridge.

- **Huda Mahmoud Hania (2008).** "Occurrence of Osteoporosis Among Menopausal Women in Gaza Strip" Islamic University – Gaza.

- **Huong T. T. Nguyen ·Bo von Schoultz , Tuan V. Nguyen · Trinh X. Thang · Tran T. Chau Pham T. M. Duc · Angelica L. Hirschberg (2014).**"Sex Hormone Levels as Determinants of Bone Mineral Density and Osteoporosis in Vietnamese Women and Men," *The Japanese Society for Bone and Mineral Research and Springer Japan.*

- **J.C.Gallaghera and Sri H (2013).**"Prevention and Treatment of Postmenopausal Osteoporosis." *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier Ltd, Volume (1), Issue (1):pages (1–16).

- **Jeri W Nieves(2005).**Osteoporosis and the role of micronutrients, *American Society for Clinical Nutrition*,pages (1232–1239) Volume 81,www.ajcn.org.

- **J Beto, J Beto (2015).** "The Role of Calcium in Human Aging," (Review Article)*TheKoreanSocietyofClinicalNutritiojournal*, Volume (1), Issue (1):pages (1-2).

- **L. Fialová & M. Vejražka. translated and edited by J. Pláteník (2017).** "Calcium and Phosphorus Metabolism of Bone Tissue ."*General Medicine journal*, Volume (1), Issue (1): pages (1-10).

- **Mahdavi, M. and Khabazi, A. (2018)** 'Serum osteocalcin levels in postmenopausal osteoporotic women receiving alendronate" *Rheumatology Research journal*, Volume (3), Issue (2): pages (83-89).

- **Marilyn Augustine & Mara J. Horwitz (2013).**"Parathyroid Hormone and Parathyroid Hormone-Related Protein Analogs as Therapies for Osteoporosis," *Curr Osteoporos Rep (2013)* , Volume (11), Issue (1): pages (400–406).

- **Mary E. Elliott, Pharm D Abrahamsen Bo Dorthe ask-lindemann, Katrine H, and Peter S(2014).** "REVIEW A Review of Lifestyle , Smoking and Other Modifiable Risk Factors for Osteoporotic Fractures." *BoneKEy Reports* 3 (July). Nature Publishing Group: pages (1–7).

- **Moreira, Marlianne Leite, Leonardo Vieira Neto, Miguel Madeira, Renata Francioni Lopes, Maria Lucia, and Fleiuss Farias(2017).**"Vitamin D Deficiency and Its Influence on Bone Metabolism and Density in a Brazilian Population of Healthy Men." *Journal of Clinical Densitometry*. Elsevier, Volume (2), Issue (4);pages (1–7).

- **Mythili Seetharaman(2007)"**Bone Mineral Density Tests" *Medicine online*, Volume (2), Issue (4); pages (1-5).

- **Nancy E. Lane. (2006).** "Epidemiology , Etiology , and Diagnosis of Osteoporosis." *American Journal of Obstetrics and Gynecology*, Volume (194), Issue (3); pages (11-15).

- **Narang A.P.S., S. Batra , S. Sabharwal and S.C. Ahuja (2004).**" 1, 25-Dihydroxycholcalciferol (1,25-(OH)2 D3) LEVELS IN OSTEOPOROSIS ", *Indian Journal of Clinical Biochemistry*, Volume(19), Issue (2);pages(111–113).

- **Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, Seibel MJ, Mason RS (2012)**.Vitamin D and health in adults in Australia and New Zealand, *Endocrine Society of Australia and OsteoporosisAustralia*, Volume(2), Issue(18); pages (1-4).

- **Opinder Sahota (2000)**."Osteoporosis and the Role of Vitamin D and Calcium Vitamin D Deficiency , Vitamin D Insufficiency and Vitamin D Sufficiency"(Review) *British Geriatrics Society journal* .Volume (25), Issue (1); pages(301–304).

- **Prentice (2004).**A.Diet nutrition and the prevention of osteoporosis, *Public Health Nutrition journal* .Elsie Widdowson Laboratory Cambridge UK,Volume (7), Issue (1); pages (227–243).

- **Sadaf Shakoor, Fasiha I, Naheed A, Muhammad A and Sana A(2015).** "Prevalence of osteoporosis in relation to serum calcium and phosphorus in aging," *J. Global Innoviation Agricultural Social Science,* Volume (2), Issue (2); pages (70-75).

- **Scottie M, Vanessa A.F (2018)** "O Steoporosis," (Text Book) , College of Agriculture, University of Arizona (Tucson, AZ). Volume (1), Issue (1);pages (0-4).

- **Shetty S, Nitin K, Joseph D B, Nihal T, and Thomas V P(2016)**."Review Article Bone Turnover Markers : Emerging Tool in the Management of Osteoporosis*." Indian Journal of Endocrinology and Metabolism* , Volume (20), Issue (6); pages (847-849).

- **Society, British Geriatrics (2017)"**clinical guidelines for prevention and treatment of osteoporosis "*The National Osteoporosis Guidelines Group (NOGG 2017).*Volume (1), Issue (3); pages (5-6).

- **Sundeep K, Merry J O and David G. M (2012).** 'Estrogen and the skeleton', *Trends in Endocrinology & Metabolism*. Elsevier Ltd, Volume (23), Issue (11); pages(576–581).

- **Tsung-R K, and Chih-H C (2017)**."Bone Biomarker for the Clinical Assessment of Osteoporosis : Recent Developments and Future Perspectives." *Kuo and Chen Biomarker Research*, Voume(5), Issue (18); pages (5–13).

- **Tulay Okman-Kilic (2015).**Estrogen Deficiency and Osteoporosis,chapter 2 "*We Are IntechOpen science , the First Native Scientific Publisher of Open Access Books TOP 1 %*."Chapter (2), Volume (1); pages (8-9).

- **Tümay Sözen,Lale Özışık, Nursel Çalık Başaran (2017)**."An Overview and Management of Osteoporosis," *Eropean journal of Rheumatology* , Volume (4), Issue (1) ;pages (46–56).

- **Vanita Suri, Varun Suri(2016)**."Menopause and Oral Health" *Journal of Mid-life Health* ,Volume (5), Issue (3):pages (115–120).

- **Wei-Peng Lin, Jinn Lin (2011)**."Formosan Journal of Musculoskeletal Disorders Parathyroid Hormone for Osteoporosis Treatment."(Review Article) *Formosan Journal of Musculoskeletal Disorders* 2 (4). Elsevier Taiwan LLC, Volume (2), Issue (4):pages (113–117).

- **Wkh D, E Txdoiw (2016)** 'SCIENTIFIC Evaluation of bone mineral density in postmenopausal women with alterations of the mandible cortical bone' *Stomatologija, Baltic Dental and Maxillofacial Journal*,Volume (18), Issue (3); pages (86–91).

- **Zhao B, Wei Z, Shengchao D, Zubin Z (2016)"**Vitamin D receptor BsmI polymorphism and osteoporosis risk in post-menopausal women" *Archives of Medical Science journal*, Volume (1), Issue (2);pages (26-27).

Appendices

Appendix (1)

Sudan University of Science and Technology

Evaluation of 25-OH Vitamin D, Total Calcium and Phosphorus in Sudanese postmenopausal women with Osteoporosis

Participant:

Name: _____ Questionnaire No ( )

Address: _____

Contact Phone Number: _____

Birth Date: _____

Marital Status:

Single ( ) Married ( ) Divorced ( ) Widowed ( )

Do you have:

- Menstrual Period Problems?
- Significant children related Problems?
- Urine loss when you cough, sneeze or lough?

Do you now or have you ever had:

- Diabetes
- High Blood Pressure
- High Cholesterol
- Hypothyroidism
- Asthma
- Kidney Diseases

Laboratory Investigations:

Blood investigation	Result	Comment
Vitamin D level (ng/ml)		
Calcium (mg/dl)		
Phosphorus (mg/dl)		