بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies

Assessment of Total Plasma 25-OH Vitamin D Level, and its Association with Bone Mineral Density Among Post-Menopausal Sudanese Women

تقييم مستوى فايتمين (د) الكلي و علاقته مع كثافة العظم المعدنية لدى النساء السودانيات بعد سن اليأس

A dissertation Submitted in partial Fulfillment for the requirement of M.Sc. Degree in Medical Laboratory Science (Clinical Chemistry)

By
Mohammedalhassan Ahmed Elammin Khairralla
B.Sc.in Medical Laboratory Sciences - Clinical Chemistry
(Omdurman Islamic University-2016)

Supervisor:
Dr: Ghada Abdelrahman Elfadil.
Medical laboratory Science - Clinical Chemistry

February-2020

الآيـــــــة

قال تعالى:

(قَالَ رَبِّ إِنِّي وَهَنَ الْعَظْمُ مِنِّي وَاشْتَعَلَ الرَّأْسُ شَيْبًا وَلَمْ أَكُن بِدُعَائِكَ رَبِّ شَقِيًّا(4)).

سورة مريم

Dedication

To our dear parents who are priceless.

To my brothers and sisters and our families.

To our dear friends in my life.

Acknowledgements

I would like firstly and finally thank the almighty Allah for give us the strength to accomplish this work. I would like to express our gratitude appreciation to our Supervisor: Dr. Ghada abdelrahman. Whose give us much of her time for suggestion and super vision of this work. Also to thank all and the lecturers of the faculty, and to all the teachers in the Department of Clinical Chemistry of Sudanese university for giving me the chance to be a part of the project.

Our thanks also extend to medical, nursing and laboratory staff whom work in Best-Care Hospital for determined bone mineral density and facilitate sample collection, and thanks to patient's whom agree to participant in study.

And to the lab staff of Lab Test Special think for Dr. Salahaldeen Ismail for sample analysis. Also to Dr. Altaf Suleman which help me in statistical analysis.

Abstract

Background: The post-menopausal stage in the women consider critical phase which effect bone mineral density. The loss of bone density in postmenopausal women has been linked to a subclinical deficiency in vitamin D. The aim of this study to assess of total 25-OH vitamin D level, and bone mineral density among postmenopausal Sudanese women.

Methodology: This study included 87 post-menopausal women age varying from (40- 80 years); DXA scan (reading T-score) was used to determine bone mineral density (Normal, osteopenia and osteoporosis). plasma total 25(OH) vitamin D was estimated by using Fortress kit and ELIZA technique. The data was analyzed by used SPSS version 19.

Result: This study showed according to bone mineral density (BMD) (Normal 33, osteopenia39 and osteoporosis 15), vitmin D level (Insufficient 7, Deficiency 46, and sever deficiency 34).

Significant decrease in mean of vitamin D levels in postmenopausal women when compared with reference range *P-value* (0.00). Insignificant changed in vit-D level when compared BMD groups, significant decrease in mean of BMD T score when compared with vit-D group P-value (0.01), and no Correlation between BMD and Vit D.

Conclusion: Our study conclude that Postmenopausal Sudanese women had a decrease in plasma total 25 OH vitamin D level, and osteopenia had most common among post-menopausal Sudanese women.

المستخلص

الخلفية: ما بعد سن اليأس في النساء تعتبر مرحله حرجه لتأثر كثافة العظم.وفقدان كثافة العظم في النساء بعد سن اليأس من الممكن ان تكون مرتبطه نوعا ما بالنقص في فايتمين (د).والهدف من الدراسة تقيم مستوى فايتمين (د) وعلاقته بكثافة العظم في النساء السودانيات بعد سن اليأس.

الطريقة المنهجية: شملت الدراسة على 87 امرأة بعد سن اليأس اعمارهم تراوحت بين (40-80 سنة) واستخدمت جهاز ال(د اكس أ الذي يقرأ تي سكور) لتقيم كثافة العظم (طبيعي وماقبل الهشاشة ومصابين بالهشاشة). واستخدمت البلازما لتقيم فايتمين (د) الكلي باستخدام محلول فور ترس وتقنية الاليزاز وحللت البينات باستخدام برنامج التحليل الاحصائي للعلوم الاجتماعية من النوع 19.

النتيجة: دراستي هذه اظهرت على حسب كثافة العظم(طبيعين 33 وماقبل الهشاشة 39 ومصابين بالهشاشة 15) فايتمين (د) (غيركافي 7 ونقص 46 والنقص حاد 34) . وجود علاقة ذات أهميه بين متوسط مستوي فايتمين(د) عند النساء بعد سن اليأس ومتوسط المدى المرجعي لفايتمين (د)(0.00).وعدم وجود علاقة ذات اهميه بين مستوى فايتمين (د) لكثافة العظم حسب المجموعات. وجود علاقة ذات اهمية بين متوسط نسبة كثافة العظم داخل مجموعات فايتمين (د)(0.01).وأيضا أظهرت الدراسة عدم وجود علاقة بين كثافة العظم ككل وفايتمين (د)

الخلاصه: دراستي تخلص الي ان النساء السودانيات بعد سن اليأس لديهم نقص فايتمين (د) الكلي، وقلة في كثافة العظم شائعة بين النساء السودانيات بعد سن اليأس.

List of contents

List of tables

List of figures

List of abbreviations

BMD	Bone mineral density
BMPs	Bone morphogenetic proteins
CAMP	Cyclic adenosine monophosphate
Ca-SR	Calcium sensing receptors
CDK	Cyclin-dependent kinase
DHC	Dehydrocholestrol
DPA	Dual energy photon absorptiometry
DXA	Dual energy-X-ray
ECF	Extra cellular fluid
ELISA	Enzyme link immune sorbent assay
FSH	Follicular stimulating hormone
GH	Growth hormone
IGF	Insulin like growth factors
IL-6	Interleukin-6
LH	Luteinize hormone
MAPK	Mitogen activated proteins kinase
MSCs	Mesenchymal stem cells
OI	Ontogenesis imperfecta
OPG	Osteoprotegerin
PHPT	Primary hyper parathyrodism
PTH	Parathyroid hormone
QCT	Quantitative computed tomography
RANK	Receptor activator of nuclear kappa-B

RANKL	Receptor activator of nuclear kappa-B ligand
RAS	Renin-angiotensin system
RP	Radiographic photo densitometry
SOC	Store-operated Calcium channel
SPA	Single energy photon absorptiometry
T3	Tri iodothyronine
T4	Titraiodothyronine\thyroxine
TMB	Tetra methyl benzidine
TSH	Thyroid stimulating hormone
UK	United kingdom
USA	United states American
UV	Ultra violet
VDCC	Voltage dependent calcium channel
VDR	Vitamin D receptors
WHO	World health organization

Chapter One

Introduction, Rationale and Objectives

1. Introduction, Rationale and Objectives

1.1. Introduction

A woman's reproductive capacity stops at the time of menopause and the average natural menopause occurs at age 51.4 years in developed countries such as the United States and the U.K. but generally occurs between the ages of 40 and 58 years (Kanis *et al*., 2008).

The ovaries no longer have follicles and their function, as a steroidogenic endocrine organ does not work anymore. Many women experience symptoms and complaints due to these hormonal changes. Although it does not cause death, menopause can decrease quality of life and lead to degenerative diseases especially bone loss or osteoporosis (WHO., 2003).

A deficiency of estrogen leads to increased bone remodeling, where bone resorption outpaces bone formation and leads to a decrease in bone mass. That estrogen may influence local factors that regulate the precursors of osteoblasts and osteoclasts (Wang *et al*., 2018).

Bone mass is lower in women, and loss of bone mass occurs earlier in women than in men, thus women aged >45 years have an increased risk for fractures. Loss of bone mass due to estrogen deficiency occurs first in spongiosa while shrinkage does not occur in trabecular bone. Deterioration of bone mass is caused by an imbalance between bone resorption and bone formation (Panchbhai., 2013).

The decrease in bone mass that increases the risk of bone fracture. The porous network of the trabecular bone beneath the cortical bone is particularly weakened in osteoporosis, so bones of the wrist, hip, and spine possessing more trabecular bone are more susceptible to fractures when exposed to sufficient forces (Lee., 2018).

The low bone mineral density in postmenopausal results from the estradiol hormone in the follicle. Estradiol is a type of estrogen that assists osteoblasts, suppresses cytokines, and inhibits osteoclast activity. Estradiol decreases during menopause; therefore, its work is replaced by another type of estrogen that can be produced by adipose tissue. But the mechanism of action of estrone is not like estradiol (ratio of estradiol: estrone = 10:5). The longer the duration of menopause, the more estradiol function decreases, which causes osteoclasts to become active in bone resorption (Susi *et al*., 2019).

The loss of bone density in postmenopausal women has been linked to a subclinical deficiency in vitamin D, which is considered to be a risk factor for fractures due to the susceptibility of this population to falls and inappropriate neuromuscular responses.Vitamin D insufficiency can result from nutritional deficiency and/or low sun exposure in confined elderly patients and in patients with chronic diseases (Pedro., *et al*., 2013).

Vitamin D deficiency has been linked to secondary hyperparathyroidism and bone loss, with reduction in BMD and an increased risk of osteoporosis (Ebeling., 2014).

1.2. Rationale

Several studies have suggested that low serum vitamin D levels are associated with low bone mineral density. There are reports of correlation of vitamin D deficiency and low BMD among postmenopausal women of many countries of the world. Also most patients with vitamin D insufficiency exhibit low bone mass and that all patients with vitamin D deficiency have bone mineral densities varying between osteopenia and osteoporosis.

The low bone mineral density as (osteoporosis, Osteopenia) is major public problem which resulting in osteoporotic fracture can lead to chronic pain, lack of independence, movement, effect economic and may death. For that I need to apply it in this country to decrease the osteoprotic fracture occure in post-menopausal women .And also not publish study in Sudan related to study the association between the level of vitamin D and bone mineral density in post-menopausal women.

1.3 Objectives

1.3.1 General objective

To assess a total plasma 25-OH Vitamin D level, and its association with bone mineral density among post-menopausal Sudanese women's.

1.3.2 Specific objectives

1. To calculate frequency of osteopenia and osteoporosis among study group.
2. To estimate a total plasma 25-OHvitamin D level in post-menopausal women.
3. To correlate between 25-OH vitamin D level, and bone mineral density, and age among study group.

Chapter Tow

Literature Review

2.Literature Review

2.1.Bone

Bone is a mineralized connective tissue that exhibits four types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts (Downey and Siegel., 2006). Bone is a highly specialized supporting framework of the body, characterized by its rigidity hardness, and power of regeneration and repair. It protects the vital organs, provides an environment for marrow (both blood forming and fat storage), acts as a mineral reservoir for calcium homeostasis and a reservoir of growth factors and cytokines, and also takes part in acid–base balance (Taichman., 2005).

2.1.1. Function of bone

Bone exerts important functions in the body, such as locomotion, support and protection of soft tissues, calcium and phosphate storage, and harboring of bone marrow (Datta et al., 2008). The function of bone lining cells is not well clear, but these cells seem to play an important role in coupling bone resorption to bone formation (Everts *et al*., 2002).

2.1.2. Bone component

The bones are composed of:

i. Bones cells:

Bone cells can be categorized into two lineages: the osteoblast lineage, representing the bone-forming axis (consisting of MSCs, pre-osteoblasts, mature osteoblasts, bone-lining cells, and osteocytes), and the osteoclast lineage, representing the bone-resorbing arm (consisting of macrophages, osteoclasts, and multinucleated giant cells, all derived from bone marrow hematopoietic stem cells). Mature osteoblasts are the only cells that can explicitly build bone by secreting bone matrix proteins and guiding mineralization. Depletion of mature osteoblasts results in an arrest of skeletal growth. However, mature osteoblasts are short-lived; a subset is encapsulated within the newly-formed bone matrix, becoming osteocytes, while the others either undergo apoptosis or become inactive bone-lining cells (Long., 2011).

Osteoblasts cells : are cuboidal cells that are located along the bone surface comprising 4–6% of the total resident bone cells and are largely known for their bone forming function (formed the bone matrix) Secrete type I collagen-rich bone matrix and regulate matrix mineralization (Capulli., 2014). Osteoblasts are derived from mesenchyme stem cells (MSC) (osteoprogenitor cells) of the bone marrow stroma and are responsible for bone matrix synthesis and its subsequent mineralization (Logan and Nusse., 2004) . Osteoblasts are responsible for regulation of osteoclasts and deposition of bone matrix (Mackie., 2003) . As

they differentiate, they acquire the ability to secrete bone matrix. Ultimately, some osteoblasts become trapped in their own bone matrix, giving rise to osteocytes which, gradually, stop secreting osteoid. The osteoblasts when activated, they have a large Golgi apparatus and endoplasmic reticulum essential for rapid osteoid synthesis. Osteoblasts have three possible fates: they can become a bone lining cell, an osteocyte or undergo apoptosis (Matic *et al*., 2016).

Bone lining cells: are quiescent flat-shaped osteoblasts that cover the bone surfaces, where neither bone resorption nor bone formation occurs. Bone lining cells functions are not completely understood, but it has been shown that these cells prevent the direct interaction between osteoclasts and bone matrix, when bone resorption should not occur, and also participate in osteoclast differentiation, producing osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL) (Andersen *et al*., 2009).

Osteocytes cells: which comprise 90–95% of the total bone cells, are the most abundant and long-lived cells, with a lifespan of up to 25 years (Franz *et al*., 2006). These cell types are situated within the bone matrix and occupy microscopic spaces called lacuna. They play a role in bone remodeling by transmitting signals to other nearby osteocytes regarding bone stress (tendons pulling on the bone). Osteocytes are also involved in regulating fluid flow within the bone, so this cellular signal may be due to changes in fluid flow in response to mechanical stresses on the bone (Elango *et al*., 2018).

Osteoclasts cells: are multinucleated cell formed by fusion of precursors derived from the monocytes/macrophage lineage. Podosomes facilitate adhesion to the bone surface and formation of a sealing zone provides an isolated acidic microenvironment within which the osteoclast can dissolve mineral and digest the bone matrix. Bone mineral is dissolved by secretion of hydrochloric acid and bone matrix is broken down by secretion of proteolytic enzymes including cathepsin K (Ross., 2013).

ii. Bone matrix:

Bone is composed by inorganic salts and organic matrix. The organic matrix contains collagenous proteins (90%), predominantly type I collagen, and noncollagenous proteins including osteocalcin, osteonectin, osteopontin, fibronectin and bone sialoprotein II, bone morphogenetic proteins (BMPs), and growth factors. There are also small leucine-rich proteoglycans including decorin, biglycan, lumican, osteoaderin, and seric proteins (Downey and Siegel., 2006). The inorganic material of bone consistsThe inorganic mineral component consists mainly of calcium hydroxyapatite [Ca10 (PO4)6(OH)2]. This mineral gives bone

strength and hardness and serves as the storehouse for 99% of the body's calcium, 85% of the body's phosphorus, and 65% of the body's sodium and magnesium (Vinay *et al*., 2013).

2.1.3. Bone formations

Bone is composed of support cells, namely, osteoblasts and osteocytes ; remodeling cells, namely, osteoclasts ; and non-mineral matrix of collagen and noncollagenous proteins called osteoid , with inorganic mineral salts deposited within the matrix. During life, the bones undergo processes of longitudinal and radial growth, modeling (reshaping), and remodeling (Clarke., 2008) . Ossification (or osteogenesis) is the process of formation of new bone by cells called osteoblasts. These cells and the bone matrix are the two most crucial elements involved in the formation of bone .If the process of formation of bone tissue occurs at an extraskeletal location, it is termed as heterotopic ossification .Three basic steps involved in osteogenesis are:

i. Synthesis of extracellular organic matrix (osteoid)

ii. Matrix mineralization leading to the formation of bone

iii. Remodeling of bone by the process of resorption and reformation. Osteoblasts are responsible for bone matrix synthesis and its subsequent mineralization (Logan and Nusse., 2004) .

2.1.4. Bone remodeling

Bones are not inert structures within the human body; they continue to change over the course of a lifespan. This process of skeletal change is known as bone remodeling (Cosman., 2018). Bone remodeling is a lifelong process wherein old bone is removed from the skeleton (a sub process called bone resorption), and new bone is added (a sub-process called ossi fi cation or bone formation). Remodeling involves continuous removal of discrete packets of old bone, replacement of these packets with newly synthesized proteinaceous matrix, and subsequent mineralization of the matrix to form new bone (Fernández *et al*., 2006).

2.1.4.1. Bone remodeling phases

On a molecular level, there are phases of bone remodeling:

i. Resting phase:in which the factors that initiate the remodeling process remain unknown.

ii. Activation phase : The first phenomenon that occurs is the activation of the bone surface prior to resorption, through the retraction of the bone lining cells (elongated mature osteoblasts existing on the endosteal surface) and the digestion of the endosteal membrane by collagenase action (Bruzzaniti and Baron., 2007).

iii. Resorption phase : In which the osteoclasts then begin to dissolve the mineral matrix and decompose the osteoid matrix.

iv. Reversal phase of the resorption signal; and formation of a new bone

v.Formation phase: formation of a new bone that fills up the resorption cavity. The cavities are not filled in completely with new bones, which results in a permanent loss of bone mass and an increase in the risk of osteoporotic fractures. Defective osteoclast activity leads to osteopetrosis and bone marrow failure, whereas excess activity can contribute to bone loss and osteoporosis (Chen *et al*., 2019).

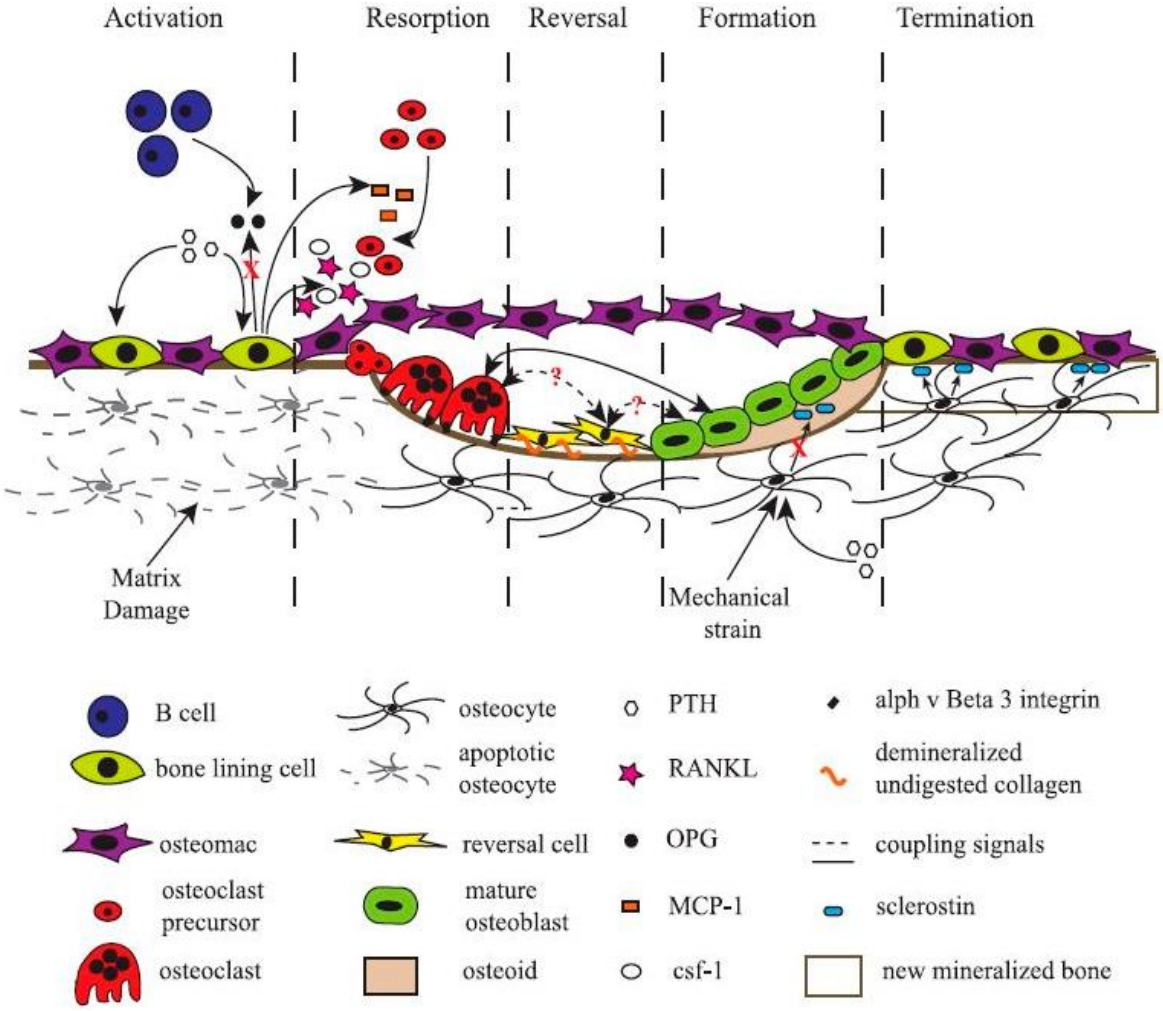


Figure (2.1). Schematic representation of a basic multicellular unit and the associated bone remodeling process (Bach Quang., 2018).

2.1.4.2. Hormonal Impact on Bone Remodeling

2.1.4.2.1. Parathyroid Hormone (PTH)

Has an indirect action on the osteoclasts by increasing the activity of receptor activator of nuclear factor kappa ligand (RANKL), which regulates the osteoclastic activity of bone resorption and leads to more calcium released into the plasma(Urano., 2018).

2.1.4.2.2.Calcitonin

Apolypeptide hormone, is released from thyroid C cells in response to elevated calcium levels. Regarding bones, calcitonin binds to calcitonin receptors on osteoclasts to inhibit bone resorption. However, calcitonin is clinically used as a treatment option to treat osteoporosis (Garnero., 2017) .

2.1.4.2.3. Calcitriol

It stimulates absorption of dietary calcium and phosphatefrom the intestine; this process involves the synthesis of a calcium-binding protein (calbindin D) in enterocytes. The binding of calcitriol to osteoblasts increases the production of alkaline phosphatase and of a calcium binding protein, osteocalcin, the exact function of which is uncertain. At high concentrations, calcitriol stimulates osteoclastic bone resorption, which releases calcium and phosphate into the ECF.In the kidneys, calcitriol inhibits its own synthesis (Marshall et al., 2012).

2.1.4.2.4. Estrogen

A deficiency of estrogen leads to increased bone remodeling, where bone resorption outpaces bone formation and leads to a decrease in bone mass. That estrogen may influence local factors that regulate the precursors of osteoblasts and osteoclasts. Estrogen may block the production and action of interleukin-6 (IL-6), which would hinder bone resorption. Also, it is believed that the survival of osteoclasts thrives in the deficiency of estrogen, where the degree of bone turnover would be greater (Wang *et al.*, 2018).

2.1.4.2.5. Growth Hormone

Acts directly and indirectly via IGF to stimulate osteoblast proliferation and activity, but it also stimulates the bone resorption activity of osteoclasts; however, the cumulative net effect of this dual activity favors bone formation (Wang et al., 2018).

2.1.4.2.6. Glucocorticoids

Glucocorticoids decrease bone formation by favoring the survival of osteoclasts and causing the cell death of osteoblasts. There is an increase in RANKL action and a decrease in osteoprotegerin (OPG). OPG is a cytokine receptor and member of the tissue necrosis factor

superfamily that acts a decoy receptor for RANKL, so it would normally hinder RANKL-RANK interaction and activity (Kucukalic and Begic., 2004).

2.1.4.2.7. Thyroid Hormone

Thyroid-stimulating hormone (TSH), thyroxine (T4), and triiodothyronine (T3) cause bone elongation at the epiphyseal plate of long bones through chondrocyte proliferation and also stimulate osteoblast activity. In states of hypothyroidism or hyperthyroidism, the degree of bone turnover is low and high respectively. The rate of bone turnover is due to the effect of T3/T4 on the number and activity level of osteoblasts and osteoclasts. For example, the high metabolic state of thyrotoxicosis causes increased osteoblast function and increased osteoclastic number and activity and leads to a higher bone turnover (Kucukalic and Begic., 2004).

2.1.5. Bone disorder s:

2.1.5.1. Congenital bones disorder:

Osteogenesis imperfecta (OI), also known as "brittle bone disease," is actually a group of genetic disorders caused by defective synthesis of type I collagen.Because type I collagen is a major component of extracellular matrix (Vinay *et al*., 2013).

Osteopetrosis is a group of rare genetic disorders characterized by defective osteoclast-mediated bone resorption. Osteopetrosis (literally, "bone-that-is-like-stone disorder") is an appropriate name, since the bones are dense, solid, and stonelike. The defects that cause osteopetrosis are categorized into those that disturb osteoclast function and those that interfere with osteoclast formation and differentiation (Vinay *et al*., 2013).

2.1.5.2. Acquired bones diseases

Osteoporosis: Is an acquired condition characterized by reduced bone mass, leading to bone fragility and susceptibility to fractures. The bone loss may be confined to certain bones or regions, as in disuse osteoporosis of a limb, or be generalized, involving the entire skeleton. Generalized osteoporosis may be primary or occur secondary to a large variety of insults, including metabolic diseases, vitamin deficiencies, and drug exposures (Vinay *et al*., 2013).

Paget Disease (Osteitis Deformans): This unique skeletal disease is characterized by repetitive episodes of frenzied, regional osteoclastic activity and bone resorption (osteolytic stage), followed by exuberant bone formation (mixed osteoclastic-osteoblastic stage), and finally by an apparent exhaustion of cellular activity (osteosclerotic stage). The net effect of this process is a gain in bone mass; however, the newly formed bone is disordered and weak, so bones may become enlarged and misshapen (Vinay *et al*., 2013).

Rickets and Osteomalacia: Both rickets and osteomalacia are manifestations of vitamin D deficiency or its abnormal metabolism. Rickets refers to the disorder in children, in which it interferes with the deposition of bone in the growth plates. Osteomalacia is the adult counterpart, in which bone formed during remodeling is undermineralized, resulting in predisposition tofractures (Vinay *et al*., 2013).

Hyper parathyroidism: Increased secretion of PTH in PHPT leads to elevated serum calcium levels due to release from the bone stores. This has been shown to increase the risk of osteoporosis by increasing the rate of bone turnover (Sneddon *et al*., 2004).

2.2. Bone minerals density (BMD)

Mechanical properties of bone are determined by various factors, such as size and shape of individual bone, cortical thickness, porosity and the orientation of collagen fibers, and, to a greater extent, degree of mineralization or bone mineral density (BMD), which is defined as "the mass of inorganic (mineral) matter per unit volume.". BMD must be analyzed in bone's three levels of biological organization: in bone material (BMDmaterial), in a bone's trabecular and cortical tissue compartments (BMDcompartment circumstances such as ageing, adaptation, healing pathologically), BMD can be modified under many different conditions, affecting bone's behavior under physical stress:

i. Disorders in bone mineral homeostasis: Rickets and Osteomalacia, Hyperparathyroidism, Hypogonadism, Hyperthyroidism, Diabetes Mellitus Type I, Cushing Disease.

ii. Disorders related to bone remodeling imbalance: Paget Disease, Disuse Osteoporosis, Osteopetrosis.

iii. Disorders related to collagen production: Osteogenesis Imperfect a, Scurvy, Marfan syndrome, and Ehlers Danlos Syndrome.

iv. Certain drugs can affect bone metabolism: Glucocorticoids, heparin, warfarin, cyclosporine, medroxyprogesterone, cytostatics, thyroide hormone, antiseizure drugs, chemotherapeutic agents, anti-rheumatics (Juan and Hector., 2017).

2.2.1. Methods of BMD measurements

Different invasive methods for measuring BMD include: Radiogrammetry (RG), Compton scattering technique, Radiographic Photodensiometry (RP), Single energy photon absortiometry (SPA), Dual energy photon absortiometry (DPA), Neutron activation analysis, Quantative computed tomography (QCT), Dual energy X-ray (Chugh *et al*., 2013).

Dual energy X-ray absorptiometry (DXA): The World Health Organization (WHO) has established DXA as the best densitometric technique for assessing BMD in postmenopausal

women and based the definitions of osteopenia and osteoporosis on its results (Table 2.1). DXA allows accurate diagnosis of osteoporosis, estimation of fracture risk and monitoring of patients undergoing treatment. Additional features of DXA include measurement of BMD at multiple skeletal sites, safety of performance, short investigation time and ease of use (WHO).

Table (2.1): WHO diagnostic criteria for osteoporosis:

Diagnosis	**T- Score**
Normal	> -1.0
Osteopenia	< -1.0, > -2.5
Osteoporosis	< -2.5
Severe osteoporosis	< -2.5 plus fragility fractures

The T-score is calculated using the formula: (patient's BMD _ young normal mean)/SD of young normal (Watts., 2004). Several large studies have shown an unacceptably high risk of fracture in postmenopausal women who have T-scores of -2.5 and below. In addition to the T-scores, DXA reports also provide Z-scores, which are calculated similarly to the T-score, except that the patient's BMD is compared with an age-matched (and race- and gender-matched) mean, and the result expressed as a SD score. In premenopausal women, a low Z-score (below -2.0) indicates that bone density is lower than expected and should trigger a search for an underlying cause(Watts., 2004).

2.2.2. Classifications of BMD

2.2.2.1. Osteopenia

Osteopenia is a clinical term used to describe a decrease in bone mineral density (BMD) below normal reference values, yet not low enough to meet the diagnostic criteria to be considered osteoporotic. BMD is diagnosed via dualenergy xray absorptiometry (DXA) bone scans. The diagnostic difference delineating osteopenia from osteoporosis as defined by the World Health Organization (WHO) is a tscore between -1 to -2.5. Values less than -2.5Are diagnostic for osteoporosis.

2.2.2.2. Osteoporosis

Osteoporosis is a worldwide disease characterized by reduction of bone mass and alteration of bone architecture resulting in increased bone fragility and increased fracture risk (Qaseem *et al*., 2017). The most common metabolic disorder of the skeleton is osteoporosis, which is a decrease in bone mass that increases the risk of bone fracture(Lee., 2018) . Osteoporosis is a bone disorder that increases a person's risk of fracture due to low bone mineral density (BMD), impaired bone microarchitecture/mineralization, and/or decreased bone strength. This asymptomatic condition often remains undiagnosed until it manifests as a low-trauma fracture of the hip, spine, proximal humerus, pelvis, and/or wrist, which frequently leads to hospitalization (Jeremiah *et al*., 2015).

2.2.2.2.1. Risk factors osteoporosis

i. Modifiable risk factors:

Nutritional deficiency: maternal diet can influence bone mass in the offspring. a high-caloric diet and heavy alcohol consumption have been associated with lower bone mass and higher rates of fracture (Levis and Lagari, 2012).

Physical activity: Because mechanical forces stimulate bone remodeling, reduced physical activity increases bone loss (Vinay *et al*., 2013).

Smoking: In heavy smoking the serum PTH showed an increasing and decrease of circulating levels of vitamin D (1,25(OH) D). Also the smoking is associated with increased follicle stimulating hormone and luteinizing hormone, which directs the estrogen levels to decrease and results in rapid bone loss (Kline *et al*., 2016).

High alcohol intake: Light alcohol consumption was inversely significantly associated with hip fracture risk, whereas heavy alcohol consumption was associated with an elevated hip fracture risk (Zhang., 2015).

Medication: synthetic glucocorticoids increase the expression of receptor activator for nuclear factor κ-B ligand (RANKL) and decrease the expression of its soluble decoy receptor, osteoprotegerin (OPG), in stromal and osteoblastic cells, leading to elevated bone resorption. Also inhibit osteoblast cell differentiation and suppress IGF-I gene transcription which is responsible for bone formation and the synthesis of type I collagen (Canalis., 2005)

ii. Non modifiable :

Age: With increasing age, the replicative and matrix production activities of osteoblasts progressively diminish. The various growth factors deposited in the extracellular matrix also

diminish with time. Unfortunately, while new bone synthesis wanes with advancing age, osteoclasts retain their youthful vigor (Vinay *et al*., 2013).

Gender: female sex; estrogen deficiency following menopause or surgical removal of the ovaries is correlated with a rapid reduction in bone mineral density, while in men, a decrease in testosterone levels has a comparable (but less pronounced) effect (Sinnesael *et al*., 2013).

Genetic factors: Vitamin D receptor polymorphisms appear to influence the peak bone mass early in life. Additional genetic variables can influence either calcium uptake or PTH synthesis and responses (Vinay *et al*., 2013).

2.2.2.2.2. Classification of osteoporosis

i. Primary osteoporosis:

Postmenopausal (typeI) osteoporosis: drop in estrogen leads to increased cytokine production (especially IL-1, IL-6, and TNF), presumably from cells in the bone. These stimulate RANK–RANK ligand activity and suppress OPG production. There is some compensatory osteoblastic activity, but it is inadequate to keep pace with osteoclastic bone resorption (Vinay *et al*., 2013).

Senile (typeII) osteoporosis: In both men and women, beginning in the third or fourth decade of life, bone resorption begins to outpace bone formation (Vinay *et al*., 2013).

ii. Secondary osteoporosis:

Osteoporosis occur secondary to a large variety of insults, including metabolic diseases, vitamin deficiencies, and drug exposures. Examples, endocrine disorder (Hyperparathyroidism, Hypo or hyperthyroidism, Hypogonadism, Pituitary tumors, addition disease and Diabetes, type 1), Gastrointestinal Disorders (Malnutrition, Malabsorption, Hepatic insufficiency and Vitamin C, D deficiencies) and Drugs (Anticoagulants, Chemotherapy, Corticosteroids and Alcohol) (Vinay *et al*., 2013).

2.2.2.3. Bone fracture

Is medical condition in which there is a partial or complete break in the continuity of the bone. In more severe cases, the bone may be broken into several pieces (Katherine and abel., 2013).The auses of The bone fracture may be the result of high force impact, stress or minimal trauma injury as a result of certain medical condition that weaken the bone such as osteoporosis, osteopenia, bone cancer or osteogenesis imperfect where the fracture is then properly termed pathologic fracture (Witmer *et al*., 2016).

2.3. Menopause

Menopause is a phase of a woman's natural life that signifies the end of the menstrual cycle. It is diagnosed after a woman has not had a menstrual period for 12 months. The average natural menopause generally occurs between the ages of 40 and 58 years (Kanis *et al*., 2008).

2.3.1. Pathophysiology of menopause

Menopause is normal physiologic process in aging women, in which the numbers of ovarian, primary follicles quickly diminish, such that there are inadequate amounts to respond to the effects of FSH. In turn there is no LH surge and ovulation doses not take place, resulting in the decline of estrogen production and the cessation of menstruation. Moreover, LH and FSH go uninhibited and remain at high levels years after the onset of menopause. Small amounts of estrogen may still be produced via conversion from testosterone released by the adrenal glands, such that symptoms other than the discontinuation of periods may be negligible in some individual (Polo and Rantala., 2019).

2.3.2. Causes of menopause

As women grow older, their ovarian follicles diminish in number. There is a decline in granulosa cells of ovary.Also the decline in estrogen levels disrupts the hypothalamic-pituitary-ovarian axis. AS result afailure of endometrial development occurs causing irregular menstrual cycles, until they stop altogether. Menopause may occur due surgical remove of ovary. Menopause can by treatment of certin conditions like endometriosis and breast cancer with antiestrogen .

2.3.3. Staging of menopause

Reproductive stage: The menstrual cycles is regulare.as well as slight change to flow and duration before entering the next stage.

Menopausal Transition stage: Is where perimenopause primarily occurs, the menstrual cycle undergoes variability of its duration such that length of time between menstruations differs by 7 or more days each cycle. As this stage progresses, women typically experience amenorrhea for aperiod of 60 or more days.In this stage the elevated of FSH due to the decline of estrogen.

Post-menopause: The menstruation has ceased.Perimenopause continues until there has been no menstruation for 1 year. Then the early post-menopause continues for another year. After 3 to 6 years,women inter into late post-menpause, in which they may experience more symptom of urogenital atrophy.

2.4. Vitamin D

Vitamin D is a fat-soluble vitamin used by the body for normal bone development and maintenance by increasing the absorptions of calcium, magnesium and phosphate (Nair and Maseehؘ. 2012).Vitamin D and its metabolite may be categorized as either cholecalciferols(vitamin D3) or ergocalciferols(vitamin D2). Vitamin D3 produced by skin from 7-dehydrocholestrol on exposure to the ultra violet of sunlight.either vitamin D2 manufactered by irradiation of ergosterol produced by yeast(Carl *et al*., 2008).

2.4.1. Synthesis and transport of vitamin D

Vitamin D, therefore, shares striking similarities in origin with steroid hormones, that is, vitamin D is a metabolic product of the cholesterol synthetic pathway. The tissues that are involved in the synthesis of vitamin D are the skin, liver, and kidneys, and the tissues it affects are the gut, bone, and parathyroids. De novo synthesis of vitamin D begins in the skin, where 7-dehydrocholesterol is transformed to vitamin D3 by the action of ultraviolet light. Vitamin D3 is biologically inert and must be further metabolized to the biologically active metabolite. An enzyme in the liver, 25-hydroxylase, metabolizes vitamin D3 to 25-hydroxy vitamin D. Hepatic 25- hydroxylase is not regulated by any component of the calcium homeostatic system and functions constitutively to hydroxylate vitamin D3 at the 25-position of the sterol ring system (Bishop *et al*., 2010).

Synthesis of endogenous vitamin D begins in the skin. The epidermis and dermis both contain 7-dehydrocholesterol (DHC). When UV radiation (280–315 nm) passes through these skin layers, 7 dehydrocholesterol absorbs UVB photons inducing their conversion to previtamin D3. This photoisomerization is followed by previtamin D3 thermal-dependent isomerization, leading to formation of the vitamin D3 molecule, also known as cholecalciferol. Skin synthesis is limited by various determinants, including pigmentation, age, zenith angle of the sun, poor air quality and % of the skin surface area available for exposure. A recent study of sun-protective behaviour in the USA showed that wearing long sleeves or staying in the shade reduced vitamin D status (Linos *et al*., 2011).

2.4.2. Functions of vitamin D

The overall function of 1,25- di OH-D3 To maintain adequate plasma level of calcium by: Increasing uptake of calcium by the intestine, Minimizing loss of calcium by kidney by increasing the reabsorption and effect on bone (indirect) : by stimulated the mobilization of calcium from bone by process that requires protein synthesis and presence of PTH (Denise., 2014) .

2.4.3. Mechanism of actions of vitamin D

An overview of vitamin D its physiological action:

i. Effects on the skeletal system:

1a,25(OH)2D enhances intestinal calcium absorption via its nuclear VDR that up-regulates the expression of the epithelial calcium channel and a calcium-binding protein (calbindin 9K) 1a,25(OH)2D indirectly stimulates osteoclastogenesis by promoting the maturation of preosteoclasts to multinucleated osteoclasts. 1a,25(OH)2D initially induces expression of membrane-bound RANKL by interacting with its VDR in osteoblasts. This osteoblast membrane factor then binds to its cognate receptor RANK localized on preosteoclast membranes. In preosteoclasts, the RANKL–RANK interaction triggers strong activation of the jb nuclear factor responsible for the maturation signal (Sylvain *et al*., 2012). In bone, 1, 25(OH) 2D stimulates terminal differentiation of osteoclast precursors to osteoclasts. 1, 25(OH) 2D also stimulates osteoblasts to influence osteoclasts to mobilize bone calcium. 1, 25(OH) 2D does not directly affect mature osteoclast physiology. 1,25(OH)2D plays an important role in mineralization of bone, and abnormal bone results when vitamin D is deficient or its metabolism is defective(Bishop *et al*., 2010).

ii. Effect on non-skeletal systems

The following is an account of some of the diseases believed to be influenced by vitamin D status:

Cancer: Experimental evidence supports a reduction of risk of many cancers through the action of 1a,25(OH)2D in suppressing the proliferation and stimulating differentiation of cancer cells. The cyclin/cyclin-dependent kinase (CDK) complex acts to ensure the phosphorylation of target proteins involved in cell cycle progression. Other antitumour effects include apoptosis. Studies report that vitamin D activity regulates pro- and antiapoptotic factors in support of the apoptotic process (Sylvain *et al*., 2012).

Immunity: Vitamin D is important for stimulation of innate immunity. Studies report that 1a,25(OH)2D enhances the antimicrobial properties of monocytes and macrophages. 1a,25 (OH)2D enhances both chemotaxis and the phagocytic capabilities of macrophages. Furthermore, it also activates both the cathelicidin gene (CAMP) and defensing b2 expression, both of which are antibacterial peptides capable of destroying the microbe cell membrane (Sylvain *et al*., 2012).

Muscle function: 1, 25(OH) 2D modulates muscle cell calcium exchange and intracellular calcium. Calcium homeostasis regulation is an essential element for muscle contraction and

relaxation. Stimulation by 1a, 25(OH) 2D results in enhanced calcium intake and release of intracellular calcium stored in muscle cells. This cytosolic calcium influx has been identified as being mediated via voltage dependent calcium channels (VDCC) and the store-operated Ca2? Channel (SOC). 1a, 25(OH) 2D modulates muscle cell proliferation, differentiation and consequently myogenesis. Mitogenactivated protein kinase (MAPK) signalling pathways relay extracellular signals to activate intracellular targets, resulting in modulation of gene expression, proliferation, differentiation or apoptosis (Sylvain *et al*., 2012).

Cardiovascular: 1, 25(OH) 2D can indirectly modulate blood pressure by decreasing PTH levels. Vitamin D interferes with the renin–angiotensin system (RAS) that regulates blood pressure. That 1a, 25(OH) 2D and its analogues can reduce renin synthesis. Renin is a protease responsible for conversion of angiotensinogen to angiotensin I, which in turn is a precursor of angiotensin II in the RAS. Consequently, 1a, 25(OH) 2D may also reduce hypertension by slowing down RAS.

Diabetes: 1, 25(OH) 2D can trigger transcription of the human insulin receptor gene in U-937 human promonocytic cells. The activities where vitamin D (as 1a, 25(OH) 2D3) may be acting include pancreatic beta cell function, insulin sensitivity in peripheral target cells and, indirectly, systemic inflammation (Sylvain *et al*., 2012).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Study design

Cross sectional descriptive study

3.2. Study setting

The study was conducted in best care hospital – Khartoum state. In period form September 2019 to February 2020.

3.3. Study population

A total numbers of 87 subjects of postmenopausal women included in the study, those are volunteers and appeare to DEX scan.

3.4. Inclusion criteria

Postmenopausal women whose accepted to participate in this study were excluded.

3.5. Exclusion criteria

Postmenopausal women with glucocorticoids and vitamin D supplement were excluded.

3.6. Ethical consideration

This study was approved with ethical committee of Sudan university of Science and technology and then approved by health ministry ethical committee and also all participants were told about the research importance during interview and all of them agreed to participate.

3.7. Sampling technique

An informed consent was obtained from patient's after were informed by the aim of study. And collected blood sample in heparin container and separated the plasma by standard procedure, they were taken into sterile plan container and stored at -20c until analysis which perfect for the job to estimate vitamin D.

3.8. Vitamin D estimation

3.8.1. 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).

3.8.2. Procedure (appendix)

3.8.3. Quality control

The precision and accuracy for each EUROIMMUN 25-OH vitamin D kit and ELISA technique was apply and calibrators and controls using during test result to check all condition performed during the test to support accuracy result.

3.8.4.Reference range (appendix)

3.9. Statistical analysis

Was performed by using SPSS version 19. The mean and standard deviation (SD) for variables of the test group was obtained .One Way ANOVA test was utilized for comparison between variables groups and p-value less than 0.05 was considered Significant.

Chapter Four

Results

4. Results

This study was conducted to assess the total plasma 25 OH vitamin D and its association to bone mineral density in postmenopausal Sudanese women. 87 Post-menopausal women age (40-80 years).and the Mean ± SD of Age (58.8 ± 10.9), Vit-D (11.98 ± 4.2) and BMD as T-score (-1.35± -0.86) of study groups.

Table (4.1)

Show 37.9% of post-menopausal women were normal BMD, and 44.8% were osteopenia, and 17.2% were osteoporosis.

Table (4.2)

Show significant decrease in the mean of vitamin D in study group when compare with the mean of vitamin D in reference value P-value (0.00).

Table (4.3)

Show insignificant difference between the means of vitamin D level according to BMD groups *P*-value(0.16).

Table (4.4)

Show significant difference decrease between mean of BMD T-score and vitamin D groups P-value (0.01).

Figure (4.1)

A scatter plot shows no correlation between Vitamin D and BMD T-score. r= (0.096) and P-value (0.37).

Figure (4.2)

A scatter plot show positive correlation between BMD T-score and age r= (0.36) and p.value (0.001).

Figure (4.3)

A scatter plot show no correlation between Age and Vit-D with R value (-0.07) and p.value (0.51).

Table (4.1) Frequencies and percentage of BMD, Vitamin D and age of study groups.

Variables	Frequency	Percentage %
BMD		
Normal BMD	33	37.9%
Osteopenia	39	44.8%
Osteoporosis	15	17.2%
Total	87	100%
VitD		
Insufficient	7	8%
Deficiency	46	52.9%
Sever deficiency	34	39.1%
Total	87	100%
Age(year)		
Adult <65	57	65.5%
Elderly >65	30	34.5%
Total	87	100%

Table (4.2): A comparison between the mean of vitamin D level in study group and the mean of reference value(40 ng\ml) of Vit D.

	Mean ± SD	P- value
Vit D(ng\ml)	11.98 ± 4.2	0.00
Test value(ng\ml)	40	

* One sample T-test was used and P- value (0.00).

*Test value indicate to mean reference range of vitamin D (ng\ml).

Table (4:3) A comparison between mean of vitamin D level according to BMD groups.

Parameter	Normal BMD (34) (Mean ± SD)	Osteopenia(38) (Mean ± SD)	Osteoporosis (15) (Mean ± SD)	P-value
Vit-D (ng/ml)	11.28 ± 3.4	12.9 ± 4.5	11.12 ±4.5	0.16

One way ANOVA test was used for comparison.

Insignificant P-value (0.16)

Table (4:4) A comparison between mean of BMD T-score according to Vit D groups.

Parameter	Insufficient (7) (Mean ± SD)	Deficiency (46) (Mean ± SD)	Sever Deficiency (34) (Mean ± SD)	P-value
BMD T-score	-2.03 ± -0.9	-1.14± -0.7	-1.49 ± -0.9	0.01

One way ANOVA test was used for comparison.

Significant P-value (0.01).

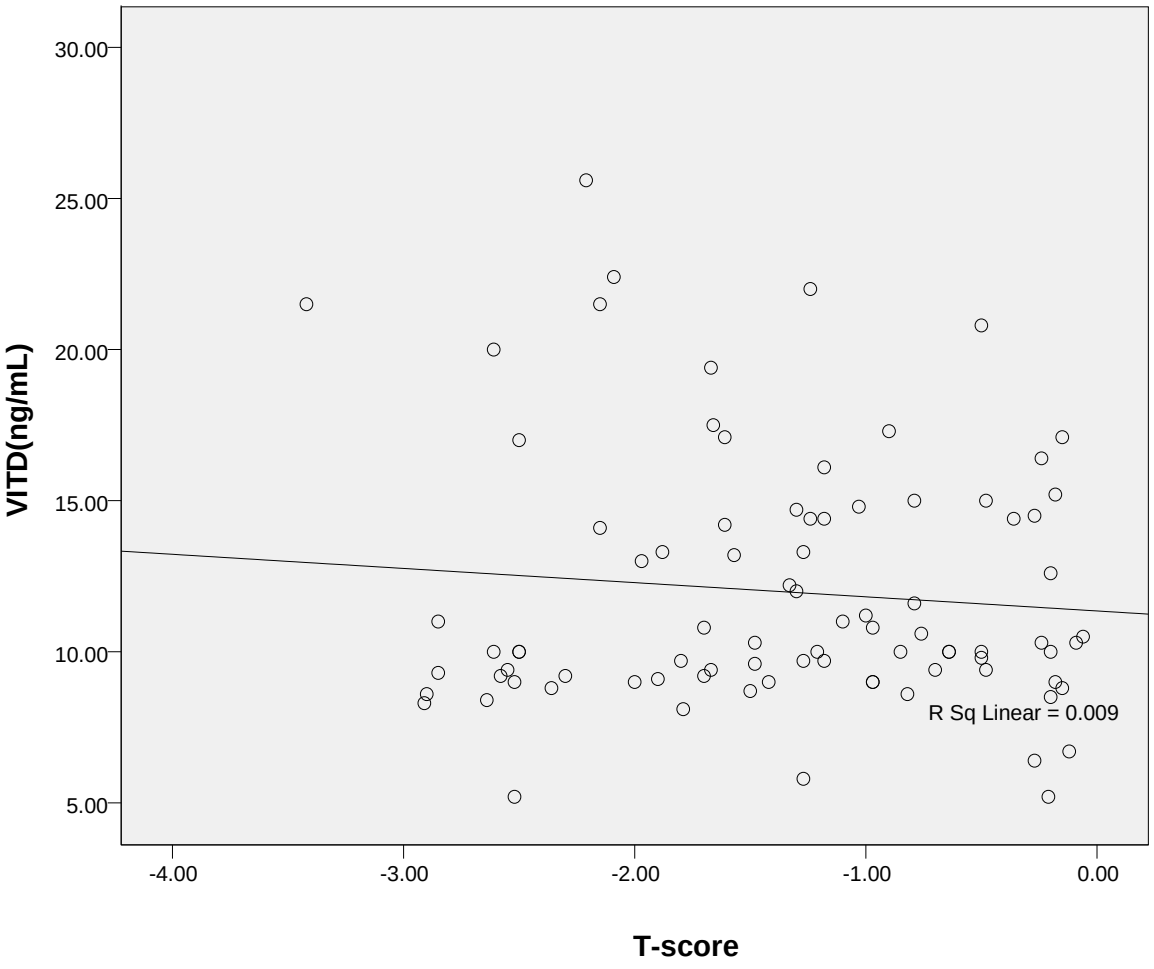


Figure (4.3) A scatter plot show no Correlation between BMD T- score and Vit-D with R value (0.096) and *p.value* (0.37).

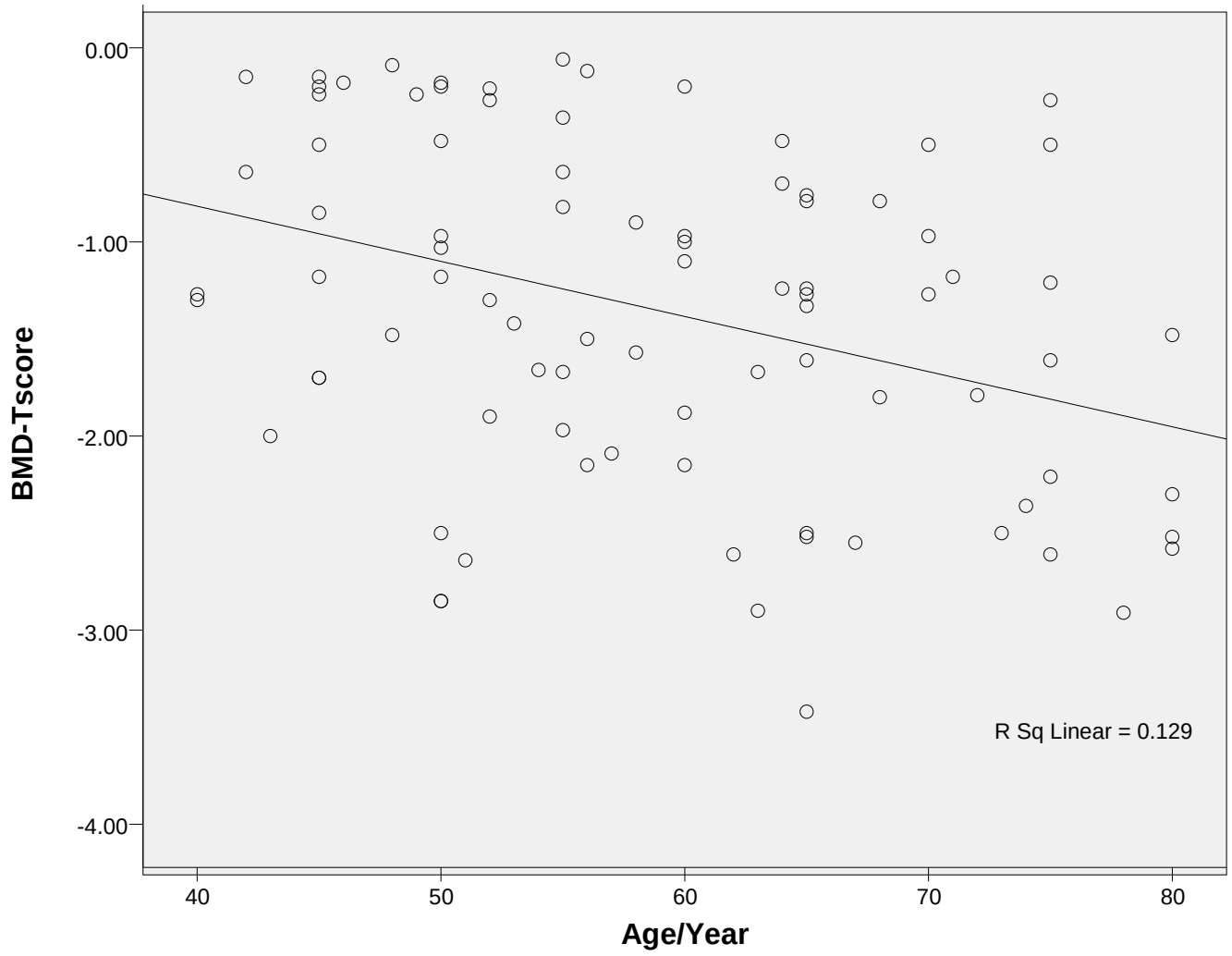


Figure (4.2) A scatter plot show positive Correlation between BMD T-score and age with R value (0.36) and *p.value* (0.001).

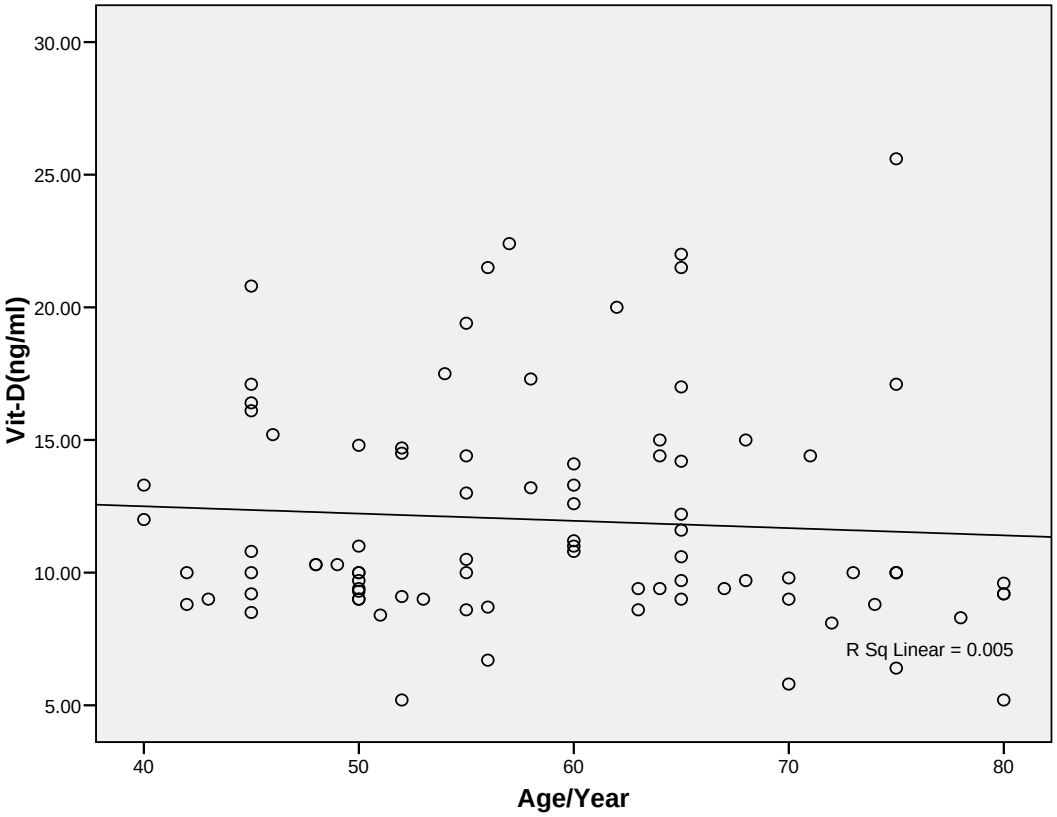


Figure (4:3): A scatter plot show no correlation between age and Vit-D with R value (-0.07) and *P.value* (0.51).

Chapter Five

Discussion, Conclusion and Recommendation

5. Discussion, Conclusion and Recommendation

5.1. Discussion

The link between vitamin D and bone mineral density is still being discussed. An evaluation of the vitamin D level in postmenopausal women with low bone mineral density is essential for two reasons. First, vitamin D deficiency in patients with osteoporosis causes demineralization that may reduce bone mass, Second, it is important to achieve suitable levels of vitamin D in patients with osteoporosis to maximize the response to antiresorptive therapy, facilitate changes in bone mineral density and efficiently manage fractures. Several studies have suggested that low serum vitamin D levels are associated with low bone mineral density. However, other studies have not supported this association (Pedro *et al*., 2013).

Our study show 45% of post-menopausal were osteopenia, and 17% were osteoporosis, this similar to study done by Pedro, *et al*., (2013). This may be due to lack of estrogen for each with lack physical activity and increase the age among osteoporosis.

Our study show significant decrease in mean of vitamin D level among postmenopausal when compared with the reference range, this agree to previous study displayed a high percentage of vitamin D deficiency among Sudanese women (Husain *et al*., 2019).The decrease may be due to lack of sun exposure (eg: color of skin, clothes and others) and lack of vitamin D in nutrients.

In current study show significant between mean of BMD T score according to vit-D groups in which the insufficient vit D more related to decrease in BMD, and this was in agreement to previous study conducted with Pedro *et al*.,(2013). The decrease may be not related to vitamin D only but with genetically.

Also presented no correlation between the low level of vitamin D and BMD T score in post- menopausal women. Similar studies done in various part of the world demonstrated that BMD had no significant relation to serum 25(OH) D status (Beg *et al*., 2014).The decrease in BMD may be due to lack of estrogen and genetically but not due to decrease in vit-D which is decrease in most Sudanese womens.

Our study current positive correlations between BMD Tscore and Age in pos-menopausal women. But have no correlation between the vit-D and Age. This a greed with study (Pedro et al., 2013).BMD correlated with the age, Increasing of age lead to replicative and matrix production activities of osteoblasts progressively diminish,

and various growth factors deposited in the extracellular matrix also diminish with time (Vinay *et al*., 2013).

5.2. Conclusion

This study conclude that Postmenopausal Sudanese women had a decrease in total plasma 25 OH vitamin D level, and osteopenia had most common among postmenopausal women's.

5.3. Recommendations

- More studies should be used in this problem by used other study design as case control to increase the significant of result and correlation.
- Sun exposure formula will be used in the questioner's to adjust decrease in vitamin D measurement.
-Could be used special vitamin D reference range for Sudanese.
- BMD and bone marker should be measured before the menopause.
-Genetic related to vitamin D and bone mineral density should be study.

References

Andersen, T., Sondergaard, E., Skorzynska, E.,(2009). A physical mechanism for coupling bone resorption and formation in adult human bone, *American Journal of Pathology*;**174**(1):239–247.

Bach Quang, L., Victor,N., Simon,M., Clemens, A., Van,B., Vanessa ,L.,(2018). The Components of Bone and What They Can Teach Us about Regeneration, *Review Materials;***11**:7-16.

Beg, M., Akhtar, N., Alam, M., Rizvi, I., Ahmad, J., Gupta, A., (2014). Vitamin D status and serum osteocalcin levels in postmenopausal osteoporosis: Effect of bisphosphonate therapy, *J Indian Acad Clin Med*; **15**: 172–176.

Bishop, M., Edward, F., Larry, S., (2010). *Clinical chemistry* .6th ed. By Lippincott Williams & Wilkins, a Wolters Kluwer ;502-503.

Bruzzaniti, A., Baron, R., (2007). *Molecular regulation of osteoclast activity*. Rev Endocr Metab Disord; **7**:123–139.

Canalis, E., (2005). *Mechanisms of glucocorticoid action in bone*. Curr Osteoporos Rep;**3**:98–102.

Capulli, M., Paone, R., Rucci, N., (2014). Osteoblast and osteocyte: games without frontiers, *Archives of Biochemistry and Biophysics*;**561**:3–12.

Carle, A., Edward, R., David, E., Barbara, G., (2008). *Tietz fundamental of clinical chemistry.*6th. Saunder imprint of Elsevier inc; 723-725.

Chen, L., Kuo, L., Chen, K., (2019). Medical Treatment for Osteoporosis: From Molecular to Clinical Opinions, *International Journal of molecular and science*;**20**:2-23.

Chugh, T., Jain, AK., Jaiswal, RK., Mehrotra, P., Mehrotra, R., (2013) Bone density and its importance in orthodontics, *Journal of Oral Biology and Craniofacial Research*; **3**(2): 9297.

Clarke, B., (2008). Normal bone anatomy and physiology, *Clinical Journal of the American Society of Nephrology*;**3**(3):131–139.

Cosman, F., (2018). Long-term treatment strategies for postmenopausal osteoporosis, *Curr Opin Rheumatol*;**30**(4):420-426.

Datta, K., Ng W, F., Walker, J., Tuck, S., Varanasi, S., (2008). The cell biology of bone metabolism, *Journal of Clinical Pathology*;**61**(5):577–587.

Denise, R., Ferrier., (2014).*Lippincott's reviews biochemistry.*6th ed.china,Philadelphia;386-388.

Downey, P., Siegel, M., (2006). Bone biology and the clinical implications for osteoporosis, *Physical Therapy*;**86**(1):77–91.

Everts, V., Delaissié, J., Korper, W., (2002). The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation, *Journal of Bone and Mineral Research*;**17**(1):77–90.

Elango, J., S., Henrotin, Y., W., M., Guo, R., Wang, C., Robinson, J., Regenstein, J., B., Wu, W., (2018). *Cross-talk between primary osteocytes and bone marrow macrophages for osteoclastogenesis upon collagen treatment*. Sci Rep;**8**(1):5318.

Fernández.,Tresguerres.,Hernández-Gil, I., Alobera., Gracia, M., Pingarrón, M., (2006). *Physiological bases of bone regeneration II. The remodeling process*.Med Oral Patol Oral Cir Bucal; **11**:151–157.

Franz-Odendaal, T., Hall, B., Witten, P., Buried alive., (2006). how osteoblasts become osteocytes, *Developmental Dynamics* ;**235**(1):176–190.

Garnero, P., (2017). *The Utility of Biomarkers in Osteoporosis Management*. Mol Diagn Ther;**21**(4):401-418.

Husain, N., Badie, S., Abdelrahman, I., Bedri, S., Musa, R., Osman, H., Mustafa, A., Gafer, N., Farah, E., Satir, A.A., Ahmed, M., Osman, M., Khalil, A., Agaimy, A., (2019). Vitamin D level and its D level and its determinants among Sudanese women: Does it matter in a sunshine African country?, *J family med prim care*;**8**(7): 2389-2394.

Jeremiah, M., Unwin, B., Greenawald, M., (2015). *Diagnosis and management of osteoporosis*. Am Fam Physician.;**92**(4):261–268.

Juan, P., G., Hector, A., (2017). Bone Mineral Density (BMD). Curr Trends Biomedical Eng & Biosci; **2**(1): 576-577.

Kanis, J., Burlet, N., Cooper, C., Delmas, P., Reginster, J., Borgstrom, F., (2008). *European guidance for the diagnosis and management of osteoporosis in postmenopausal women*. Osteoporos Int ; **19**(4):399.

Katherine and Abel., (2013). *Official CPC certification study guide, American medical association.* P. 108

Kim, Y., Han, J., Lee, J., Choi, H., Kim, J., Lee, T., (2017). *The correlation between bone mineral density/trabecular bone score and body mass index, height, and weight*. Osteoporos Sarcopenia ; **3**(2):98-103.

Kline, J., Tang, A., Levin, B., Smoking.,(2016). *Alcohol and caffeine in relation to two hormonal indicators of ovarian age during the reproductive years*. Maturitas;**92**:115–122.

Kucukalic-Selimovic.E., Begic A.,(2004). *Value of bone scintigraphy for detection and ageing of vertebral fractures in patients with severe osteoporosis and correlation between bone scintigraphy and mineral bone density*. Med Arh;**58**(6):343-4.

Lee BS.,(2018). *Myosins in Osteoclast Formation and Function. Biomolecules*;**8**(4) .

Levis,S., and Lagari, V.,(2012). *The role of diet in osteoporosis prevention and management*. Curr Osteoporos Rep;**10**:296–302.

Linos, E., Keiser, E., Kanzler, M., Sainani, K., Lee, W., Vittinghoff, E.,Chren, M., Tang, J., (2011). *Sun protective behaviors and vitamin D levels in the US population*. Cancer Causes Control;1–8.

Logan, C., and Nusse, R., (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol*;**20**:781–810.

Long, F., (2011). Building strong bones: Molecular regulation of the osteoblast lineage. *Nat. Rev. Mol. Cell Biol*.**13**: 27–38.

Mackie, E., (2003). Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol*;**35**:1301–1305.

Maresz, K., (2015). *Proper calcium use: vitamin K2 as a promoter of bone and cardiovascular health*. Integr Med (Encinitas);**14**:34–39.

Matic, I., Matthews, B., Wang, X., (2016). *Quiescent bone lining cells are a major source of osteoblasts during adulthood*. Stem Cells; **34**:2930–2942.

Nair, R., Maseed, A., (2012). Vitamin D:the (sunshine) vitamin. *J Pharmacol pharmacother*;(2): 118-126.

Panchbhai, A., (2013). *Quantitative estimation of vertical heights of maxillary and mandibular jawbones in elderly dentateand edentulous subjects*. Spec Care Dentist; **33**(2):62-69.

Pedro, J., Saulo, S., FRL., BE., JSF., JMF., Robinson, E., (2013). Vitamin D and its relation to bone mineral density in post-menopausal women. *J Revista Brasileira de ortopedia;***48**(3): 228-235.

Qaseem, A., Forciea, M., McLean, R., Denberg, T.,(2017). Clinical Guidelines Committee of the American College of Physicians.Treatment of low bone density or osteoporosis to prevent fractures in men and women.Intern Med ;818–839.

Ross, F.,(2013). *Osteoclast biology and bone resorption*. In: Rosen CJ, et al.(eds) Primer on the metabolic bone diseases and disorders of mineral metabolism.New Jersey, USA: John Wiley & Sons, Inc; pp.25–33.

Sinnesael, M., Claessens, F., Boonen, S., Vanderschueren, D., (2013). *"Novel insights in the regulation and mechanism of androgen action on bone"*. Current Opinion in Endocrinology, Diabetes and Obesity;**20**(3): 240–244.

Susi, R., Pitw, W., LSK., SCM., HI., Elza, I., (2019). Relationship of age, BMI, BMD and menopause duration with alveolar bone resorption in post-menopausal women.*Pesquisa Brasileira em odo notopediatria*. clinical integrada; **19**:191-192.

Sylvain, B., Susan, J., Sebastien, P., Jean, M., (2012). Vitamin D metabolism, functions and needs: from science to health claims. *European Journal of Nutrition;* DOI 10.1007/s00394-012-0430-5.

Taichman, R., (2005). *Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem cell niche*. Blood; **105**:2631–2639.

Urano, T., Shiraki, M., Kuroda, T., Tanaka, S., Urano, F., Uenishi, K., Inoue S., (2018). Bisphosphonates prevent age-related weight loss in Japanese postmenopausal women. *J. Bone Miner*. Metab;**36**(6):734-740.

Viany, K., Abul, K,Abbas., Jon,C., (2013). *Robbins basic pathology*.9th ed. Saunders, an imprint of elsevier Inc;765-772.

Wang,L., Dong, J., Xian, C., (2018). *Computational Investigation on the Biomechanical Responses of the Osteocytes to the Compressive Stimulus*: A Poroelastic Model. Biomed Res Int;407.

Watts, N.,(2004). *Fundamentals and pitfalls of bone densitometry using dual-energy X-ray absorptiometry (DXA)*. Osteoporos .Int; **15**:847–854.

World Health Organization(2003). *Prevention and Management of Osteoporosis*: Report of a WHO Scientific Group.(WHO technical report series; 921). Geneva.

Zhang, X., Yu Z., Yu M., Qu X.,(2015). *Alcohol consumption and hip fracture risk*. Osteoporos Int.;**26**:531–542.

Appendices

Assessment of total plasma 25-OH vitamin D level and its relation to bone mineral density among postmenopausal Sudanese women

Questionnaire

No ……. .Date……..

Name……Age…….. Tel……

In menopause: Yes ( ) No ( )

Clinical data:

BMD as (T-score):…….

Normal ( ) Osteopenia( ) Osteoporosis( )

- Hyperparathyroidism ( ) - Lack of exercise ( )
- Lack of sun exposure ( ) - Surgical of ovary ( )
- Excessive alcohol ( ) - Smoker ( )

Site of fracture if present: …….

Interference factors:

- Vitamin D supplement ( ) – Calcium ( ) – Glucocorticoid ( )

Laboratory investigations:

- Vit. D …….ng/ml.
- Comment:……

Signature ……

EUROIMMUN Medizinische Labordiagnostika AG

25-OH Vitamin D ELISA
Test instruction

ORDER NO.	ANTIGEN	SUBSTRATE	FORMAT
EQ 6411-9601	25-OH vitamin D	Ab-coated microplate wells	96 x 01 (96)

Indication: Suspected vitamin D deficiency or overdosing, reduced intestinal vitamin D uptake, hypocalcaemia, hypophosphataemia, hypocalciuria, elevated alkaline phosphatase, reduced bone mineral content.

Principles of the test: This ELISA test 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 patient sample 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. Results for the samples can be calculated directly using a standard curve.

Contents of the test kit:

Component	Colour	Format	Symbol
1. **Antibody-coated microplate wells** 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	—	12 x 8	STRIPS
2. **Calibrator 1** 0 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 1
3. **Calibrator 2** 4 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 2
4. **Calibrator 3** 10 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 3
5. **Calibrator 4** 25 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 4
6. **Calibrator 5** 60 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 5
7. **Calibrator 6** 120 ng/mL 25-OH vitamin D	red-brown	1 x 1.0 ml	CAL 6
8. **Control 1,** 25-OH vitamin D	red-brown	1 x 1.0 ml	CONTROL 1
9. **Control 2,** 25-OH vitamin D	red-brown	1 x 1.0 ml	CONTROL 2
10. **Biotin**, 100x concentrate	blue	1 x 1.2 ml	BIOTIN 100x
11. **Sample buffer**	yellow	1 x 100 ml	SAMPLE BUFFER
12. **Enzyme conjugate,** ready for use	blue	1 x 12 ml	CONJUGATE
13. **Wash buffer** 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
14. **Chromogen/substrate solution** TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
15. **Stop solution** 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
16. **Test instruction**	—	1 booklet	
17. **Quality control certificate**	—	1 protocol	

LOT Lot description — CE — Storage temperature

IVD In vitro diagnostics — Unopened usable until

EUROIMMUN Medizinische Labordiagnostika AG · 23560 Lübeck (Germany) · Seekamp 31 · Tel. +49 451 58550 · Fax +49 451 5855 591

Preparation and stability of the patient samples

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Severely haemolytic or lipaemic serum samples should not be used.

Please **note:** Diluted samples should only be used for one test run and subsequently discarded. Always use fresh samples and calibrator dilutions for every test run!

Performance: The calibrators/controls and patient samples for analysis are diluted 1:26 in working strength biotin.
Always pipette the samples and calibrators into the dilution tubes first (not included in kit; adequate are common commercial available single-use tubes consisting of borosilicateglas, polypropylene or polystyrene). Then add the working strength biotin within 5 minutes and leave to act for 10 minutes at room temperature.

Example: Add 0.5 ml of working strength biotin to 20 µl of sample and mix thoroughly (vortex). Incubate the mixture for at least 10 minutes at room temperature (+18°C to +25°C). The samples can subsequently be pipetted into the reagent wells according to the pipetting scheme.

Incubation

(Partly) manual test performance

Sample incubation: (1. step)	Pipette **200 µl** of sample diluted in biotin/sample buffer into each of the microplate wells. Incubate for **2 hours** at room temperature (+18 °C to +25 °C).
Washing:	Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash. Automatic: Wash reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus"). Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.
Enzyme conjugate incubation: (2. step)	Pipette **100 µl** of enzyme conjugate (streptavidin-peroxidase) into each of the microplate wells and incubate for **30 minutes** at room temperature (+18 °C to +25 °C).
Washing:	Empty the wells. Wash as described above.
Substrate incubation: (3. step)	Pipette **100 µl** of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to 25°C) (protect from direct sunlight).
Stopping the reaction:	Pipette **100 µl** of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.
Measurement:	**Photometric measurement** of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

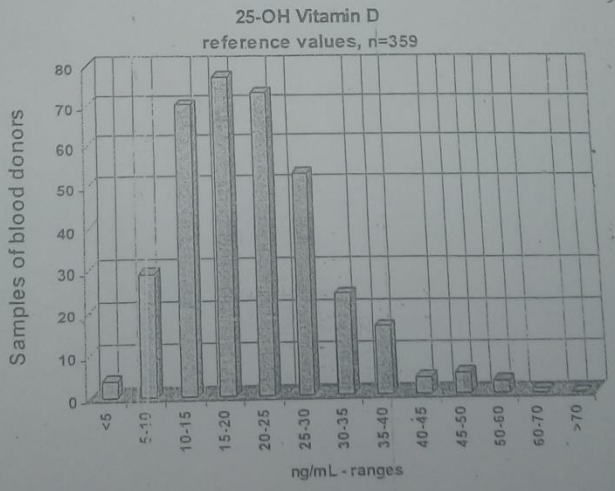
Method comparison: The EUROIMMUN ELISA was compared with various reference tests and showed the following correlations:

Diasorin, 25-OH Vitamin D Total Liaison	EUROIMMUN = 0.985 x Diasorin + 3.8 ng/ml; n = 108; $R^2 = 0.854$
IDS, 25-OH Vitamin D ELISA	EUROIMMUN = 1.147 x IDS - 0.5 ng/ml; n = 231; $R^2 = 0.931$
LC-MS/MS	EUROIMMUN = 1.001 x LC-MS - 1.2 ng/ml; n = 100; $R^2 = 0.926$

Reference range: 359 serum samples from apparently healthy blood donors in the age range of 13 to 99 years old were investigated using the EUROIMMUN ELISA. The mean 25-OH vitamin D concentration was 20.9 ng/ml with a 5-95% percentile range of 8.2 to 37.4 ng/ml.

25-OH vitamin D concentration	Number (n = 359)	ng/ml
Very severe vitamin D deficiency	4	<5
Severe vitamin D deficiency	29	5-10
Vitamin D deficiency	147	10-20
Suboptimal vitamin D provision	126	20-30
Optimal vitamin D level	49	30-50
Upper norm	4	50-70
Overdose, but not toxic	0	70-150
Vitamin D intoxication	0	>150
Mean value		20.9
5.0 – 95.0 % percentile		8.2 – 37.4
2.5 – 97.5 % percentile		6.9 – 44.4

The evaluation method used was modified according to Hollick, MF Vitamin D Deficiency. N Engl J Med 2007; 357:266-281



Every laboratory should use their own normal values established under specific ambient conditions.

Calculation:
25-OH vitamin D_3 (ng/mL) x 2.5 = 25-OH vitamin D_3 (nmol/L)