1.1. Introduction:

1.1.1. Vitamin D

Is a fat-soluble vitamin that is produced when ultraviolet rays from sunlight strike the skin and trigger vitamin d synthesis. Vitamin D is also naturally present in some food like oily fish and sun-dried mushroom. The vitamin D from skin synthesis or digestive tract enters the blood circulation and is hydroxylated by specific enzyme forming 25- hydroxyvitamin D (25(OH)D) in the liver. 25(OH) D is the main circulating form of vitamin D in the body. It is not bio-active but its serum concentration of 25(OH) D. The 25(OH)D is formed in the liver then reaches the kidney via the circulation. In the kidney, 25(OH) D is further hydroxylated to form hydroxyvitamin D ($1,25(OH)_2D$), which is the active metabolite in the body. $1,25(OH)_2D$ binds to the vitamin D receptor (VDR) in target cells to form a vitamin - receptor complex, which has its biological effect via gene expression. It is well know that the characteristic biologic roles of vitamin D are to affect the bone, kidney and small intestine to regulate the metabolism of calcium and phosphors. However, the finding that many tissues synthesize $1,25(OH)_2D$, and that VDR is extensively expressed throughout the body, led to the concept that adequate vitamin D levels are important for many non-skeletal functions in the body, including the cardiovascular and immune system. Emerging data indicated that $1,25(OH)_2D$ might play a role in immunoregulation, Cellular proliferation, differentiation, and including apoptosis procedures by direct and indirect gene regulation. So, Vitamin D deficiency could be a pathological condition for many disease including cardiovascular disease, cancer and autoimmune diseases (Yan, 2014).

1.1.2. Calcium (Ca^{2+}):

Calcium is the fifth most abundant element in the human body. It is an essential element that is only available to the body through dietary source (Munro, 2010). The main sources of calcium are: mill, cheese, eggs, fish, green vegetables and fruits (Kamal and Salam, 2007).

Current dietary calcium recommendations range from 1000 to 1500 mg/d, depending on age. In some individuals, particularly the elderly, calcium supplements may be needed to achieve the recommended dietary calcium intake (Munro, 2010).

1.1.2.1. Physiology of Calcium (Ca^{2+}):

Calcium Physiology In 1883, Ringer showed that Ca^{2+} was essential for myocardial contraction. While attempting to study how bound and free forms of Ca^{2+} affected frog heart con-traction, McLean and Hastings showed that the ionized/free Ca^{2+} concentration was

proportional to the amplitude of frog heart contraction, whereas protein-bound and citrate bound Ca^{2+} had no effect, from this observation, they developed the first assay for ionized/free Ca^{2+} using isolated frog hearts. Although the method had poor precision by today's standards, the investigators were able to show that blood-ionized Ca^{2+} was closely regulated and had a mean concentration in humans of about (1.18 mmol/L). Because decreased ionized Ca^{2+} impairs myocardial function, it is important to main an ionized Ca^{2+} at a near normal concentration during surgery and in critically ill patients. Decreased ionized Ca^{2+} concentrations in blood can cause neuromuscular irritability, which may become clinically apparent as irregular muscle spasms, called tetany (Bishop et al., 2010).

1.1.3. Menopause:

Puberty is the time in which a child's sexual and physical characteristics mature. It occurs due to hormone changes. Menopause, also known as the climacteric, is the time in most women's lives when menstrual periods stop permanently, and they are no longer able to bear children (Ahmed, 2017).

Menopause typically occurs between 49 and 52 years of age. Medical professionals often define menopause as having occurred when a woman has not had any vaginal bleeding for a year (O'Connor et al., 2009) .

It may also be defined by a decrease in hormone production by the ovaries. In those who have had surgery to remove their uterus but they still have ovaries, menopause may be viewed to have occurred at the time of the surgery or when their hormone levels fell. Following the removal of the uterus, symptoms typically occur earlier, at an average of 45 years of age. Before menopause, a woman's periods typically become irregular, who means that periods may be longer or shorter in duration or be lighter or heavier in the amount of flow. During this time, women often experience hot flashes; these typically last from 30 seconds to ten minutes and may be associated with shivering, sweating, and reddening of the skin. Hot flashes often stop occurring after a year or two. Other symptoms may include vaginal dryness, trouble sleeping, and mood changes (Ahmed, 2017).

The severity of symptoms varies between women. While menopause is often thought to be linked to an increase in heart disease, this primarily occurs due to increasing age and does not have a direct relationship with menopause. In some women, problems that were present like endometriosis or painful periods will improve after menopause (Melby et al., 2005).

Menopause is usually a natural change. It can occur earlier in those who smoke tobacco(Warren, 2009).

Other causes include surgery that removes either ovaries or some types of chemotherapy (O'Connor et al., 2009).

At the physiological level, menopause happens because of a decrease in the ovaries' production of the hormones estrogen and progesterone. While typically not needed, a diagnosis of f menopause can be confirmed by measuring hormone levels in the blood or urine (Ahmed, 2017).

Menopause is the opposite of menarche, the time when a girl's periods start (Wood and James, 2017).

Specific treatment is not usually needed. Some symptoms, however, may be improved with treatment. With respect to hot flashes, avoiding smoking, caffeine, and alcohol is often recommended. Sleeping in a cool room and using a fan may help (Ahmed, 2017).

The following medications may help: menopausal hormone therapy (MHT), clonidine, gabapentin, or selective serotonin reuptake inhibitors (Krause and Nakajima, 2015).

Exercise may help with sleeping problems. While MHT was once routinely prescribed, it is now only recommended in those with significant symptoms, as there are concerns about side effects. High-quality evidence for the effectiveness of alternative medicine has not been found (Ahmed, 2017) .

There is tentative evidence for phytoestrogens (Franco et al., 2016).

1.2. Rationale:

Up to 20% of bone loss happens in the 5 to 7 years just after menopause. The two major causes of Vitamin D and calcium loss during this time are estrogen deficiency and age related processes. Bone turnover increases to high levels and estrogen deficiency may induce Vitamin D and calcium loss by indirect effects on extra skeletal calcium homeostasis. This loss may develop Osteoporosis, eye damage, and an abnormal heartbeat. Nowadays, vitamin D has been considered, due to its various effects on health, and numerous studies have been conducted on its various effects on different parts of body and proper functioning of different organs and systems. The benefit of the research is to make postmenopausal females who are at risk of hypovitaminosis D aware that a deficiency may lead to major risks of health problems such as osteoporosis, cardiovascular disease, diabetes and different types of cancers.

In Sudan there is no much studies about menopause health, so more researches need to be carried out, to understand the medical condition of menopausal women, and accordingly,

there is no published study about calcium status in menopausal women in recent years in Sudan.

1.3. Objectives:

1.3.1. General objective:

To evaluate vitamin D and serum calcium levels among postmenopausal women.

1.3.2. Specific objectives:

1- To Estimate serum vitamin D and calcium levels compared to premenopausal group with postmenopausal females.

2- To correlate between vitamin D and serum calcium levels in premenopausal and postmenopausal females.

3- To correlate between vitamin D and serum calcium levels with some variables and their effect (age, weight, height, body mass index and exposure to sunlight) in postmenopausal women.

1.4. Hypothesis

We hypothesize that postmenopausal females aged above 50 years who have been living in Khartoum may have vitamin D deficiency as a result of multiple risk factors.

1.5. Null Hypothesis

Postmenopausal females' populations have been living in Khartoum would have normal vitamin D levels.

Chapter Two

2.1 Literature Review:

2.1.1 Overview of Vitamin D:

The generic term vitamin D designates a group of chemically related compounds that possess antirachitic activity. The two most prominent members of this group are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) and vitamin D does not meet the classical definition of a vitamin. A more accurate description of vitamin D is that it is a prohormone and thus, vitamin D is metabolized to a biologically active form that functions as a steroid hormone (Zempleni et al., 2007).

2.1.2 Vitamin D Structure

Vitamin D refers to a family of structurally related compounds that display anti rachitic activity. Members of the D-family are derived from the cyclopentanoperhydrophenanthrene ring system, which is common to other steroids, such as cholesterol, vitamin D has only three intact rings; the B ring has undergone fission of the 9, 10-carbon bond resulting in the conjugated triene system that is present in all the vitamins (Zempleni et al., 2007).

2.1.3 Vitamin D Nomenclature

Vitamin D is named according to the new revised rules of the International Union of Pure and Applied Chemists (IUPAC). Vitamin D is designated seco because its B ring has undergone fission. Asymmetric centers are named using R, S notation and Cahn's rules of priority. The configuration of the double bonds is notated E, Z; E for Trans, Z for cis. The formal name for vitamin D3 is 9,10-seco(5Z,7E)-5,7,10(19)-cholestatriene- 3b-ol and for vitamin D2 it is 9,10-seco (5Z,7E)-5,7,10(19), 21-ergostatetraene-3b-ol (Zempleni et al., 2007).

2.1.4 Chemical properties

Vitamin D3 (C27H44O) Three double bonds; melting point, 848C 858C;Ultra violet (UV) absorption maximum at 264–265 nm with a molar extinction coefficient of 18,300 in alcohol or hexane, insoluble in H2O; soluble in benzene, chloroform, ethanol, and acetone; unstable in light; will undergo oxidation if exposed to air at 248C for 72 h; best stored at 08C. Vitamin D2 (C28H44O) Four double bonds; melting point, 1218C; UV absorption maximum at 265 nm with a molar extinction coefficient of 19,400 in alcohol or hexane, same solubility and stability properties as D3 (Zempleni et al., 2007).

2.1.5 Physiology of vitamin D

Vitamin D functions through its vitamin D endocrine system, vitamin D3 must be sequentially hydroxylated at the C-25 position and then the C-1 position to generate the steroid hormone, 1a, 25(OH) 2D3, before it can produce any biological effects. The activation of vitamin D2 occurs via the same metabolic pathway as that of vitamin D3,vitamin D2 has only 25%–30% of the biological activity of vitamin D3 (Zempleni et al., 2007).

2.1.6 Sources and Synthesis of Vitamin D:

The primary sources of vitamin D is synthesis of vitamin D_3in the skin on exposure to sunlight. One study showed that the average individual synthesis 80% of their total vitamin D from sunlight (Nowson et al., 2012).

However many factor can effect vitamin D_3 synthesis in people. Vitamin D in the body may come both from dietary sources and from synthesis in the skin triggered by sun exposure, or more specifically ultraviolet B (UVB) irradiation. UVB irradiation stimulates cutaneous (skin) synthesis of cholecalciferol, which is stored in adipose tissue or undergoes hydroxylation in the liver to 25(OH)D, and then further hydroxylation in the kidney to the biologically active form, 1,25-dihydroxyvitamin D (Vidailhet et al., 2012).

2.1.6.1 Food sources of vitamin D

For the most part, vitamin D is present in unfortified foods in only very small and variable quantities. The vitamin D that occurs naturally in unfortified foods is generally derived from animal products. Salt-water fish such as herring, salmon, and sardines contain substantial amounts of vitamin D, and fish-liver oils are extremely rich sources. However, eggs, veal, beef, unfortified milk, and butter supply only small quantities of the vitamin. Plants are extremely poor sources of vitamin D; fruits and nuts contain no vitamin D; and vegetable oils contain only negligible amounts of the provitamin (Zempleni et al., 2007).

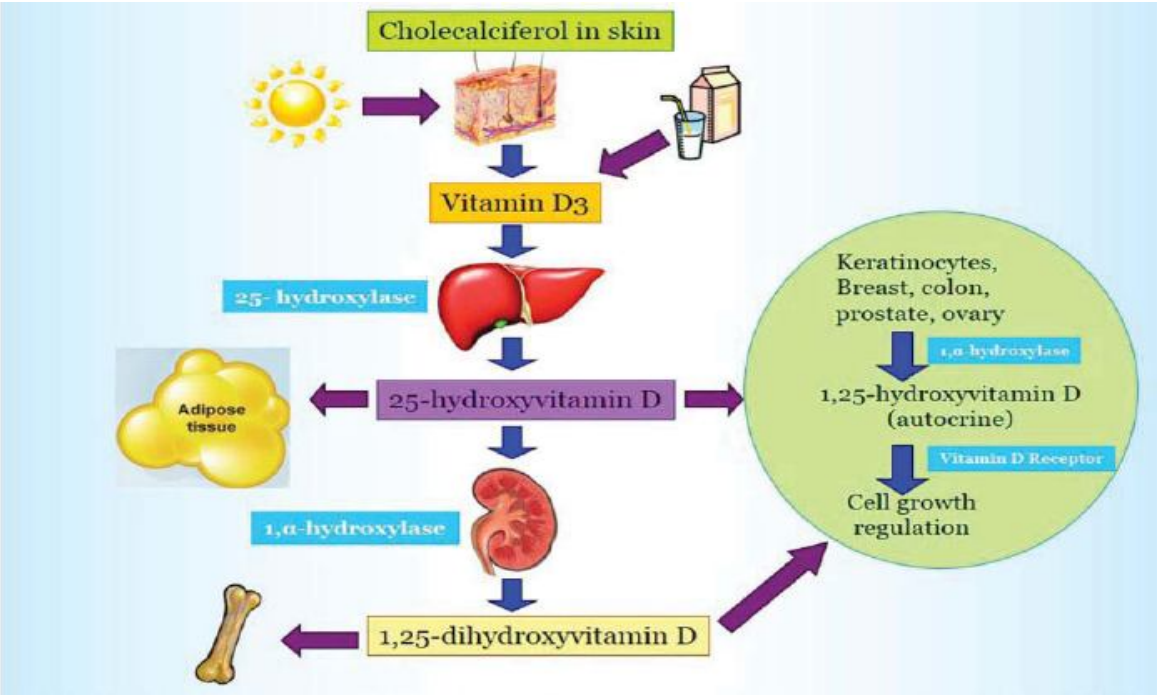

Figure 1: Synthesis of vitamin D3 and the skeletal and extra- skeletal effects of vitamin D3 (Yan, 2014).

2.1.7 Vitamin D Synthesis

Chemical Synthesis of vitamin D is that vitamin D is derived from cholesterol, the first synthesis of vitamin D resulted from the first chemical synthesis of cholesterol, as a consequence of a hydrogen shift the top panel depicts the dynamic changes occurring within the seco-B conjugated triene framework of the hormone (C5, 6, 7, 8, 9, 10, 19). Photochemical Production of Vitamin D3 although the body can obtain vitamin D from the diet, the major source of this prohormone can be its production in the skin from dehydrocholesterol. The highest concentrations of 7-dehydrocholesterol are found in the stratum basale and the stratum spinosum (Smith et al., 2004, Nowson et al., 2012, Zempleni et al., 2007).

2.1.8 Formation and ingestion of vitamin D

The two forms of vitamin D are vitamin D2 (ergocalciferol) and vitamin D3(cholecalciferol). Vitamin D2 is different from vitamin D3 by an additional double bond between the 22-23 carbon and an extra 24-methyl group (Figure 2.3). Vitamin D is not easily found in the food supply. Vitamin D2 is synthesized by the ultraviolet irradiation of yeast and the plant sterol, ergosterol. Vitamin D3 is naturally synthesized in the skin and is found in fatty fish and cod liver oil. Evidence suggests that ingestion of vitamin D3 is more efficient than vitamin D2 in raising serum 25-hydroxyvitamin D [25(OH)D] concentrations in humans Vitamin D3 is produced naturally in the skin by the ultraviolet (UV) irradiation of 7- dehydrocholesterol, a precursor of cholesterol. With sunlight exposure, ultraviolet B photons with wavelengths between 290 and 315 nm penetrate the skin and are absorbed by

the epidermal and dermal stores of 7-dehydrocholesterol (provitamin D3).This causes the cleavage of the 9-10 carbon bond of 7-dehydrocholesterol to form a 9,10-secosterol called previtamin D3, which is biologically inert and must undergo an isomerization via the skin's temperature to form vitamin D3 in the skin. Once vitamin D3 is formed it enters the dermal capillary bed where it binds to the vitamin D binding protein (DBP) and enters the circulation (Taha, 2012).

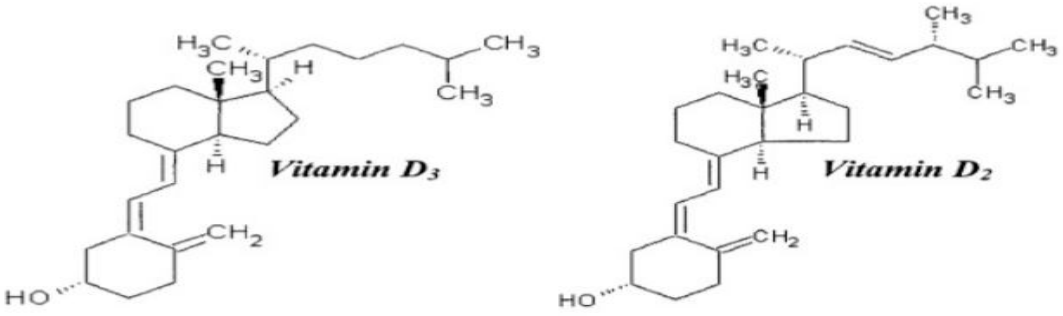

Figure 2: The chemical structures of vitamin D3 (on the left) and D2 (on the right) (Taha, 2012)

2.1.9 Isolation of vitamin D metabolites

Since vitamin D is a steroid, it is isolated from tissue by methods that extract total lipids, the technique most frequently used for this extraction is the method of Bligh and Dyer, over the years a wide variety of chromatographic techniques have been used to separate vitamin D and its metabolites. These include paper, thin-layer, column, and gas chromatographic methods (Zempleni et al., 2007).

2.1.10 Transport of Vitamin D in the Blood

2.1.10.1 Storage of Vitamin D

Following intestinal absorption, vitamin D is rapidly taken up by the liver thus blood has the highest concentration of vitamin D when compared with other tissues (Zempleni et al., 2007).

2.1.10.2 Metabolism of vitamin D

Before vitamin D can exhibit any biological activity, it must first be metabolized to its active forms. 1α, 25(OH) 2D3 is the most active metabolite known, but there is evidence that 24, 25(OH) 2D3 is required for some of the biological responses attributed to vitamin D, vitamin D undergoes its initial transformation with the addition of a hydroxyl group to the 25-carbon to form 25(OH)D3, the major circulating form of vitamin D, the production of 25(OH) D3 is catalyzed by the cytochrome P450 enzyme, vitamin D3 25-hydroxylase, the kidney is considered the primary source of circulating 1α,25(OH)2D3. The major controls on the production of 1α, 25(OH) 2D3 are 1α, 25(OH) 2D3 itself, PTH, and the serum concentration of calcium and phosphate (Bender et al., 2003) (Zempleni et al., 2007)

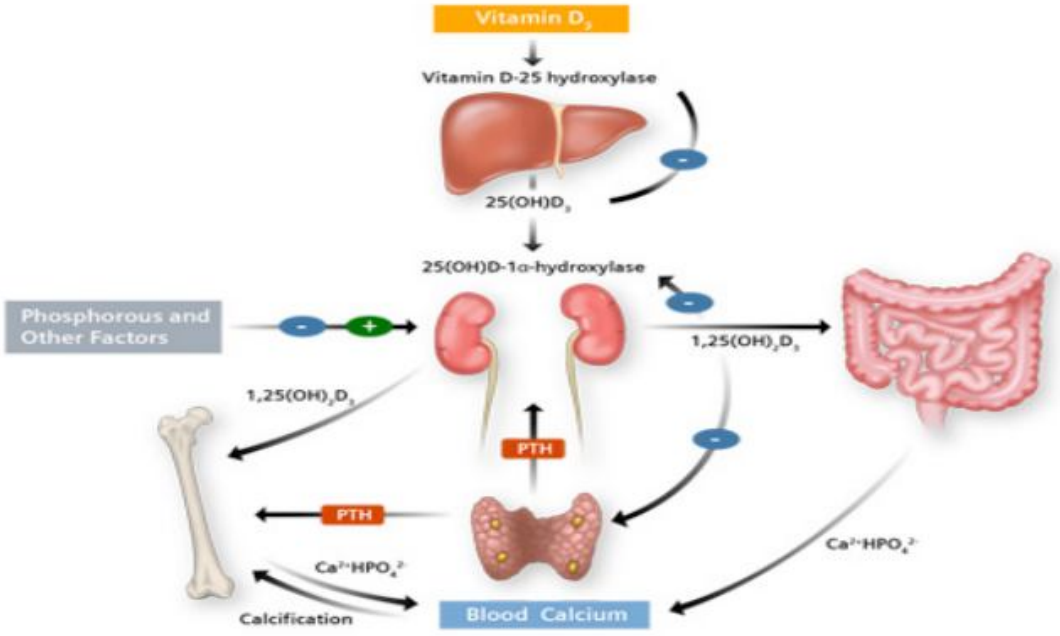

Figure 2.3 Schematic diagram of the metabolism of vitamin D .Vitamin D_3 is converted in the liver by the enzyme vitamin D25-hydroxylase to 25(OH) D (Taha, 2012)

2.1.10.3 Mechanism of action and health outcomes

The 1, 25$(OH)_2$ D hormone has a high affinity for the intracellular vitamin D receptor (VDR). VDR is widely expressed in the vitamin D target organs (intestine, bone, kidney and parathyroid glands) and in various non-calcium regulating organs including the skin, muscle, prostate, breast, colon, pancreas, and immune cells. 1, 25$(OH)_2$ D enters the cell by diffusion, facilitated entry (i.e. via megalin), or is locally synthesized 8(i.e. autocrine pathway), and then binds to VDR in the cytoplasm. This complex binds to the retinoic acid receptor (RXR) to form a heterodimer. The VDR-RXR heterodimer can then bind to vitamin D response elements (VDREs) in target genes and control their expression (Taha, 2012)

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2.1.10.4 Catabolism and excretion of vitamin D

The catabolic pathway for vitamin D is obscure, but it is known that the excretion of vitamin D and its metabolites occurs primarily in the feces with the aid of bile salts, very little appears in the urine (Zempleni et al., 2007).

2.1.11 Physiological action of vitamin D

2.1.11.1 Action of vitamin D in Endocrine System

The most clearly established effects of vitamin D are to maintain calcium and phosphate homeostasis, and to optimize bone health and muscle function. The hormonal form, 1, 25-(OH) 2D, increases active intestinal calcium (and phosphate) absorption, when calcium concentrations decrease below normal, even slightly, coupled to a G protein system, stimulate the secretion of parathyroid hormone (PTH). Parathyroid hormone then proceeds to the osteoblasts and to the proximal convoluted tubule cells within seconds. Most importantly, in the convoluted tubule cells that serve as the endocrine gland for the vitamin D hormone, 1- hydroxylase concentrations are markedly elevated. This signals the vitamin D hormone, which by itself stimulates intestinal absorption of calcium or together with parathyroid hormone, at higher concentrations, stimulates mobilization of bone calcium and renal reabsorption of calcium, the increase in serum calcium concentrations exceeds the set point of the calcium sensing system, shutting down the parathyroid gland-induced cascade of events (Norman, 2008, Katsilambros et al., 2010) (Harvey and Ferrier, 2011).

2.1.11.2 Non genomic action of vitamin D

The rapid or non-genomic responses mediated by 1α, $25(OH)_2 D_3$ were originally postulated to be mediated through the interaction of 1α, $25(OH)_2 D_3$ with a novel protein receptor located on the external membrane of the cell, this membrane receptor has now been shown to be the classic VDR (heretofore largely found in the nucleus and cytosol) associated with caveolae present in the plasma membrane of a variety of cells (Zempleni et al., 2007)

2.1.11.3 Vitamin D in Non-Classical System

Nuclear receptors for 1α, $25(OH)_2 D_3$ are found in a variety of tissues and cells not directly involved in calcium homeostasis, thus, the role of the vitamin D endocrine system has expanded to include a broader range of effects on cell regulation and differentiation, the expression of more than 100 proteins is known to be regulated by 1α, $25(OH)_2 D_3$, including several oncogenes by far extending the classical limits of vitamin D actions on calcium homeostasis, the presence of muscle weakness or myopathy during metabolic bone diseases related to vitamin D deficiency(Zempleni et al., 2007).

2.1.12 Functions of vitamin D

Vitamin D is essential for bone health throughout life. The hormonally active metabolite of vitamin D is 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), which regulates calcium absorption from the bowel, mediates the mineralization of osteoid tissue within bone, and plays an important role in muscle function. The major source of vitamin D is cutaneous production, following exposure to ultraviolet radiation. It has been suggested that, in temperate latitudes, exposure of the hands, arms and face to sunlight without the use of sun block for 5–10 min, two or three times weekly from April to October, will produce sufficient vitamin D to supply nutritional requirements.11 The diet provides smaller amounts of vitamin D, but this source is essential when cutaneous production is limited because of lack of exposure to sunlight. (FRANCIS et al., 2006)

2.1.12.1 Specific Functions of Active Vitamin D

Active vitamin D (1α, 25 (OH) $_2D_3$) and minerals metabolism, the classical target tissues for 1a,25 (OH) $_2D_3$ are those that are directly involved in the regulation of mineral homeostasis, serum calcium and phosphorous, actions on Intestine, deficiency of vitamin D severely impairs intestinal transport of both calcium and phosphorus, although calcium uptake is usually accompanied by phosphate uptake, the two ions are transported by independent mechanisms, both of which are stimulated by 1, 25(OH) $_2D_3$. Actions on bone, although the most obvious consequence of vitamin D deficiency is decreased mineralization of bone, $1,25(OH)_2D3$ apparently does not directly increase bone formation or calcium phosphate deposition in osteoid, actions on kidney, 1, 25(OH) $_2$D3 increases reabsorption of both calcium and phosphate.PTH secretion is increased in vitamin D deficiency, and hence tubular reabsorption of phosphate is restricted. actions on the parathyroid glands, the chief cells of the parathyroid glands are physiological targets for $1,25(OH)_2D3$ and respond to it in a manner that is characteristic of negative feedback Immunoregulatory Roles of 1α, 25(OH) $_2$D3,1α, 25(OH) $_2$D3 has been shown to affect cells of the immune system in a variety of ways.1α, 25(OH) 2D3 reduces the proliferation of HL-60 cells and also induces their differentiation to monocytes and macrophages. The actions of 1α, 25(OH) $_2$D3 on normal monocytes is controversial but it appears that it may enhance monocyte function. 1α, 25(OH) $_2$ D3 appears to reduce levels of HLA-DR and CD4 class II antigens on monocytes or macrophages with no effect on the expression of class I antigens (Zempleni et al., 2007).

2.1.13 Mechanism of Action

2.1.13.1 Nutritional requirements and recommended dietary allowance of vitamin D

The vitamin D3 requirement of healthy adults has never been precisely defined. Since vitamin D3 is produced in the skin on exposure to sunlight and can be retained in vertebrate tissues, humans may not have a requirement for vitamin D when sufficient sunlight is available. The international unit (IU) of

vitamin D3 is defined as ‘‘the vitamin D activity of 80.025 mg of the international standard preparation of crystalline vitamin D3. Thus, 1.0 IU of vitamin D3 is 0.025 mg (Zempleni et al., 2007).

The adequate intake allowance of vitamin D is 200 IU=day (5 mg=day) for infants, children, adult males, and females (including during pregnancy and lactation) up to age 51. For males and females ages 51–70 or more than 70, the adequate indicated level is set at 400 IU=day (10 mg=day) or 600 IU=day (15 mg=day), respectively (Goodman, 2002) (Zempleni et al., 2007).

2.1.14 Vitamin D Deficiency

A deficiency of vitamin D results in inadequate intestinal absorption and renal reabsorption of calcium and phosphate, as a consequence, serum calcium and phosphate levels fall and serum alkaline phosphatase activity increases, in response to these low serum calcium levels, hyperparathyroidism occurs. Increased levels of PTH, along with whatever 1α, 25(OH) 2D3 is still present at the onset of the deficiency, result in the demineralization of bone and this ultimately leads to rickets in children and osteomalacia in adults (Zempleni et al., 2007).

2.1.15 Hypervitaminosis D

Excessive amounts of vitamin D are not available from natural sources. However, vitamin D intoxication is a concern in those patients treated with vitamin D or vitamin D analogs for hypoparathyroidism, vitamin D-resistant rickets, renal osteodystrophy, osteoporosis, psoriasis, some cancers, or in those who are taking supplemental vitamins. Hypervitaminosis D is a serious problem as it can result in irreversible calcification of the heart, lungs, kidneys, and other soft tissues (Bender et al., 2003) (Zempleni et al., 2007).

2.1.16 Safety and Toxicity

Humans have a high physiological capacity to produce vitamin D3 in the skin.

Exposure to UVB light can generate the equivalent of 10,000 - 20,000IU of vitamin D in the skin. It is common for people living in sunny areas to have 25(OH)D levels over 100 nmol/ L, and up to 225 nmol/L. Farmers in Puerto Rico and lifeguards in St. Louis were shown to have 25(OH)D levels above 130nm/L. Therefore, serum 25(OH)D concentrations <225nmol/L are common in sunny environments, even without the use of supplements, and should be regarded as natural and safe. Vitamin D toxicity is manifested as hypercalcemia or hypercalciuria. Published literature suggests no adverse effects in vitamin D3 intakes up to 40,000 IU/day. Clinical trials that administered oral vitamin D3 intakes of 4000 IU. 10,000 IU phase 2 trials exploring the effects of high-dose (10,000 IU/day) vitamin D_3 in breast cancer patients with bone metastases. Cancer 40,000IU did not report any adverse events. Vitamin D intoxication is very rare but can be caused by the ingestion of excessively

high doses. Prolonged excessive intakes can lead to hypercalcemia, dehydration, kidney damage, and soft tissue calcification. Increased calcium intake, reduced renal function, reduced estrogen levels, and granulomatous conditions such as sarcoidosis can all predispose an individual to vitamin D intoxication. The first manifestation of vitamin D intoxication is hypercalciuria (i.e. excess calcium in the urine) followed by raised serum calcium concentrations.The urinary calcium: creatinine ratio was shown to be an effective screening tool to detect hypercalciuria caused by abnormalities in calcium metabolism. Vitamin D toxicity that results from hypercalcemia reflects the role of calcium in many tissues and targets, including bone, the cardiovascular system, nerves and cellular enzymes. Initial symptoms of hypervitaminosis D include generalized muscle weakness and fatigue, nausea, vomiting, constipation, confusion, drowsiness, difficulty in concentration, and depression. The probable mechanism for the toxicity of vitamin D is that high 25(OH)D concentrations causes excessive production of 1,25(OH)D. Along with the vitamin D and its other metabolites, 25(OH)D causes displacement of the hormone from DBP therefore increasing the amount of free, circulating 1,25(OH)D that is accessible to target cells Also, at toxic doses, the freely circulating vitamin D and its metabolites accumulate in adipose tissue and muscle. Thus far, the reported cases of vitamin D intoxication have been industrial accidents or were poisonings from an unknown source (Taha, 2012).

The US Institute of Medicine (IOM) recommendations for vitamin D (IOM 2011), based on a review of the evidence, concluded that:

- Serum 25(OH)D <30 nmol/l is deficient;
- Serum 25(OH)D of 30–50 nmol/l may be ‘inadequate’ in some people;
- Serum 25(OH)D >50 nmol/l is ‘sufficient’ for almost the whole population (97.5%).

The UK National Osteoporosis Society (NOS 2013) recently proposed that IOM vitamin thresholds should be adopted by UK practitioners (Spiro and Buttriss, 2014).

2.2 Overview of Calcium:

2.2.1 Definition and Formation of Calcium

Calcium is the fifth most common element and is the most prevalent cation in the human body. A healthy adult contains approximately 1–1.3 kg of calcium, and 99% of this is in the form of hydroxyapatite in the skeleton. The remaining 1% is contained in the extracellular fluid (ECF) and soft tissues. Additionally, less than 1% of the skeletal content of calcium is in bone fluid and exchanges freely with the ECF (Mundy and Guise, 1999).

2.2.2 Total Calcium Distribution

About 99% of calcium in the body is part of bone. The remaining 1% is mostly in the blood and other ECF. Little is in the cytosol of most cells. In fact, the concentration of ionized calcium in blood is 5,000 to 10,000 times higher than in the cytosol of cardiac or smooth muscle cells. Maintenance of this large gradient is vital to maintain the essential rapid inward flux of calcium (Bishop et al., 2010).

Calcium in blood is distributed among several forms. About 45% circulates as free calcium ions (referred to as ionized calcium), 40% is bound to protein, mostly albumin, and 15% is bound to anions, such as HCO_3, citrate, PO_4, and lactate. Clearly, this distribution can change in disease. It is noteworthy that concentrations of citrate, HCO_3, lactate, PO_4, and albumin can change dramatically during surgery or critical care. This is why ionized calcium cannot be reliably calculated from total calcium measurements, especially in acutely ill individuals (Bishop et al., 2010).

2.2.2.1Cellular Distribution

Ionic cytosol Ca is maintained at about 10^{-6}M. Because blood and ECF Ca is 10^{-3}M, the 1000-fold chemical gradient favors Caentry into the cell. The differential electrical charges across the cell plasma membrane of 50-mV gradient (cell interior negative) creates an electrical gradient that also favors Ca entry. Therefore, the major threat to cell viability is excessive Ca influx from the extracellular space along the electrochemical gradients. The defense against excess Ca influx into cells includes extrusion of Ca from the cell through energy-dependent Ca channels, Ca dependent ATP-driven Ca pumps, and Na-Ca exchangers and active uptake into organelles including the endoplasmic reticulum and mitochondria. Ca-binding proteins and Ca transport proteins facilitate Ca transport into these cellular organelles. They also serve to buffer excess calcium preventing cell death. Ca-rich mitochondria and endoplasmic reticulum also serve as reservoirs to maintain cytosolic Ca when levels fall. Ca bound to the plasma membrane and the organelles may also be released in pulses in response to activation of receptors on the external surface of the plasma membrane. Cell Ca homeostasis varies by cell function. For example, Ca facilitates the linking of excitation and contraction in skeletal and cardiac muscle through mobilization of the large Ca intracellular stores of the sarcoplasmic reticulum. In non skeletal cells, Ca serves as a signal transducer, mediating signaling from activated plasma membrane receptors to

carry out a variety of functions such as hormone secretion, neurotransmission, and kinase phosphorylation (Murray et al., 2006).

2.2.3 Functions and Physiological Actions of Calcium:

Calcium is an integral component of the skeleton, and the skeleton provides a reservoir of calcium for other essential calcium-dependent functions throughout the body. The skeleton serves at least three main functions. First, calcium, as part of the mineral hydroxyapatite, deposited into the organic matrix of the skeleton, is critical for its structure and is necessary for tissue rigidity, strength, and elasticity. This function allows for normal movement and exercise. Second, the skeleton functions as a source of minerals and alkali and therefore is critical for overall mineral homeostasis. The skeleton is the principal depot for calcium, containing 98 percent of total body calcium. It can be called on repeatedly, through the processes of bone formation and resorption (referred to as remodeling, as discussed below), to maintain circulating levels of calcium at a constant level. While the same qualitative processes apply to skeletal calcium metabolism across the life cycle, there are quantitative differences by age and hormonal status. These life cycle differences for skeletal growth and remodeling are discussed in a section below. Excessive calcium resorption can compromise the integrity and strength of the skeletal tissues. Third, the marrow cavity of bone serves as a major site for the development of hematopoietic cells and as a major compartment of the immune system. Several of the cell types involved in bone remodeling originate in the bone marrow compartment. Stromal or connective tissue cells are found in the bone marrow; at one time, these were thought to be inert, but they are now considered multi-potent stem cells that can become either fat or bone cells under the influence of specific differentiation factors. A principal physiological function of calcium apart from its role in maintaining the skeleton, is as an essential intracellular messenger in cells and tissues throughout the body. Although this pool of calcium is quantitatively small, the ionized calcium present in the circulatory system, extracellular fluid, muscle, and other tissues, is critical for mediating vascular contraction and vasodilatation, muscle function, nerve transmission, and hormonal secretion. Ionized calcium is the most common signal transduction element in biology, owing to its ability to reversibly bind to proteins and to complex with anions such as citrate and bicarbonate (Ross et al., 2011).

2.2.3.1 The Effect of Calcium on Menopause

Studies of bone histomorphometry and markers of bone remodeling indicate that bone remodeling is accelerated in the perimenopausal and postmenopausal periods. The span of 5

to 10 years surrounding menopause is characterized by a decrease in estrogen production and an increase in resorption of calcium from bone, resulting in a marked decrease in bone density. For example, measured changes in markers of bone mineral density (BMD) in a cohort of 281 women who were 45–57 years of age, and found the BMD in lumbar spine and femoral neck was decreased by 20 percent in perimenopausal and postmenopausal women compared with premenopausal women. The bone loss is most rapid in the early years of menopause, and then approximately 6 to 7 years postmenopause the loss continues at a slower rate. The bone loss associated with menopause results from uncoupling in the bone remodeling units, such that resorption of bone is greater than formation of new bone. Over time, such changes lead to skeletal fragility and decreased bone mass. Some cohort studies demonstrate that accelerated bone loss is an independent risk factor for fracture, such that the combination of low bone mass and high rates of bone turnover markedly increase the potential for a future fracture. Bone remodeling in postmenopausal osteoporosis includes changes in osteoid thickness, surface area, and volume. determined that defective osteoblast recruitment in women with osteoporosis resulted in decreased osteoid thickness, a characteristic of osteoporosis. Considerable variability exists among women regarding the effects of menopause on bone loss, and such effects vary according to body mass index and ethnicity. The effect of estrogen/progesterone treatment on preventing bone loss and reducing fracture risk is well established. However, the use of such therapy has declined as a result of recent reports of adverse non-skeletal effects. Because rapid bone loss occurs after estrogen treatment is discontinued, the potential impact on subsequent fracture rates is of interest but remains unclear (Ross et al., 2011).

2.2.3.2 The Relation between Calcium, Osteoporosis and Fractures

Osteoporosis is a skeletal disorder associated with aging and characterized by compromised bone strength due to reduced bone mass and reduced bone quality. Reduced bone mass—as measured by low BMD increases bone fragility and, in turn, predisposes a person to an increased risk of fracture, notably at the vertebrae, hip, and forearm. The relationship between BMD measures and the incidence of fractures is notable. Overall, osteoporosis-related morbidity and mortality, as well as health care costs, are a significant public health concern. Osteoporosis is most commonly associated with women, but the condition also occurs in men. Bone mineral density (BMD), osteoporotic fracture rate, and number of women with fractures (Ross et al., 2011). Menopause can initiate osteoporosis through elevated bone remodeling, which occurs characteristically in postmenopausal women.

Remodeling activity, although designed to repair weakened bone, actually makes it temporarily weaker when remodeling is excessive. It can lead to enhanced skeletal fragility. Although it is unclear to what extent calcium intake can mitigate such bone loss, inadequate calcium intake can exacerbate the situation. Men experience age-related bone loss as well, although not due to menopause. This, in turn, can result in osteoporosis. However, the incidence of fracture risk increases some 5 to 10 years later in men than it does in women (Ross et al., 2011).

2.2.4 Calcium Regulation

Three hormones, PTH, vitamin D, and calcitonin, are known to regulate serum Ca^{2}+ by altering their secretion rate in response to changes in ionized Ca^{2+}. PTH secretion in blood is stimulated by a decrease ionized Ca^{2+} and, conversely, PTH secretion is stopped by an increase in ionized Ca^{2+}. PTH exerts three major effects on both bone and kidney. In the bone, PTH activates a process known as bone resorption, in which activated osteoclasts break down bone and subsequently release Ca^{2+} into the ECF. In the kidneys PTH con-serves Ca^{2+} by increasing tubular reabsorption of Ca^{2+} ions, PTH also stimulates renal production of active vitamin D. Vitamin D3, a cholecalciferol, is obtained from the diet or exposure of skin to sunlight. Vitamin D3 is then converted in the liver to 25-hydroxycholecalciferol (25-OHD_3), still an inactive form of vitamin D. In the kidney, 25-OH-D3is specifically hydroxylated to form1, 25-dihydroxycholecalciferol (1, 25-$[OH]_2$-D_3), the biologically active form. This active form of vitamin D in-creases Ca^{2+} absorption in the intestine and enhances the effect of PTH on bone resorption. Calcitonin, which originates in the medullary cells of the thyroid gland, is secreted when the concentration of Ca^{2+} in blood increases. Calcitonin exerts itsCa^{2+} lowering effect by inhibiting the actions of both PTH and vitamin D. Although calcitonin is apparently not secreted during normal regulation of the ionized Ca^{2+} concentration in blood, it is secreted in response to a hypercalcemic stimulus (Bishop et al., 2010).

2.2.5 Clinical Applications

2.2.5.1 Hypocalcemia and Hypercalcemia

Hypocalcemia and hypercalcemia are terms used clinically to refer to abnormally low and high serum calcium concentrations. It should be noted that, because about one half of serum calcium is protein bound, abnormal serum calcium, as measured by total serum calcium, may occur secondary to disorders of serum proteins rather than as a consequence of changes in ionized calcium (Munro, 2010).

2.2.5.2 Causes of Hypocalcemia

Primary hypoparathyroidism glandular aplasia, destruction, or removal, Hypomagnesemia, Hypermagnesemia, Hypoalbuminemia (total calcium only, ionized not affected by) chronic liver disease, nephritic, syndrome, malnutrition, Acute pancreatitis, Vitamin D deficiency, Renal disease, Rhabdomyolysis and Pseudohypoparathyroidism (Bishop et al., 2010).

2.2.5.3 Causes of Hypercalcemia

Primary hyperparathyroidism adenoma or glandular, hyperplasia, Hyperthyroidism, Benign, Familial hypocalciuria Malignancy, Multiple myeloma, Increased and Prolonged vitamin D, immobilization Thiazide diuretics (Bishop et al., 2010).

2.2.6 Homeostasis

Because diet Ca intake and skeletal Ca requirements vary widely from day to day and across the various stages of the lifecycle, the homeostatic system is constantly adjusting to deliver sufficient Ca, Mg, and PO_4 from intestine and kidney into the ECF and blood and then to bone to meet changing skeletal growth requirements without disturbing the serum ionized Ca concentration [Ca^{2+}]. The serum Ca^{2+}fraction controls cellular biological functions, and therefore the homeostatic system maintains serum Ca^{2+} at the expense of BMC. Serum Ca^{2+}may increase from Ca influx from intestinal absorption or bone resorption and decrease with Ca efflux into bone mineralization sites, secretion into the intestinal lumen, or filtration at the renal glomerulus and secretion along selected segments of the nephron. A decline in serum Ca^{2+} is potentially more likely and is therefore defended against by all of the actions of PTH. PTH secretion is regulated by the parathyroid cell plasma membrane Ca-sensing receptor (CaSR), which detects ambient serum $Ca^{2+}$and so regulates minute to minute PTH secretion. PTH increases Ca influx into the extracellular space through enhanced renal tubule reabsorption of filtered Ca that occurs within minutes; increased osteoclastic and osteocytic-mediated bone resorption that appears within minutes to hours; and stimulation of intestinal Ca absorption indirectly through increased renal proximal tubule $1,25(OH)_2D3$ synthesis that appears by 24 h after PTH secretion. Hypercalcemia suppresses CaSR signaling and there by suppresses PTH secretion. Elevated serum Ca^{2+} stimulates distal nephron CaSR, which reduces net tubule Ca reabsorption, increases urine Ca excretion, and thus lowers serum Ca^{2+} to normal (Murray et al., 2006).

2.2.7 Overview of Vitamin D Regulation of Calcium

2.2.7.1 Vitamin D and Intracellular Calcium Homeostasis

Calcium homeostasis has long been linked to blood pressure regulation; however, this concept evolved with the demonstrations that intracellular calcium concentrations were positively associated with blood pressure and that the flux of calcium into vascular smooth muscle cells may be facilitated by $1,25(OH)_2D$. This suggests that vitamin D may play a role in regulating vascular tone by influencing the concentration of calcium in vascular smooth muscle cells (Forman and Wiliams, 2010) .

2.2.7.2 Secondary hyperparathyroidism

There are also other mechanisms involved in the relationship between blood pressure and vitamin D. Secondary hyperparathyroidism, commonly seen in vitamin D deficiency, could be the reason for hypertension. The mechanism is not completely clear, but it is a well known association that high PTH levels affect vascular smooth muscle cells and increase vascular stiffness and promotes hypertension (Jafari and Paknahad, 2012) .

2.2.8 Calcium and Vitamin D in the Prevention of Osteoporotic Fractures

Osteoporosis has been defined as a skeletal disorder characterized by compromised bone strength, predisposing a person to increased risk of fracture. The three major osteoporotic fractures are those of the forearm, vertebra and hip, but fractures of the humerus, pelvis and ribs are also common. The incidence of these fracture rises steeply with age, such that most occur in people aged >65 years, where they are associated with excess mortality, substantial morbidity, and significant health and social services expenditure (FRANCIS et al., 2006).

2.3 Overview of Menopause:

2.3.1 Definition of Menopause:

Menopause is defined as the permanent cessation of menstruation and ovulation due to ovarian failure. After 12 months of amenorrhea without pathological etiology, menopause is considered “natural” or “spontaneous.” Menopause can also be induced prematurely (before age 40 years) or early (before age 45 years), through medical interventions such as surgery (e.g., bilateral oophorectomy with or without hysterectomy), chemotherapy, or radiation. It occurs naturally between the ages of 42 and 58 years and is a consequence of reproductive senescence. The average age at onset appears fixed, as it has been unchanged since ancient Greece.4 In the United States, the number of women entering menopause (approximately 2 million per year5) will remain generally stable or even decline as baby boomers age. But

given the continued improvement in life expectancy at age 50, the number of menopausal years will increase both for individual women and the population as a whole (Grant et al., 2015).

2.3.2 The Symptoms of Menopause:

The most prominent symptoms of menopause tend to be the following according to (Center, 2009):

2.3.2.1 Hot Flashes and Night Sweats:

Women often experience hot flashes as an intense build-up in body heat, starting in the chest and face, lasting minutes, followed by sweating and chills, and especially common at night. Some women report accompanying anxiety as the sensation builds. In most cases, hot flashes resolve within two years of menopause, although in some women they May persist for years.

2.3.2.2 Palpitations: (heart pounding or racing) can occur, with or independent from hot flashes.

2.3.2.3 Difficulty Sleeping: Insomnia is also common during menopause; it may be caused by

The hot flashes or it may be an independent symptom of hormonal changes.

2.3.2.4 Mood Changes: Mood changes are most likely to be a combination of sleeplessness, hormonal swings, and psychologic. Once a woman has reached a menopausal state, however, depression is no more common than before, and women with a history of premenstrual depression often experience significant mood improvement.

2.3.2.5 Forgetfulness: This appears to be one of the few symptoms that are common across most cultural and ethnic groups.

2.3.2.6 Vaginal Dryness: Thinning of the vaginal lining results from estrogen deficiency, sometimes causing itching, burning, or pain on intercourse.

2.3.2.7 Sexuality: Sexual responsiveness tends to decline in most women after menopause, although other aspects of sexual function, including interest, frequency, and vaginal dryness vary. It is useful to remember that the symptoms of menopause will eventually go away.

2.3.2.8 Urine Leakage: This may occur with coughing, straining, or with sudden urges to urinate, all resulting from decreased elasticity of the vaginal and urethral tissues

2.3.3 Causes of Menopause:

Age: The typical age of menopause (last period from natural causes) is between 40 and 55 (Minkin et al., 1997) .

Premature ovarian failure: Premature ovarian failure (POF) is diagnosed or confirmed by high blood levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) on at

least three occasions at least four weeks apart Known causes of premature ovarian failure include autoimmune disorders, thyroid disease, diabetes mellitus, chemotherapy, being a carrier of the fragile X syndrome gene, and radiotherapy (Kalantaridou et al., 1998)

Surgical menopause: Menopause can be surgically induced by bilateral oophorectomy (removal of ovaries), which is often, but not always, done in conjunction with removal of the Fallopian tubes (salpingo-oophorectomy) and uterus (hysterectomy) (Ahmed, 2017) .

2.3.4 Stages of Menopause:

2.3.4.1 Premenopause:

Premenopause is a term used to mean the years leading up to the last period, when the levels of reproductive hormones are becoming more variable and lower, and the effects of hormone withdrawal are present (Ahmed, 2017) .

2.3.4.2 Perimenopause:

The term "perimenopause", which literally means "around the menopause", refers to the menopause transition years, a time before and after the date of the final episode of flow. This transition can last for four to eight years. During perimenopause, estrogen levels average about 20–30% higher than during premenopause, often with wide fluctuations (Prior and Jerilynn, 2013) .

These fluctuations cause many of the physical changes during perimenopause as well as menopause (Chichester et al., 2011) .

Some of these changes are hot flashes, night sweats, difficulty sleeping, vaginal dryness or atrophy, incontinence, osteoporosis, and heart disease. During this period, fertility diminishes but is not considered to reach zero until the official date of menopause. The official date is determined retroactively, once 12 months have passed after the last appearance of menstrual blood (Prior and Jerilynn, 2013).

The menopause transition typically begins between 40 and 50 years of age (average 47.5) (McNamara et al., 2015) .

The duration of perimenopause may be for up to eight years (McNamara et al., 2015) .

Women will often, but not always, start these transitions (perimenopause and menopause) about the same time as their mother did (Ahmed, 2017) .

2.3.4.3 Postmenopause:

The term "postmenopausal" describes women who have not experienced any menstrual flow for a minimum of 12 months, assuming that they have a uterus and are not pregnant or lactating (Ahmed, 2017).

2.4 Diseases Affected by Vitamin D on Elderly Females:

2.4.1 Osteoporosis (OP):

Osteoporosis is defined as a metabolic bone disease ‘characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (Susan and Lanham, 2007).

Osteoporosis has been defined as a skeletal disorder characterized by compromised bone strength, predisposing a person to increased risk of fracture. The three major osteoporotic fractures are those of the forearm, vertebra and hip, but fractures of the humerus, pelvis and ribs are also common. The incidence of these fracture rises steeply with age, such that most occur in people aged 65 years, where they are associated with excess mortality, substantial morbidity, and significant health and social services expenditure (FRANCIS et al., 2006)

Is characterized by low bone mass, deterioration of microarchitecture of bone tissue and bone fragility increase with consequent susceptibility to fracture. This bone pathology can be classified in primary or secondary forms. Primary osteoporosis is characterized by a progressive mineral bone lost as a function of people aging and it is influenced by changes of sex hormone. Instead, different pathologies as well as the use of specific medications, which affect skeletal health, can induce secondary osteoporosis. Primary form of osteoporosis comprises postmenopausal or senile disease (typeIor type II respectively). Although osteoporosis has long been considered a disease of women, an increase in age-related fractures has been observed also in men. Nowadays, the number of males with osteoporosis is unknown, probably because of the infrequency of screening and controversies in bone mineral density (BMD) testing standards in men. Approximately the 50% of women and the 25% of men aged 50 and older will have an osteoporotic fracture in the lifetime. Although many national and international organizations indicate to realize osteoporosis screening and treatment for men in their clinical guidelines, male osteoporosis remains recurrently not diagnosed and not treated (Paola Pisani et al., 2016).

2.4.1.1 Prevention from Osteoporosis

To reach optimal peak bone mass and continue building new bone tissue as you age, you should consider several factors according to (Shriver, 2017).

Calcium: An inadequate supply of calcium over a lifetime contributes to the development of osteoporosis. Many published studies show that low calcium intake appears to be associated with low bone mass, rapid bone loss, and high fracture rates. National nutrition surveys show that many people consume less than half the amount of calcium recommended to build and

maintain healthy bones. Food sources of calcium include low-fat dairy products, such as milk, yogurt, cheese, and ice cream; dark green, leafy vegetables, such as broccoli, collard greens, bok choy, and spinach; sardines and salmon with bones; tofu; almonds; and foods fortified with calcium, such as orange juice, cereals, and breads. Depending on how much calcium you get each day from food, you may need to take a calcium supplement.

Calcium needs change during one's lifetime. The body's demand for calcium is greater during childhood and adolescence, when the skeleton is growing rapidly, and during pregnancy and breastfeeding. Postmenopausal women and older men also need to consume more calcium. Also, as you age, your body becomes less efficient at absorbing calcium and other nutrients. Older adults also are more likely to have chronic medical problems and to use medications that may impair calcium absorption.

Vitamin D: Vitamin D plays an important role in calcium absorption and bone health. Food sources of vitamin D include egg yolks, saltwater fish, and liver. Many people obtain enough vitamin D naturally; however, studies show that vitamin D production decreases in the elderly, in people who are housebound, and for people in general during the winter. Adults should have vitamin D intakes of 600 IU (International Units) daily up to age 70. Men and women over age 70 should increase their uptake to 800 IU daily.

Exercise: Like muscle, bone is living tissue that responds to exercise by becoming stronger. Weight-bearing exercise is the best for your bones because it forces you to work against gravity. Examples include walking, hiking, jogging, climbing stairs, weight training, tennis, and dancing.

Smoking: Smoking is bad for your bones as well as your heart and lungs. Women who smoke have lower levels of estrogen compared with nonsmokers, and they often go through menopause earlier. Smokers also may absorb less calcium from their diets.

Alcohol: Regular consumption of 2 to 3 ounces a day of alcohol may be damaging to the skeleton, even in young women and men. Those who drink heavily are more prone to bone loss and fracture, because of both poor nutrition and increased risk of falling.

Medications that cause bone loss: Several medications can contribute to bone loss. For example, the long-term use of glucocorticoids (medications prescribed for a wide range of diseases, including arthritis, asthma, Crohn's disease, lupus, and other diseases of the lungs, kidneys, and liver) can lead to a loss of bone density and fracture. Bone loss also can result from long-term treatment with certain antiseizure drugs, such as phenytoin1 and barbiturates; gonadotropin-releasing hormone (GnRH) drugs used to treat endometriosis; excessive use of

aluminum-containing antacids; certain cancer treatments; and excessive thyroid hormone. It is important to discuss the use of these drugs with your doctor and not to stop or change your medication dose on your own.

All medicines can have side effects. Some medicines and side effects are mentioned in this publication. Some side effects may be more severe than others. You should review the package insert that comes with your medicine and ask your health care provider or pharmacist if you have any questions about the possible side effects.

2.6 Previous Studies:

2.5.1 In a study done by Bhale DV, Ansari HA , in 2014 a total of 30 postmenopausal women and 30 normal controls (pre-menopausal women) were included .The results showed that Serum calcium level was significantly deficient in post postmenopausal women than in pre-menopausal women (Bhale and Ansari, 2014).

2.5.2 In another previous study done by Qureshi HJ, et al, A total of 45 postmenopausal women and 45 pre-menopausal women were included. The results showed serum calcium was significantly lower in postmenopausal women as compared to that in pre-menopausal women (Qureshi et al., 2010).

2.5 .3 On the other hand in a study conducted by The North American Menopause Society 2010 in North America. Management of Osteoporosis in Postmenopausal Women. Including 100 pre-menopausal and 100 postmenopausal women with evidence of osteoporosis. The results showed that Osteoporosis, which is especially prevalent among older postmenopausal women, increases the risk of fractures. Hip and spine fractures are associated with particularly high morbidity and mortality in this population (Society., 2010).

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Study Approach:

Quantitative methods used to measure plasma vitamin D and calcium level in Sudanese women in Yastabshiroon, during period from March to May 2017.

3.1.2 Study Design

A quantitative, Descriptive Analytical cross-sectional Hospital based study.

3.1.3 Study Area

This study was carried out in Yastabshiroon Hospital at Khartoum state.

3.1.4 Study Population

Ninety females were enrolled in this study, and then classified based on age into two groups, group one less than 45 years (45 females as premenopausal) considered control, group two more than 50 years (45 females as postmenopausal).

3.1.5 Inclusion Criteria

Specimens were collected from Postmenopausal females and healthy volunteer were included.

3.1.6 Exclusion Criteria

Postmenopausal women with (Hypertension, Diabetes Mellitus, Bones Disorder, Liver Diseases, Renal Diseases and Parathyroid Disorder) which can effect on vitamin D and calcium level in the blood have been excluded from the study.

3.1.7 Ethical Considerations

All individual included in this study told for study purpose and asked for agreement and verbal Consent was taken regarding acceptance to participate in the study and re-assurance of confidentiality. Before the specimen was collected.

3.1.8 Data Collection:

Interview a questionnaire: was used for each participant in this study to obtain the clinical data.

3.1.9 Blood Samples Collection:

Local antiseptic 70% ethanol was used to clean the skin, venous blood (3ml) were taken from each participant by standard procedure in heparin anticoagulant container, and then centrifuged at 3000 rpm for 3 minutes and plasma obtained for vitamin D and calcium, the plasma was separated in Eppendorf tubes and kept in refrigerator (-20°) until used.

3.1.10 Study Variables:

Age, weight, height, body mass index and exposure to sunlight) these variables for both groups, but in postmenopausal group we add other questions if menopause occur before 50 years old and number of children.

3.2 Methods:

3.2.1 Measurement of Vitamin D:

3.2.1.1 Biochemical Measurement:

ELISA was used for estimation of Vitamin D.

3.2.1.2 Reagent Composition:

Preparation of working reagent and assay condition for measurement of plasma Vitamin D**.**

3.2.1.3 Principle of Vitamin D by using ELISA:

The ELISA test provides a quantitative in vitro determination of 25 OH Vitamin D in human serum or plasma. First step calibrators and volunteer's samples are diluted with biotin - labeled 25–OH vitamin D and added to microplate wells coated with monoclonal anti- 25–OH vitamin D antibodies. During in the incubation an unknown amount of 25–OH vitamin D in patient sample and known amount of biotin - labeled 25–OH vitamin D compete for antibody binding sites in the microplate wells plate. Unbound 25–OH vitamin D is removed by washing. For the detection of bound biotin - labeled 25–OH vitamin D a secound incubation is performed using peroxidase – labelled streptavidin. In a third incubation using the peroxidase substrate tetramethylbenzidine (TMB) the bound peroxidase promotes colour reaction. The colour intensity is inversely proportional to the 25–OH vitamin D concentration in the samples.

3.2.1.4 Procedures:

As the leaflet.

3.2.2 Measurement of Plasma of Calcium

3.2.2.1 Biochemical Measurement:

Biosystem-semi automated was used for estimation of calcium.

3.2.2.2 Reagent Composition

Preparation of working reagent and assay condition for measurement of plasma calcium.

3.2.2.3 Principle of Calcium Estimation:

Connerty and Briggs described methods using alizarin 3 –Sulphonate and Vitro calcium reagent is based on the Cresolphthalein Complexone (CPC) method of Moorehead and Briggs . CPC reacts with calcium and Magnesium in alkaline solution to form a deeply colored Complex. 8- hyddroxyquinoline is incorporated into the reagent to preferentially bind magnesium and prevent interference from this

cation. CPC is an acid – Base indicator necessitating the use of a strong buffer to stabilize the pH. calcium reacts with Cresolphthalein Complexone to form Purple color complex in alkaline medium

Calcium + O – Cresolphthalein Complexone alkaline medium > Calcium - Cresolphthalein Complexone complex.

The intensity of color formed is proportional to the calcium concentration in the sample.

3.2.2.4 Procedure

As the leaflet.

3.2.2.5 Calculation:

The calcium concentration in sample was calculated using Formula:

$$\text{Calcium (mmol\L): } = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{conc of standard.}$$

3.2.3 Quality Control:

The precision and accuracy of al methods were checked each time; a batch was analyzed by using commercially control sera.

3.2.4 Statistical Analysis:

Statistical Package of Social Science (SPSS Version 23) software was used for data analysis used for descriptive statistics mean, standard deviation of plasma of vitamin D and calcium level were calculated, Paired sample t- test employed to compare mean concentrations and Linear regression analysis was used to assess Parson´s correlation between the variables of the study. The Results were expressed as (mean ± SD), and significance difference were consider as (P-value <0.05) and the result were presented in the form of tables and figures.

Chapter Four
Results

4.1 Demographical Data:

The blood concentration levels of vitamin D and calcium were estimated in two groups of women after and before menopause, Age, weight, height, body mass index and exposure to sunlight were taken in consideration; to find out if they effect on the levels of vitamin D and calcium.

The numbers of participants in this study were 90 individuals 45 Premenopausal and 45 Postmenopausal Females.

4.2 The mean levels of serum Vitamin D and calcium among Females with Postmenopausal compared to Premenopausal females (control group):

The study showed that, there were significant decreased in the mean of serum Vitamin D and serum calcium levels in Postmenopausal Females compared to premenopausal (23 ± 8.33) verse (14 ± 6.19) and (9.6 ± 1.16) verse (7.8 ± 0.94) respectively with (P-value 0.000) which presented in Table (4.1).

4.3 Correlations:

Person's correlation showed in serum vitamin D level is correlated with Age, exposure to sunlight, occupation, medication, weight, height and body mass index (r = 0.497, p-value 0.000), (r = - 0.085, p-value 0.426), (r = 0.132, p-value 0.214), (r = - 0.300, p-value 0.004), (r = 0.240, P-value 0.023) and (r=0.333, P -Value 0.001) respectively.

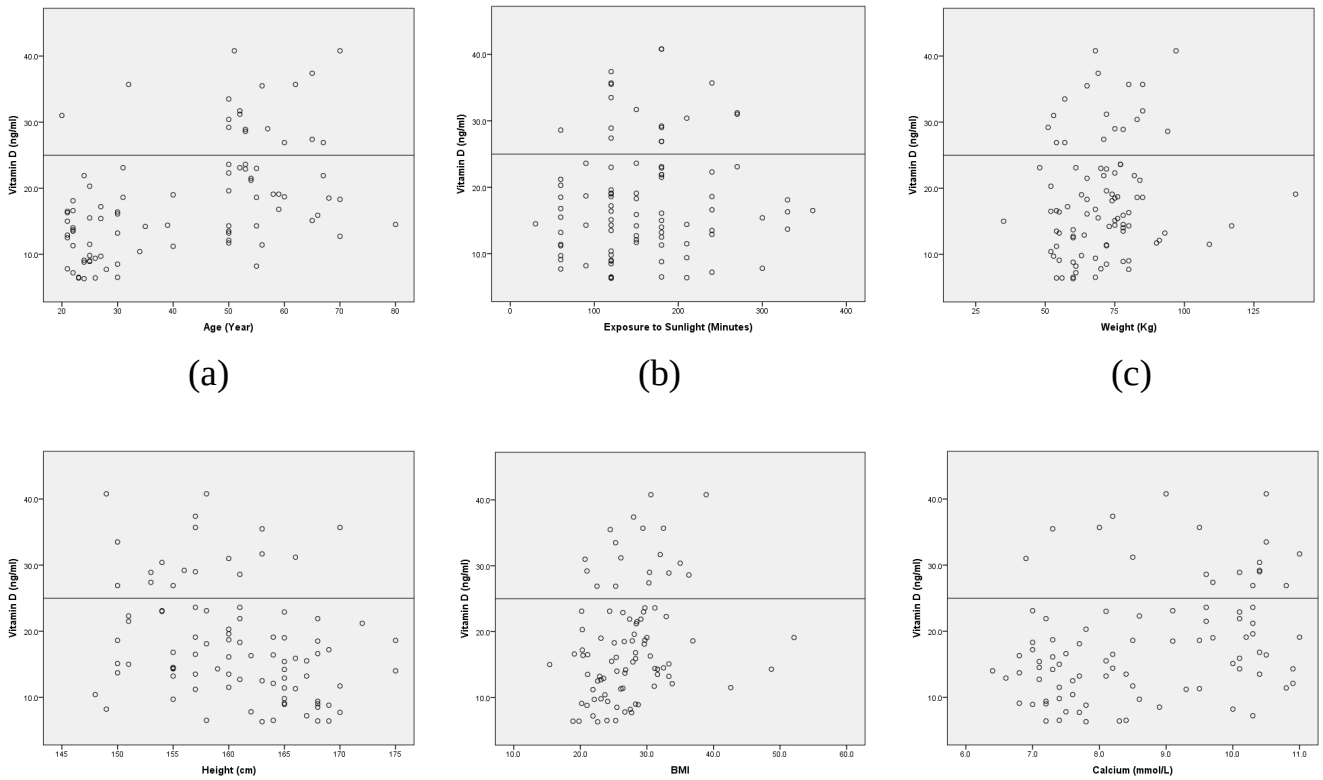
Person's correlation showed in serum Calcium level is correlated with Age, exposure to sunlight, weight, height and body mass index (r = 0.333, p-value 0.001), (r = - 0.246, p-value 0.019), (r = - 0.231, p-value 0.028), (r = 0.289, p-value 0.006) and (r = 0.385, p-value 0.000) respectively.

Person's correlation showed in the participants according to their age less than 50 years old and 50 and more years old is correlated with vitamin D and serum Calcium levels (r = 0. 019, p-value 0.000) and (r = 0.084, p-value 0.000) respectively.

Table 4.2: The mean levels of Age, exposure to sunlight, weight, height, body mass index, vitamin D and Calcium among postmenopausal females compared to control group (premenopausal females).

Variable	Premenopausal Females Mean ± SD	Postmenopausal Females Mean ± SD	P. Value
Age	26 ± 5.2	57 ± 7.4	0.013
Exposure to Sunlight	3.1 ± 1.5	2.2 ± 0.92	0.001
Weight	65 ± 13	76 ± 16	0.935
Height	163 ± 6.2	158 ± 6.0	0.898
Body Mass Index	24 ± 4.5	30 ± 5.8	0.453
Vitamin D	23 ± 8.3	14 ± 6.19	0.019
Calcium	9.6 ± 1.2	7.8 ± 0.94	0.084

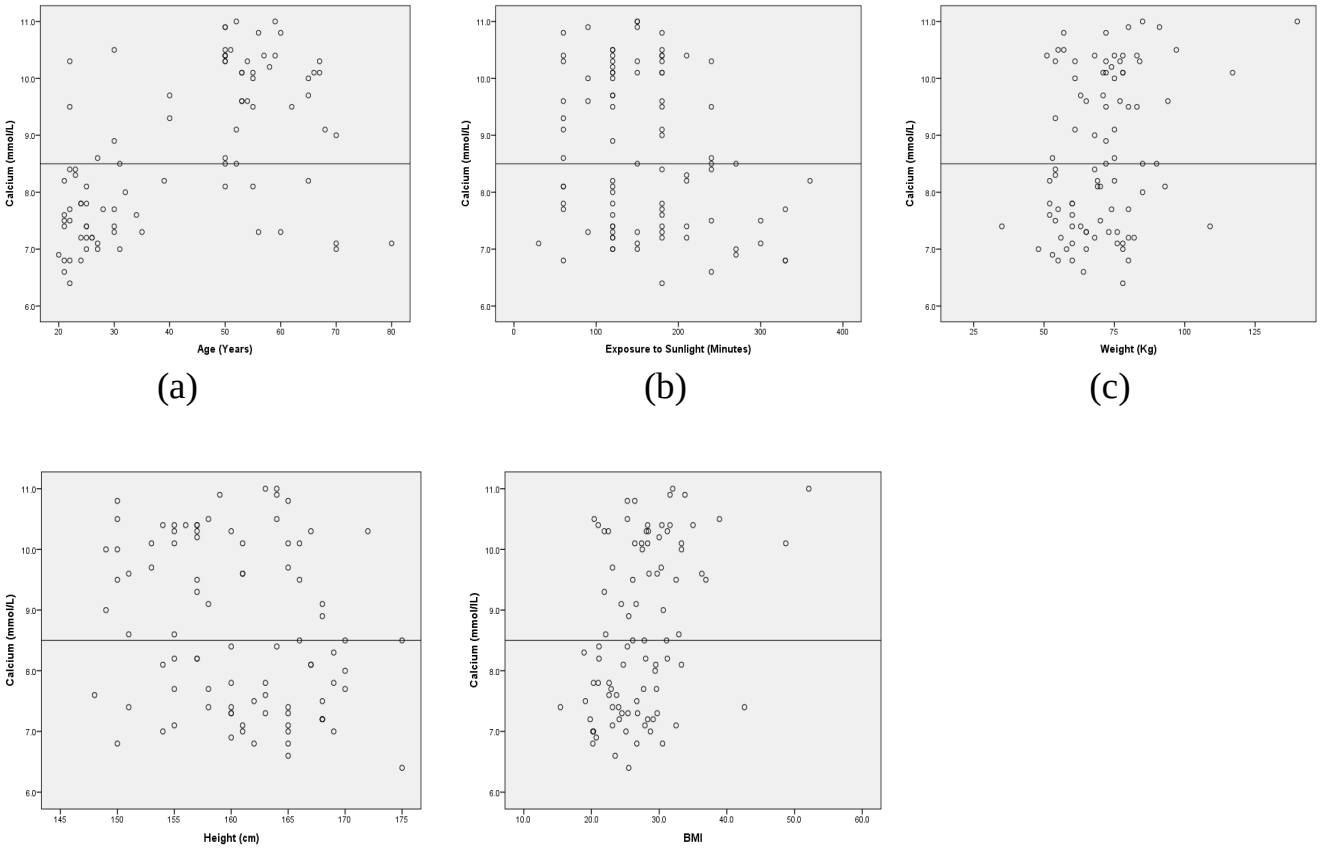
4.3.1 Correlation of vitamin D and Calcium between study variables



(a) (b) (c)

(d) (e) (f)

Figure (4.1): Correlation between vitamin D with (a) Age (r = 0.497, P-value 0.000), (b) Exposure to sunlight (r = 0.085, P-value 0.426), (c) Weight (r = 0.132, P-value 0.214), (d) Height (r = - 0.300, P-value 0.004), (e) Body mass index(r = 0.240, P-value 0.023) and (f) Calcium (r = 0.333, P-value 0.001)



(a) (b) (c)

(d) (e)

Figure (4.2): Correlation between Calcium and (a) Age (r = 0.333, P-value 0.001), (b) Exposure to Sunlight (r = - 0.246, P-value 0.019) (c) Weight (r = - 0.231, P-value 0.028), Height (r = 0.289, P-value 0.006) and (e) Body Mass Index (r = 0.385, P-value 0.000).

Chapter Five

Discussion, Conclusion and Recommendation

5.1 Discussion:

This study is descriptive cross sectional hospital based study was conducted in order to evaluate serum vitamin D and calcium levels among postmenopausal females, in addition to compare vitamin D and calcium levels with premenopausal females risk factors (Age, Regularity of Menstruation, Menstrual cycle, dietary intakes of calcium, fractures, exposure to sunlight, occupation, medication, weight, height and body mass index).

In postmenopausal women, the prevalence of 25(OH) vitamin D concentrations ≤20 ng/ml ranged from 1.6% to 86% for community-living and institutionalized women, respectively. The most common factors associated with inadequate vitamin D levels included limited sun exposure, lack of dietary vitamin D intake, nursing home environment, wintertime, and increasing age (over 70 years).The prevalence of inadequate vitamin D levels appears to be high in post-menopausal women, especially in those with osteoporosis and history of fracture. Vitamin D supplementation in this group might offer scope for prevention of falls and fracture, especially in elderly and osteoporotic populations (Gaugris et al., 2005) .

In other study show that 91.9% of cases had 25OHD serum levels below 30 ng/ml (74.8% had VD deficiency, 17.1% VD insufficiency). Only 8.1% had sufficient VD levels. A history of fragility fractures was present in 45.83% of the osteoporotic patients, 27.27% of the osteopenic ones and 15.15% of the women with normal BMD. 32 women (26%) were on VD supplementation at the time of evaluation. Among these subjects, the 25OHD level was significantly higher in those with prior fragility fractures (p=0.018) and osteoporosis (p=0.008). 25OHD concentration negatively correlated with PTH, alkaline phosphatase (ALP) and osteocalcin. The bone markers evaluated had a significant inverse correlation with the radius BMD, T and Z scores (p=0.004). 27.17% of the cases with VD deficiency had secondary hyperparathyroidism. The 25OHD concentration was significantly lower in these cases (p=0.000) (Ross et al., 2011).

In This study measured in 44 normal subjects and 27 osteoporotic patients. For all normals, calcium absorption and serum 1,25(OH)2D were positively correlated ($r = 0.50$, $P < 0.001$). In nonelderly normal subjects (ages 30-65 yr), dietary calcium intake correlated inversely with both calcium absorption ($r = -0.39$, $P < 0.01$) and with serum 1,25(OH)2D ($r = -0.50$, $P < 0.01$). Both osteoporotic patients and elderly normal subjects (ages 65-90 yr) differed from nonelderly normals in that these correlations were not present. In addition although serum

25-OH-D was normal, serum 1,25(OH)2D was significantly decreased in both osteoporotic patients and elderly normals ($P < 0.001$). In osteoporotic patients, calcium absorption increased significantly ($P < 0.001$) after 7 d administration of a small dose (0.4 μg/d) of synthetic 1,25(OH)2D3. In osteoporotics mean serum immunoreactive parathyroid hormone was either normal (COOH-terminal assay) or low (NH2-terminal assay) relative to age-matched controls (Gallagher et al., 1979).

In other study showed that radiocalcium absorption decreased with age ($P = 0.018$); it was 28% lower in the 25 women aged >75 y than in the rest ($P < 0.001$). It was significantly related to serum 1,25-dihydroxyvitamin D [1,25(OH)2D] in the whole set and in both the younger and older subsets, but it was not related to either 25-dihydroxyvitamin D [25(OH)D] or PTH or to any other measured variable. No decrease in 1,25(OH)2D was seen with age to account for the decrease in calcium absorption, so radiocalcium absorption corrected for serum 1,25(OH)2D decreased significantly after age 75 y. On multivariate analysis, the serum 1,25(OH)2D concentration was a positive function of 25(OH)D ($P < 0.001$), albumin ($P = 0.010$), and PTH ($P = 0.012$) and a negative function of serum creatinine ($P = 0.003$). PTH was a negative function of calculated ionized calcium ($P = 0.004$) and 25(OH)D ($P = 0.009$) and a positive function of weight ($P = 0.011$) and age ($P = 0.028$) (Christopher et al., 2004).

In addition study observed that Postmenopausal female are more susceptible to vitamin D deficient than Premenopausal it is possible that due to life style changes such as working indoors, occlusive clothing, and increase use of sunscreen creams, so premenopausal female expose less to the sun less often than Postmenopausal female, resulting in reduced synthesis of vitamin D.

5.2 Conclusion:

The finding of this study concluded that, Plasma vitamin D and calcium levels are decreased in postmenopausal women.

5.3 Recommendation:

1. Vitamin D and calcium level should be regularly monitored in the blood of postmenopausal women.
2. Health education for the community to understand that the menopause is not a medical condition, but a life transition or a symbol of aging.
3. More extensive investigation should be done to monitor the bone diseases such as Osteoporosis in postmenopausal women such as Bone density test.

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Questionnaire

Measurement of Vitamin D and Calcium in females with (Pre – Post) Menopausal:

Name:…… Mobile No: ……

Serial No: ……

Date: ……

Age: ……Years

Medical Information: ……

Menopause Cycle: **Pre** ◯ **Post** ◯

Menstruation: **Regular** ◯ **Irregular** ◯

Fractures: **Yes** ◯ **No** ◯

Occupation **Yes** ◯ **No** ◯

Medication: **Yes** ◯ **No** ◯

If yes identify……

Exposure to sunlight:……Hours/day

Residence:……

Weight:…… Kgs

Height: …… Cm

Laboratory Investigations:

Vitamin D: ……. nmol/l

Calcium: ……. mmol/l

BMI:……