1. Introduction and Literature review

1.1 Introduction:

Breast cancer is the most commonly diagnosed cancer among women, it is one of the leading causes of death worldwide (Lu, Jing et al. 2016). Recently a considerable research effort is directed towards identifying preventable breast cancer risk factors (Imtiaz and Siddiqui 2014). Vitamin D (VD) appears to have a role in many cancers, including breast cancer (Holick 2008). 1α,25-Dihydroxyvitamin D3 (VD3) is the biologically active form of VD3, its known to regulate calcium and bone homeostasis. It has also shown diverse biological effects relevant to immune system and carcinogenesis (Imtiaz and Siddiqui 2014). VD has emerged as a potential treatment for cancer, it modulates proliferation, apoptosis, invasion and metastasis by binding to a specific high-affinity receptor, the VD Receptor (VDR) (Deeb, Trump et al. 2007).

Experimental studies have found that the biologically active form of vitamin D (1,25(OH)2 D), can prevent breast cancer development and progression by inhibiting cell proliferation and angiogenesis (Krishnan, Swami et al. 2012, Lopes, Paredes et al. 2012, Mohr, Gorham et al. 2012). Other studies report that high serum VD levels at early breast cancer diagnosis correlate with lower tumor size, and improved breast cancer-specific outcomes, especially in postmenopausal patients (Vrieling, Hein et al. 2011, Hatse, Lambrechts et al. 2012). The breast cells have VDRs in their nuclei and it is postulated that polymorphism of genes for these VDRs may result in increased risk for breast cancer (Chen, Bertone-Johnson et al. 2005). Several factors affect the cutaneous VD synthesis; the principle determinant is the angle of the sun. The more directly overhead the position of the sun the more UV is available for VD synthesis. It has also been shown in various studies that obese people with a body mass index (BMI) >30 kg/m² generally have low serum levels of VD (Holick 2007). Thus, being obese (BMI ≥30) is a serious
health problem, since this condition is risk factor for many somatic disorders, with cardiovascular disease (Romero-Corral, Montori et al. 2006), type 2 diabetes mellitus (Walker, Zariwala et al. 2007) and cancer being the most important ones (Bjorge, Engeland et al. 2007, Ceschi, Gutzwiller et al. 2007, Giovannucci and Michaud 2007).

1.2 Breast cancer

Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the duct with milk (Sariego 2010). It results from unregulated cell growth and the new growth of undifferentiated tissue (Anderson, Cotterchio et al. 2011). Breast tissue consists of mammary glands and ducts, made up of epithelial tissue, in addition to adipose, connective tissue and vessels of the lymphatic and blood system. Development begins during puberty, is generally quite advanced by menarche and last differentiation occurs during pregnancy and lactation (Colditz 2005).

1.2.1 Epidemiology of breast cancer

In developing countries, breast cancer is the first leading cause of death among women accounting for about 14.3% of all deaths (Balekouzou, Yin et al. 2016). According to WHO reports in 2012, the breast cancer-related mortality was the leading cause of death (24.5%) among Central African women (Balekouzou, Yin et al. 2016). Breast cancer is most common cancer in Sudanese women living in Khartoum state during the period 2009-2010, with an incidence rate of 25.1 per 100,000 followed by leukemia, lymphoma, prostate cancer, colorectal, oral cancer, cancer of esophagus, liver cancer, stomach cancer, and cancer of cervix (Saeed, Weng et al. 2014). The incidence rate of breast cancer was substantially higher than other types of cancer in adults aged 25–64 years (Saeed, Weng et al. 2014). Sudan International Conference on Breast Cancer 2011 reported that, breast cancer constitutes 29-34.5% of all the cancers seen at
Radio and Isotope Center in Khartoum (RICK). Most cases presented with late advanced disease, only 5-7% presented with stage 1 and 13-15% presented with stage II diseases (Mohammed, S 2012). Regarding the hormonal status, the majority of breast cancer seen in women in the Sudan is estrogen receptors negative (68%), progesterone receptors negative (70%). Her-2 receptors examination is not done routinely at RICK. However, results obtained from women traveled aboard or who afford the cost of private laboratory showed that 25-30% of women are positive for Her-2 (Mohammed, S 2012).

1.2.2 Risk factors of breast cancer

a) Genetic factors

Studies of breast cancer risk among twins propose some breast cancer is attributable to hereditary factors (Anderson, Cotterchio et al. 2011). Most inherited cases of breast cancer are associated with two abnormal genes, the BRCA1 and BRCA2 (Kaminska, Ciszewski et al. 2015). Mutations in BRCA1 and BRCA2 genes lead to high-risk genotypes associated with up to 80% lifetime risk of developing breast cancer (Anderson, Cotterchio et al. 2011). A meta-analysis study by Antoniou et al announced that BRCA1 mutation carriers have a 65% lifetime risk of breast cancer, while BRCA2 mutation carriers have a 45% lifetime risk of breast cancer (Antoniou, Pharoah et al. 2003); however, there is much variety in penetrance which is likely due to non hereditary factor (Anderson, Cotterchio et al. 2011).

b) Reproductive factors

It is demonstrated that nulliparity, older age at first full-term pregnancy, younger age at menarche and older age at menopause are all associated with increased breast cancer risk (Anderson, Cotterchio et al. 2011). Nulliparous women have a 20–70% increased risk of breast cancer compared to parous women and increase risk is seen among women greater than 30 years
of age at first full-term pregnancy. Among parous women, breast cancer risk diminishes 7% on average for each additional birth (Anderson, Cotterchio et al. 2011). Women who begin menstruating early in life or who have a late menopause have an increased risk of developing breast cancer (McPherson, Steel et al. 2000).

c) Risk factors by hormone receptor status

Studies are proposing that risk factors may vary by breast cancer subtype. Triple negative breast cancers (ER-, PR-, and HER2-) are related with a poorer prognosis than hormone receptor positive or luminal type cancers (Anderson, Cotterchio et al. 2011).

Other possible factors observed to be associated with increased risk of breast cancer are Diet, obesity, alcohol intake, smoking (McPherson, Steel et al. 2000) and as of late vitamin D deficiency (Imtiaz and Siddiqui 2014).

1.3 Vitamin D

Vitamin D is fat soluble vitamin, refers to a group of related metabolites used for appropriate skeleton formation and minerals homeostasis (Bishop et al. 2005). Actually vitamin D is a hormone. Vitamin D share similarities in source with steroid hormones, that is, vitamin D is a metabolic result of the cholesterol synthetic pathway. The tissues that are included in the synthesis of vitamin D are the skin, liver, and kidneys (Bishop et al. 2010).

1.3.1 Metabolism of vitamin D

Synthesis of vitamin D takes place in the skin from 7-dehydrocholesterol following exposure to sunlight. During exposure to sunlight, UV radiation (290–315 nm) is absorbed by 7-dehydrocholesterol to form vitamin D3 (Holick, Tian et al. 1995). The VD-binding protein in the dermal capillary bed has an affinity for vitamin D3 and draws it into the circulation (Holick, MacLaughlin et al. 1981, Haddad, Matsuoka et al. 1993, Holick, Tian et al. 1995). The
production of vitamin D3 in skin is a function of the amount of UV radiation achieving the
dermis and additionally the availability of 7-dehydrocholesterol (Ross, Taylor et al. 2011). As
such, the level of synthesis is affected by several factors, including season of the year, skin
pigmentation, latitude, use of sunscreen, clothing, and amount of skin exposed. Age is also a
factor, in that synthesis of vitamin D decrease with increasing age, due to decline in 7-
dehydrocholesterol levels and due to alterations in skin morphology (MacLaughlin and Holick
1985).
Small proportion of VD is obtained from dietary sources. Dietary VD2 (ergocalciferol) and D3
(cholecalciferol) are absorbed through a bile acid dependent process whereby VD is incorporated
into micelles in the intestinal lumen, then absorbed by enterocytes and packaged into
chylomicrons then transported to the venous circulation via lymphatic drainage (Kitson and
Roberts 2012). The efficiency of absorption of VD is dependent upon the presence of fat in the
It has been noted that only about 50% of a dose of VD is absorbed. However, considering that
adequate amounts of VD can be produced daily by exposure to sunlight (Collins 2001).
The VD binding protein (DBP) is the major transporter protein of VD and its metabolites, since
VD metabolites are lipophilic molecules with low water solubility that must be transported in the
circulation bound to plasma proteins (Gallieni, Cozzolino et al. 2009). In fact that over 99% of
circulating vitamin D compounds are protein bound and has major physiological implications.
DBP-bound VD metabolites have limited access to target cells and, therefore, are less susceptible
to hepatic metabolism and subsequent biliary excretion, leading to a longer circulating half-life
(Andreassen 2006).
Vitamin D is taken up rapidly by the liver. Since it has been known that the liver serves as a storage site for retinol, fat-soluble vitamin, it was believed that the liver also functioned as a storage site for vitamin D (Compston, Horton et al. 1979). Studies carried out on the distribution and storage of vitamin D and its metabolites found that human muscle (Girgis, Mokbel et al. 2014) and adipose are major storage sites for vitamin D (Rosenstreich, Rich et al. 1971, Mawer, Backhouse et al. 1972).

The active metabolite of vitamin D is 1α,25-dihydroxyvitamin D (1,25(OH)2D). Conversion of VD to 1α,25(OH)2D occurs in two consecutive hydroxylation steps (DeLuca 1974). The first is in the liver where vitamin D is hydroxylated to 25-hydroxyvitamin D [25(OH)D] which is the major circulating metabolite of vitamin D. The second hydroxylation step is in the kidney and different tissues where 25(OH) D is converted to 1α,25(OH)2D.

In the liver, hydroxylation of vitamin D to 25(OH)D is mediated by a cytochrome P450 (CYP) enzyme, distinguished as CYP2R1 (Henry 2011). CYP2R1 appears to hydroxylate vitamin D2 and D3 with equal efficiency (Strushkevich, Usanov et al. 2008). Other 25-hydroxylase enzymes have been recognized in the liver; a mitochondrial hydroxylase, CYP27A, and a microsomal enzyme, CYP2D25 (Anderson, May et al. 2003). In addition to the liver, a report exhibits that the expression of CYP27A is widespread in different tissues including kidney, intestine, bone, skin, lung and spleen (Theodoropoulos, Demers et al. 2003).

25(OH) VD is the major circulating metabolite of VD due to its stability when bound to vitamin D-binding protein in blood and greater water solubility compared to the active form of VD (Hollis, Pittard et al. 1986, Cooke and Haddad 1989, Cooke, McLeod et al. 1991). When VD metabolites are bound to VD binding protein they are protected from photo-degradation and thus are stable in blood for significant periods even at room temperature (Anderson, May et al. 2003).
Thus the estimation of serum 25OH VD is the accepted indicator of vitamin D status. Following hydroxylation in the liver, 25(OH) VD is secreted into the circulation where it binds to DBP and is transported to the kidney and some tissues for activation or breakdown (Nykjaer, Fyfe et al. 2001).

25(OH)D is further hydroxylated to 1,25(OH)2D (biologically active form) in the kidney (Norman 2008). This conversion catalyzed by CYP2B1, a mitochondrial P450 enzyme with 1α-hydroxylase activity which is produced in the proximal renal tubule (Prentice, Ceesay et al. 2008, Bikle 2009). 25(OH)D is also hydroxylated to 24,25 dihydroxyvitamin D (24,25(OH)2D). Production of 24,25(OH)2D is catalyzed by the hydroxylase enzyme, CYP24 and usually it is the first step in the metabolic pathway to inactivate 25(OH)D which prevents vitamin D intoxication (Norman 2008). Serum concentration of 24,25(OH)2D is directly related to 25(OH)D concentration (Prentice, Ceesay et al. 2008, Bikle 2009). CYP27B1 activity depends on the absolute intracellular concentration of 25(OH)D. There is little correlation between serum concentrations of 25(OH)D and 1,25(OH)2D (Need, Horowitz et al. 2000, Lips 2001, Vieth, Ladak et al. 2003).

Renal production, which is the principle source of 1α,25(OH)2D in the serum, mediates the functions of the vitamin D endocrine system. However, a number of other tissues also have the ability to produce 1α,25(OH)2D (van Driel, Koedam et al. 2006, Kogawa, Anderson et al. 2010, Zhou, LeBoff et al. 2010). Extra-renal 1α,25(OH)2D production does not generally increase 1,25(OH)2D concentrations in the circulation and its effects appear to be restricted to paracrine and autocrine functions within these tissues (Norman 2008).

VD compounds are catabolized primarily by oxidation of the side chain (Okuda, Usui et al. 1995). The 24-hydroxylation of 1,25-(OH)2D3 is the first catabolic step in the elimination of
active hormone leading to the formation of 1,24,25-trihydroxyvitamin D3 (DeLuca 2008), which is 10 times less potent than 1,25-(OH)2D3 (Okuda, Usui et al. 1995). Degradation of both is catalyzed by the 24-hydroxylase enzyme CYP24 (produced in the kidney) in a series of four successive reactions to produce inactive water-soluble compounds which are excreted in bile (Henry 2011). Further oxidative reactions of 1,24,25-trihydroxyvitamin D3 lead to progressive loss of biological activity and finally to the production of water-soluble calcitroic acid, which is excreted in urine (Makin, Lohnes et al. 1989). The products of vitamin D metabolism are excreted through the bile into the feces, and very little is eliminated through the urine (Ross, Taylor et al. 2011).

1.3.2. Regulation of 1α,25(OH)2D production

Regulation of 1α,25(OH)2D in the kidney is tightly managed. Up-regulation is through the action of parathyroid hormone (PTH); down regulation is through fibroblast growth factor 23 (FGF23) and direct negative feedback by 1α,25(OH)2D itself (Henry 2011). PTH secretion is stimulated by a decrease in serum ionized calcium concentration. PTH stimulates production of the CYP27B1 enzyme in the proximal cells of the kidney which increases renal synthesis of 1α,25(OH)2D (Bajwa, Forster et al. 2008). 1α,25(OH)2D exerts a direct negative feedback by down regulating the expression of the gene for CYP27B1 (Henry 2011). It also exerts an indirect negative feedback by reducing secretion of PTH (Norman 2008, Holick 2011). In addition, 1α,25(OH)2D induces its own degradation by stimulating production of the CYP24A1 enzyme, a 24-hydroxylase which converts 1α,25(OH)2D and 25(OH)D to water-soluble compounds which are excreted through bile (Jones, Strugnell et al. 1998). FGF23 mediates the regulatory effect of serum phosphate concentrations on lowering 1,25(OH)2D concentrations (Shimada, Urakawa et al. 2004). FGF23 is secreted by bone osteoblasts and osteocytes in response to increase the serum
phosphate concentrations (Henry 2011) and down regulates 1α,25(OH)2D synthesis by inhibiting renal transcription of CYP27B1 (Perwad, Zhang et al. 2007). Extra-renal CYP27B1 enzyme activity is not regulated by calcium and phosphate regulating hormones but rather might be influenced by changes particular to the cell’s environment or function (Henry 2011).

1.3.3 Vitamin D receptor

Vitamin D receptor (VDR) is a member of the nuclear receptors superfamily, and it is the only protein that binds with high affinity to 1α,25-dihydroxivitamin D (calcitriol) (Carlberg and Dunlop 2006, Carlberg and Seuter 2009). The nuclear receptors represent a group of important transcription factors, where the 48 members of this superfamily belong to an identified group of mammalian genes involved in transcriptional regulation (Carlberg and Seuter 2009).

VDR have two zinc fingers which forms a DNA-binding domain (DBD) of 66 amino acids. Furthermore, the C-terminal of the protein contains a ligand-binding domain (LBD) of approximately 300 amino acids formed by 12 α-helices (Carlberg and Dunlop 2006). Therefore, VDR is a transcription factor which creates an active signal transduction complex that forms a heterodimer of calcitriol-liganded VDR and unoccupied retinoid X receptor (RXR).

In mammals, VDR expression is higher in metabolic tissues such as intestine, kidneys, skin and thyroid gland, being moderately expressed in almost all tissues. Furthermore, VDR is expressed in most of malignant tissues of colon, breast and prostate (Bouillon, Eelen et al. 2006, Carlberg and Seuter 2009, Haussler, Jurutka et al. 2011).

Therefore, vitamin D/VDR signaling is involved in mineral and bone homeostasis, modulation of growth, cardiovascular events, cancer prevention and immune responses regulation, including autophagy. A VDR polymorphisms or a vitamin D deficiency in the human body can cause poor
bone development and health, as well as increase the risk of developing many chronic diseases, including cancer (Wu and Sun 2011).

1.3.4 Biological action of vitamin D

1α,25(OH)2D evokes a biological response through genomic and non-genomic response that mediated by binding with VDR (DeLuca 2004, Norman, Mizwicki et al. 2004). Classically the main role of 1,25(OH)2D3 is the homeostasis of calcium and phosphorous concentrations in blood via actions in bone, parathyroid gland, kidney and intestine. In addition, 1α,25(OH)2D3 has several other biological responses (non-classical actions of vitamin D) that are not related to the control of mineral homeostasis. In addition to the genomic actions, 1α,25(OH)2D3 is also able to generate rapid biological responses, which do not require any protein synthesis as genomic actions do (Baran 1994, Norman, Song et al. 1999). It has been suspected that putative cell membrane receptor for 1α,25(OH)2D3 (VDRmem) mediates those responses by activating a variety signal transduction systems, such as several protein kinases and phospholipase C pathways (Nemere, Schwartz et al. 1998, Sitrin, Bissonnette et al. 1999). There is also evidence that rapid responses are able to modulate the genomic pathway of 1α,25(OH)2D3 actions via phosphorylation of nuclear VDR (Norman, Ishizuka et al. 2001). Receptor phosphorylation could increase the affinity of VDR to coactivator complexes and thus enhance gene activation (Barletta, Freedman et al. 2002).

A previous study showed that calcitriol regulates as much as 5 percent of the human genome (Zella, Shevde et al. 2008). It is evident that the VDR is available in the nucleus of many tissues that are not included in calcium and phosphate homeostasis. As the VDR is expressed in epidermal keratinocytes, B and T lymphocytes, antigen-presenting cells, macrophages and monocytes, cytotoxic T cells, adipose tissue, pancreatic beta-cell, bone marrow, and cancer cells
(Zella, Shevde et al. 2008, Gallieni, Cozzolino et al. 2009). In these locations, receptors can be stimulated by circulating calcitriol, that mediates Cell differentiation, replication and apoptosis (Gallieni, Cozzolino et al. 2009). Therefore, noncalcemic activities of the vitamin D system may include the immune system, insulin secretion by the pancreatic beta-cell, heart function and blood pressure regulation, brain and fetal development (Gallieni, Cozzolino et al. 2009).

1.3.5 Molecular effects of VD/VDR

Activation or repression of gene transcription is mediated by binding of 1α,25(OH)2D3 activated VDR/RXR complex to particular DNA sequences (vitamin D response elements) (Christakos, Dhawan et al. 2016). Gene expression regulation via VD signaling is dependent upon the ability of VDR/RXR heterodimers to recruit coregulatory protein complexes that alter chromatin structure, by forming a complex with histone acetyl transferase (HAT) activity or ATP-dependent remodeling activity (Fleet, DeSmet et al. 2012). Regarding target genes expression that depends on VDR actions, vitamin D produces anti-proliferative effects, increased apoptosis and increased differentiation of cells in a number of ways (Field and Newton-Bishop 2011). According to this function of VD there is an association between VD/VDR biological effects and cancer, so the study of their molecular effects is very important (Welsh 2012).

1.3.6 Deficiency of vitamin D

Many factors contribute in VD deficiency; the most important one is the sunlight, as the major source of vitamin D is adequate exposure to sun light. So anything that interfere with transmission of sun light to the earth or skin’s surface will affect the cutaneous synthesis of vitamin D (Holick and Chen 2008). Dark skinned people who have much melanin amounts which is a natural sun blocker, require longer exposures to sunlight to produce the same amount
of vitamin D3 produced by the light skinned people. Furthermore the cutaneous production of vitamin D3 is dramatically affected by the time of day, season, and latitude (Holick 2004). Aging also affects production of vitamin D3 as the amount of 7 dehydrocholesterol starts to decline later in life. It has also been found that VD levels are influenced by body weight, the large body fats sequester VD, that is why obesity is associated with vitamin D deficiency (Holick 2004). The liver is the primary site for the production of 25(OH)D and the synthesis of plasma DBP, it also facilitates the intestinal absorption of vitamin D by producing the bile salts. Hence, liver diseases can interfere with the absorption, transport, and metabolism of vitamin D (Avioli, Lee et al. 1967). The kidney plays a very important role, it functions as the endocrine gland for 1α,25(OH)2D3. Kidney diseases reduce the 1-hydroxylase activities and results in substantial loss of 25-hydroxyvitamin D in urine (Holick 2007). VD levels are also influenced by the PTH, since the PTH stimulates the production of 1α,25(OH)2D3 in the kidney, any disease affecting the PTH secretion will have an effect in VD metabolism (Haussler, Baylink et al. 1976).

Genetic mutations resulting in impaired expression of the renal 25-(OH)-D3-1-hydroxylase is a condition referred to as vitamin D-dependent rickets type I, which can be managed using low doses of 1α,25-(OH)2-D3. Expression of a nonfunctional VDR and impairing the transcription of vitamin D-regulated genes involved in Calcium and phosphorus homeostasis is a condition referred to as vitamin D-dependent rickets type II, the management of which requires relatively high doses of 1α,25-(OH)2-D3. Other causes of hypovitaminosis are insufficient intake of VD from the diet and the exposure to certain drugs (Holick 2007).
1.3.7 Hypervitaminosis D

High concentrations of vitamin D are not available from natural sources. However, hypervitaminosis D can be a concern in patients being 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 major issue since it can result in irreversible calcification of the heart, lungs, kidneys, and other soft tissues (Jacobus, Holick et al. 1992, Pettifor, Bikle et al. 1995). Hypervitaminosis D is thought to occur as a result of high 25(OH)D levels rather than high 1α,25(OH)2D levels (Counts, Baylink et al. 1975).

1.3.8 VDR polymorphisms

The different biological effects of vitamin D in the human are mediated by its receptor, VDR. The VDR gene, located on chromosome 12q12-q14, includes eight protein coding exons (exons 2-9) and one untranslated exon (exons 1a-1f) (Lemos, Fagulha et al. 2008, Raimondi, Johansson et al. 2009). By screening with various restriction enzymes, just some limited areas of VDR gene could be analysed to verify DNA sequence variations (Kostner, Denzer et al. 2009). The genetic polymorphisms of this receptor may influence their function (Gyorffy, Vasarhelyi et al. 2002, Buttigliero, Monagheddu et al. 2011). The most commonly investigated VDR single nucleotide polymorphisms (SNPs) are FokI (rs10735810), located in exon 2 at the 5’ end of VDR gene, BsmI (rs1544410) and ApaI (rs7975232), located in intron 8 at the 3’ end of the gene and TaqI (rs731236), located in exon 9 of VDR at the 3’ end of the gene (Lemos, Fagulha et al. 2008, Kostner, Denzer et al. 2009, Buttigliero, Monagheddu et al. 2011).
1.3.9 Vitamin D/VDR and breast cancer

The association of vitamin D and breast cancer has been proven by several studies. A previous study have shown that 1,25(OH)2D3 and its analogs slow down cancer cell growth by arresting cells in the G0/G1 phase of the cell cycle, by inducing their differentiation or by the induction of apoptotic cell death (Christakos, Dhawan et al. 2016). Furthermore, 1α,25(OH)2D3 influences angiogenesis, alters cell adhesion and migration, and reduces the invasiveness of cancer cells (Christakos, Dhawan et al. 2016). Epidemiologically, two major studies have been conducted to evaluate vitamin D status: first, the study that focused on the association between sun exposure and breast cancer risk; second, conducted to evaluate vitamin D intake and breast cancer risk (van der Rhee, Coebergh et al. 2009, Anderson, Cotterchio et al. 2010). One Study noted that inadequate sunlight exposure and decreased vitamin D production by the skin, was associated with higher breast cancer incidence and mortality (van der Rhee, Coebergh et al. 2009). Another study reported that in inadequate sun exposure, VD deficiency was common in a young adult healthy Lebanese population, whether rural or urban during winter season (Gannage-Yared, Chemali et al. 2000). Conversely a study in North India noted that, significant differences in the vitamin D levels of rural and urban individuals, the rural has higher VD level duo to adequate exposure to sun light (Goswami, Kochupillai et al. 2008). Some studies done in female wearing style as a main blocker to sun exposure and therefore 25(OH)D synthesis and status; found that, VD deficiency was more common in veiled women compared with non veiled women (Gannage-Yared, Chemali et al. 2000). Also a comparative study done by (Al-Horani, Abu Dayyih et al. 2016) reported that, a significant differences was found between Jordanian western styles wearing female students and covered Jordanian female students, the western styles wearing females has higher VD level.
A meta-analysis of 6 studies concluded there was no overall association between vitamin D from diet and supplements and breast cancer risk, but a significant association was observed when intakes 400 IU/day of VD were compared to <150 IU/day (Gissel, Rejnmark et al. 2008). In contrast results from two European studies found strong significant reductions in breast cancer risk at relatively low intakes of vitamin D from food alone. Vitamin D intake from food alone >400 IU/day was significantly associated with a 50% reduced breast cancer risk among a German population (Abbas, Linseisen et al. 2007) and intake >190 IU/day was associated with a 64% reduced risk among women living in Southern Italy (Rossi, McLaughlin et al. 2009).

Some epidemiological data support an inverse association between vitamin D status (including sunlight exposure, dietary and supplement intake, and direct measurement of circulating vitamin D levels) and breast cancer risk (Lipkin and Newmark 1999, Cui and Rohan 2006, Bertone-Johnson 2007, Rohan 2007, Colston 2008, Bertone-Johnson 2009, Perez-Lopez, Chedraui et al. 2009), which has resulted in increased interest in the use of vitamin D for breast cancer prevention.

Vitamin D is fat soluble and is readily stored in adipose tissue, it could be sequestered in the fat so, lower levels will be released to the circulation (Lagunova, Porojnicu et al. 2009). Several studies demonstrated that inverse correlation between VD levels and BMI, a low VD level was found in obese health individuals and patients (Wortsman, Matsuoka et al. 2000, Parikh, Edelman et al. 2004, Konradsen, Ag et al. 2008, Garanty-Bogacka, Syrenicz et al. 2011, Minambres, Sanchez-Hernandez et al. 2012, Tamer, Mesci et al. 2012, Boonchaya-anant, Holick et al. 2014, Imtiaz and Siddiqui 2014).

Variants in genes on the vitamin D pathway may result in functional changes that may affect endogenous vitamin D levels resulting in modification of breast cancer risk. A comprehensive
review of the literature identified three genes in the vitamin D pathway that have been studied in relation to breast cancer risk: vitamin D receptor (VDR), vitamin D binding protein (Gc), and CYP24A1 (involved in degradation of 1,25-dihydroxyvitamin D) (McCullough, Bostick et al. 2009). There is some support suggesting that VDR polymorphisms may be directly associated with breast cancer risk or may modify the association between vitamin D exposure and breast cancer risk (as reviewed in Slattery 2007, McCullough, Bostick et al. 2009).

Studies conducted in the two most commonly VDR polymorphisms (Fok1 (rs2228570) and Bsm1 (rs1544410) found that Fok1 was associated with increased breast cancer risk, while no association was observed with Bsm1 polymorphism (Chen, Bertone-Johnson et al. 2005, Sinotte, Rousseau et al. 2008, McKay, McCullough et al. 2009). Four studies reported increased risk of breast cancer with Bsm1 bb genotype (Ruggiero, Pacini et al. 1998, Guy, Lowe et al. 2004, Lowe, Guy et al. 2005, Trabert, Malone et al. 2007), while other studies did not report a similar association (Ingles, Garcia et al. 2000, Hou, Tien et al. 2002, Buyru, Tezol et al. 2003).

A meta-analysis of 39 studies done in VDR-Fok1 polymorphism demonstrated that, individuals that were homozygous for minor allele genotype were more likely to develop breast cancer (Zhang and Song 2014). One recent study reported a protective effect of the Fok1 polymorphism in breast cancer. For VDR-Taq1 polymorphism Reimers et al. noted, Taq1 polymorphisms was significantly associated with reduced risk of breast cancer (Reimers, Crew et al. 2015). Conversely other studies reported no significant association between the Taq1 polymorphism and risk of breast cancer (Hou, Tien et al. 2002, Newcomb, Kim et al. 2002, Buyru, Tezol et al. 2003, McCullough, Stevens et al. 2007, Wang, He et al. 2013). A study done to evaluate the three VDR-polymorphisms (Bsm1, Taq1 and Apa1) among breast cancer females found that, no association between VDR-SNPs (Bsm1 and Taq1) and breast cancer risk, while VDR-Apa1 was
associated with increased risk of breast cancer (Guo, Jiang et al. 2015). Other studies reported, the four polymorphisms of VDR (Fok1, Bsm1, Apa1, and Taq1) were not associated with breast cancer risk (McCullough, Stevens et al. 2007, Yang, Liu et al. 2014). In VDR polymorphisms genotypes and vitamin D levels, some studies observe that VD levels in plasma are not directly correlated with genotypic frequencies difference (Guo, Jiang et al. 2015). A study conducted in Indian population reports, a significant correlation was seen between serum vitamin D levels and Taq1 SNP, whereas no correlation was observed with Fok1 SNP (Bhanushali, Lajpal et al. 2009). Another study noted that no differences in serum 25(OH) VD concentrations by Fok1 and Taq1 VDR genotypes (Abbas, Nieters et al. 2008). A study conducted in females with breast cancer reported that, low levels of VD alone and in combination with the VDR-Bsm1 homozygous genotype for minor allele have been associated with risk breast cancer (Lowe, Guy et al. 2005).
1.4 Rationale:

Breast cancer is one of the major causes of death by cancer in women worldwide and the most common malignancy in Sudan. Vitamin D is considered to be a modifiable factor that may reduce breast cancer risk via binding to specific vitamin D receptor (VDR). Some studies have demonstrated a low level expression of VDR in breast cancer cells compared to normal breast cells. There is evidence that variants of VDR genes are associated with risk of breast cancer. Those variants may alter the ability of the active form of VD to induce biological effects on target cells. Most studies in Sudan measured only vitamin D levels, so the main focus of this study was to evaluate VDR gene polymorphisms beside vitamin D level.
1.5 Objectives

General objective

To assess the serum vitamin D levels and vitamin D receptor gene polymorphisms in Sudanese females with breast cancer attending the Radio and Isotope Center in Khartoum.

Specific objectives

1- To measure serum vitamin D levels (25 (OH)2D3) in study groups.

2- To detect genotypes and alleles frequencies of different VDR-polymorphisms (BsmI, FokI, TaqI, ApaI) in study groups.

3- To correlate serum vitamin D levels with different genotypes.

4- To confirm some of RFLP–genotyping results using sequencing techniques

5- To correlate the vitamin D levels with age, BMI, parity, educational levels and occupation status in study group.
2. Materials and methods

2.1 Study Design
This study is a hospital base-case control study.

2.2 Study area and Period
The study was conducted in Radio and Isotope Center in Khartoum (RICK), Khartoum state - Sudan. The study was carried out over 3 years (2013 - 2016).

2.3 Study Population
The study was conducted on Sudanese females diagnosed with breast cancer as case group and apparently healthy Sudanese females as controls. Control group was matched with respect to age and sex.

2.4 Sample Size
The study consisted of 190 Sudanese females diagnosed with breast cancer as case group and 107 apparently healthy Sudanese females as control group.

2.5 Inclusion Criteria
Sudanese females who were diagnosed with breast cancer were enrolled in this study after their approval.

2.6 Exclusion Criteria
Females with breast cancer were excluded from this study if they had sever illness, renal disease, malabsorption and hyperparathyroidism, any other cancer , use of calcium lowering therapy and taking vitamin D supplements.

2.7 Ethical consideration
The study was approved by the ethics committee of the Faculty of laboratory Sciences of the Sudan University of Science and Technology.
All participants gave informed oral consent after explaining the aims of the study to them.

2.8 Data collection and Clinical Examination

Clinical data was collected by a questionnaire and a patient form and authorized clinician carried out the clinical examination in RICK.

2.9 Blood sample Collection

After consent a sample of venous blood (5mL) was collected into two containers, plain container to obtain serum after centrifugation and into EDTA container (whole blood) for DNA extraction.

2.10 Measurement of vitamin D (25(OH)-vitamin D):

**Principle:**

The assay was performed by a competitive Enzyme-Immuno-Assay (EIA) technique (Euroimmun microplate ELISA) with a selected monoclonal antibody recognizing 25(OH)-vitamin D. Calibraters, controls and samples were incubated after the extraction step with the detection antibody. The pre-incubated solution was transferred to the microplate coated with 25(OH)-vitamin D. During this incubation step, 25(OH)-vitamin D in the sample bound to the microtiter well competes for the binding of the detection antibodies. Then a peroxidase-conjugated antibody was added into each microplate well and a complex of 25(OH) VD detection antibodies – peroxidase conjugate was formed. Tetramethylbenzidine (TMB) was used as a peroxidase substrate. Finally, an acidic stop solution was added to terminate the reaction, whereby the color changed from blue to yellow. The intensity of the yellow color is inversely proportional to the concentration of 25(OH)-vitamin D. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration was generated using the values obtained from the standards. 25(OH)-vitamin D in the samples was determined from this curve (Appendix 2).
**Procedure:**

1. 200 µl of diluted calibrators, controls and serum were pipetted in microplate wells, and then they were incubated for 2 hours at room temperature.

2. The content of the wells were discarded and manually washed 3 times using 300 µl of working strength wash buffer for each wash.

3. 100 µl of enzyme conjugate was pipetted into each of the microplate wells and was incubated for 30 minutes at room temperature.

4. Manually discarded and washed 3 times as the above step.

5. 100 µl of substrate solution was pipetted into each of the microplate wells and was incubated for 15 minutes at room temperature.

6. 100 µl of stop reaction was pipetted into each of the microplate wells.

7. The color intensity was measured at a wavelength of 450 nm and a reference wavelength of 620 nm within 30 minutes of adding the stop solution.

8. The absorbance’s were plotted vs corresponding standard concentration and the curve was used to reading the samples.

**2.11 Gene polymorphisms**

VDR gene has multiple gene polymorphisms, and four important single nucleotide polymorphisms (SNPs) in exon 2 and 3'UTR region. These were VDR-FokI, VDR-BsmI, VDR-TaqI and VDR-ApaI.

**2.11.1 DNA extraction method**

DNA was extracted from whole blood using Guandine hydrochloride protocol.
Procedure:

1. 10 ml of red cell lysis buffer (RCLB) was added to each sample, and then samples were centrifuged for 5 min at 6000rpm.
2. The above step was repeated 2 times until a clear pellet of white blood cell appeared.
3. Supernatant was discarded then 2ml of white cell lysis buffer (WCLB), 1ml of Guanidine Hydrochloride (57.2 gram dissolved in 100 ml D.W), 300 µl of NH4 acetate ( 57.81’gram dissolved in 100 ml D.W), and 10 µl of protinase K were added .
4. The samples were incubated over night at 37 ºC.
5. After overnight incubation the samples were Cooled at room temperature, and then 2 ml of pre-chilled Chloroform was added, after that samples were centrifuged for 5 min at 6000rpm.
6. Upper layer was collected to a new falcon tube, 10 ml of cold absolute Ethanol was added to the collected samples, and then kept at -20 c for 2 hours.
7. After incubation at -20 c samples were centrifuged for 10 min at 6000 rpm. Then the supernatant was drained.
8. Pellet was washed with 4 ml of 70% ethanol. Then was centrifuged for 10 min at 6000 rpm.
9. Supernatant was poured off and pellet was allowed to dry.
10. Pellet was dissolved in 100µl of ddH2O, and then was incubated at 4ºC.

2.11.2 Genotyping of VDR

Vitamin D receptor genotyping for all four single-nucleotide polymorphic sites (SNPs) were done by the PCR-RFLP method (polymerase chain reaction- restriction fragment length polymorphism analysis). The primers used for the polymerase chain reaction (PCR) to amplify VDR gene fragments, were shown in (Table2. 1). PCR (Techne/ TC-312 peltier thermal cycle) conditions were as follows: denaturation at 94ºC for 5 min, followed by 40 cycles of PCR at
94°C (30 sec), 60°C–63.5°C (30 sec), and 72°C (30 sec). Annealing temperature for BsmI, ApaI, TaqI and FokI PCR were 63.5°C, 61°C, 61°C and 59.5°C, respectively. Following PCR, the amplified PCR products were digested with BsmI, ApaI, TaqI and FokI in accordance with the manufacturer's specifications. The presence (lowercase) or absence (uppercase) of the enzyme recognition site was identified by ethidium bromide staining of fragments separated in a 2% agarose gel.

Table 2.1: PCR-RFLP pattern of FokI, BsmI, TaqI and ApaI polymorphisms of vitamin D receptor gene

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Primers</th>
<th>PCR product bp</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fokl T/C</td>
<td>F 5’GATGCCAGCTGGCCTGGCACTG3’ R 5’ATGGAAACACCTTGCTTCTTCTCCCTC 3’</td>
<td>273</td>
<td>FF (CC) 273</td>
</tr>
<tr>
<td>Exon 2</td>
<td></td>
<td></td>
<td>Ff (CT) 273,196,79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ff (TT) 196, 79</td>
</tr>
<tr>
<td>BsmI G/A</td>
<td>F 5’CAACCAAGACTACAAGTACCGCGTCAGTGA-3’ R 5’-AACCAGCGGAAGAGGTCAGGG-3’</td>
<td>823</td>
<td>BB (AA) 823</td>
</tr>
<tr>
<td>Exon7/intron8</td>
<td></td>
<td></td>
<td>Bb (AG) 823, 648, 175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bb (GG) 648, 175</td>
</tr>
<tr>
<td>TaqI C/T</td>
<td>F 5’- AGAGCATGGGACAGGGAGCAAG- 3’</td>
<td>745</td>
<td>TT (TT) 291,249,205</td>
</tr>
<tr>
<td>Intron 8/exon 9</td>
<td></td>
<td></td>
<td>Tt (TC) 496,291,249,205</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tt (CC) 496,249</td>
</tr>
<tr>
<td>ApaI C/A</td>
<td>F 5’-GCAACTCCTCATGGCTAGGTCTCA- 3’</td>
<td>745</td>
<td>AA (AA) 745</td>
</tr>
<tr>
<td>Intron 8/exon 9</td>
<td></td>
<td></td>
<td>Aa (AC) 745, 531, 214</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aa (CC) 531, 214</td>
</tr>
</tbody>
</table>

2.12 Sequencing

Genotyping of some samples were confirmed by sequencing (Macrogen Company, Seoul, Korea). BioEdit software was used for multiple sequence alignment.

2.14 Statistical Analysis

SPSS software was used to analyze the data. The Chi-square test was used to determine the statistically differences in frequency distribution of VDR and the association of the different
variables with VD. 2-sided exact p-value ≤ 0.05 was considered statistically significant. An association between vitamin D genes polymorphisms, vitamin D levels among breast cancer patients and other risk factors was estimated using the Logistic Regression test at 95% confidence interval.
3. Results

A total of 297 Sudanese females were enrolled in this study, 190 females with breast cancer attending Radio and Isotope Center in Khartoum and 107 apparently healthy controls, with mean age (45.6 ±10.8 years and 45.7±9.1 years) respectively (Table 3.1). Table (3.1) also shows means of BMI and serum vitamin D levels in patients and controls. There was no significant differences between the two means of vitamin D levels p-value= 0.069. VD deficiency was found in 99.5% of the patients and 96.3% of the controls. There was inverse significant correlation between the BMI and serum vitamin D levels in patients (r= -0.509, p= 0.000) (Figure 3.1 & Table 3.2) and negative correlation but not significant among controls (Figure 3.2 & Table 3.2). Vitamin D levels revealed no significant differences with age group in patients and controls (Table 3.2). Parity showed no significant correlation with vitamin D levels in patients (r= 0.190, p= 0.060) (Figure 3.3). Educational levels of patients and controls were divided into four groups shown in (Figure 3.4). 42.2% of the patients and 34.45% of the controls were illiterate while 7.76% of patients and 21.3% of controls had high education. There was no significant difference between educational level and vitamin D levels in patients and controls (p= 0.123, p= 0.159) respectively (Figure 3.6). The majority of the patients and controls were housewives (88%, 56.9%) respectively, while the rest were employed (Figure 3.5). The difference between the two groups regarding vitamin D levels was nearly significant in patients and significant in controls (p=0.053, p=0.032) respectively (Figure 3.7).

The genotypes and alleles frequencies of VDR-polymorphisms (Fok1, Taq1, Bsm1 and Apa1) for study groups are summarized in (Table 3.3 and 3.4). No significant differences were found in genotypic and allelic frequencies in VDR-Fok1 in patients and controls (P =0.119, P= 0.345) respectively. No association was found with VDR-Fok1 and risk of breast cancer. VDR- Taq1
shows significant differences in genotypic and allelic frequencies between patients and controls (P = 0.01, P= 0.002) respectively. VDR-Taq1 found to have lower risk of breast cancer, OR at 95% CI 0.5 (0.3- 0.7).

A typical run of VDR-Fok1 on 1% and 2% agarose gel electrophoresis, a) VDR-Fok1 PCR product, b) Fok1/ Taq1 restriction enzyme digest, c) Multiple sequence alignment of VDR-Fok1 PCR product presented in (Figure 3.8). Similarly figure (3.9) shows a VDR- Taq1 run on 1% and 2% agarose gel electrophoresis, a) VDR-Taq1 PCR product, b) Taq1 restriction enzyme digest, c) Multiple sequence alignment of VDR-Taq1 PCR product.

There were significant differences in VDR-Bsm1 and VDR-Apa1 genotypes frequencies in patients and controls (P= 0.006, p=0.002) respectively, while there were no significant differences in allelic frequencies (P= 0.917, p= 0.196) respectively. No associations were found with VDR-Bsm1, Apa1 and risk of breast cancer, OR at 95% CI [1.0 (0.6 – 1.7), 0.7 (0.5 – 1.2)] respectively (Table 3.4).

Figure (3.10) shows typical run of VDR-Bsm1 on 1% and 2% agarose gel electrophoresis a) PCR product b) Bsm1 restriction enzyme digest. Figure (3.11) shows typical run of VDR-Apa1 on 1% and 2% agarose gel electrophoresis a) VDR-Apa1 PCR product, b) Apa1 restriction enzyme digest, c) Multiple sequence alignment of VDR-Apa1 PCR product.

The means of serum vitamin D levels in different genotypes showed no significant differences with VDR-polymorphisms (Fok1, Bsm1, Apa1) (P>0.05), while there was significant difference with VDR-Taq1 genotypes; when tt+Tt (dominant model) compared with TT, TT had higher VD level than dominant model (P=0.016) (Table 3.5).
Table 3.1 Comparison of the means ± SD of age, BMI, VD level and VD status in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Patients n= 190</th>
<th>Controls n= 107</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.6 ± 10.8</td>
<td>45.7 ± 9.1</td>
<td>0.925</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.4 ± 5.4</td>
<td>26.5 ± 5.3</td>
<td>0.349</td>
</tr>
<tr>
<td>Serum VD level (ng/mL)</td>
<td>13.6 ± 6.5</td>
<td>12.2 ± 6.8</td>
<td>0.069</td>
</tr>
<tr>
<td>Serum VD status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient (&lt;30 ng/mL)</td>
<td>189 (99.5)</td>
<td>103 (96.3)</td>
<td>0.058</td>
</tr>
<tr>
<td>Optimal (≥ 30 ng/mL)</td>
<td>1 (0.5)</td>
<td>4 (3.7)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Correlation of age and BMI of patients and controls with of vitamin D levels:

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Patients n= 187 n (%)</th>
<th>Mean±SD</th>
<th>p-value</th>
<th>Controls n= 107 n (%)</th>
<th>Mean±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;45</td>
<td>109 (57.7)</td>
<td>13.6 ± 6.7</td>
<td>0.742</td>
<td>57 (53.3)</td>
<td>11.3 ± 5.3</td>
<td>0.114</td>
</tr>
<tr>
<td>&gt;45</td>
<td>78 (42.3)</td>
<td>13.9 ± 6.4</td>
<td>0.002</td>
<td>50 (46.7)</td>
<td>13.3 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>n= 60</td>
<td></td>
<td></td>
<td>n= 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under weight</td>
<td>6 (9.7)</td>
<td>21.3±6.4</td>
<td></td>
<td>2 (4)</td>
<td>14.9 ± 10.4</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>20 (32.3)</td>
<td>17.9±6.9</td>
<td>0.002</td>
<td>20 (40)</td>
<td>13.5 ± 5.4</td>
<td>0.622</td>
</tr>
<tr>
<td>Over weight</td>
<td>23 (37.1)</td>
<td>13.5±6.1</td>
<td></td>
<td>15 (30)</td>
<td>13.1 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>11 (17.7)</td>
<td>10.3±6.2</td>
<td></td>
<td>13 (26)</td>
<td>11 ± 4.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Correlation between the serum vitamin D levels and BMI in patients.

Figure 3.2: Correlation between the serum vitamin D levels and BMI in controls.
Figure 3.3: Correlation between the serum vitamin D levels and parity in patients

$r = 0.190$
$p = 0.060$
Figure 3.4: Frequencies of education levels in A) patients and B) controls.
Figure 3.5: Frequencies of occupation status in A) patients and B) Controls
Figure 3.6: Means of vitamin D level across education levels in A) patients and B) controls
Figure 3.7: Means of vitamin D level across Occupation status in A) patients & B) controls
Table 3.3 Comparison of VDR-Fok1 and Taq1 polymorphisms genotypes and allele frequencies in patients and controls

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Genotypes/ Alleles</th>
<th>Patients n(%)</th>
<th>Controls n(%)</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fok1 rs2228570</td>
<td>FF</td>
<td>102 (74.5)</td>
<td>45 (65.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ff</td>
<td>32 (23.4)</td>
<td>24 (34.8)</td>
<td></td>
<td></td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>ff</td>
<td>3 (2.2)</td>
<td>00 (00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>236 (86.1)</td>
<td>114 (82.6)</td>
<td>1.3</td>
<td>(0.7 – 2.1)</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>38 (13.9)</td>
<td>24 (17.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n= 137</td>
<td>n= 69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq1 rs731236</td>
<td>TT</td>
<td>16 (13.2)</td>
<td>13 (21.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tt</td>
<td>25 (20.7)</td>
<td>22 (36.1)</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>tt</td>
<td>80 (66.1)</td>
<td>26 (42.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>57 (23.6)</td>
<td>48 (39.3)</td>
<td>0.5</td>
<td>(0.3 – 0.7)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>t</td>
<td>185 (76.4)</td>
<td>74 (60.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.8: Fok1 polymorphism. L: DNA ladder 100 bp. a) 1% gel electrophoresis images of PCR product: Lane 1 negative control, lanes 2 and 3 bands with size 273 bp. b) 2% gel electrophoresis images of PCR product after digestion with restriction enzymes. Lane 1 homozygous FF genotype (273 bp), lanes 2, 3 heterozygous Ff genotype (273, 196 and 79 bp). c) Multiple sequence alignment of VDR-Fok1 gene polymorphism (presence of C instead of T).
Figure 3.9: Taq1 polymorphism. L: DNA ladder 100 bp. a) 1% gel electrophoresis images of PCR product: lane 1 negative control, lanes 2, 3 and 4 bands with size 745 bp. b) 2% gel electrophoresis images of PCR product after digestion with restriction enzymes. Lanes 1, 5 homozygous tt wild type (496, 249 bp), lane 3 heterozygous Tt genotype (496, 291, 249 and 205 bp), lanes 6, 7 homozygous TT mutant (291, 249 and 205 bp). c) Multiple sequence alignment of VDR-Taq1 gene polymorphism (presence of T instead of C).
Table 3.4 Comparison of VDR-Bsm1, Apa1 polymorphisms genotypes and alleles frequencies in patients and controls

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Genotypes/Alleles</th>
<th>Patients n(%) n= 88</th>
<th>Controls n(%) n= 61</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bsm1 rs1544410</td>
<td>BB</td>
<td>38 (43.2)</td>
<td>19 (31.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bb</td>
<td>39 (44.3)</td>
<td>41 (67.2)</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>bb</td>
<td>11 (12.5)</td>
<td>1.0 (1.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>115 (65.3)</td>
<td>79 (64.8)</td>
<td>1.0</td>
<td>(0.6 – 1.7)</td>
<td>0.917</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>61 (34.7)</td>
<td>43 (35.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n= 122</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n= 63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apa1 rs7975232</td>
<td>AA</td>
<td>55 (45.1)</td>
<td>27 (45.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aa</td>
<td>48 (39.3)</td>
<td>36 (57.1)</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>aa</td>
<td>19 (15.6)</td>
<td>00 (00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>158 (64.8)</td>
<td>90 (71.4)</td>
<td>0.7</td>
<td>(0.5 – 1.2)</td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>86 (35.2)</td>
<td>36 (28.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3.10: Bsm1 polymorphism.** L: DNA ladder 100 bp. **a)** 1% gel electrophoresis images of PCR product: lane 1 negative control, lanes 2 and 3 and bands with size 823bp. **b)** 2% gel electrophoresis images of PCR product after digestion with restriction enzymes. Lanes 1, 2 shows heterozygous Bb genotype (823, 648, and 175 bp), lane 3 homozygous BB genotype (823), lane 4 homozygous bb genotype (648, 175 bp).
Figure 3.11: **Apa1 polymorphism.** L: DNA ladder 100 bp. a) 1% gel electrophoresis images of PCR product: lane 1 negative control, lanes 2, 3 and 4 bands with size 745bp. b) 2% gel electrophoresis images of PCR product after digestion with restriction enzymes, lanes 1-6 heterozygous Aa genotype (745, 531 and 214 bp), lane 7 homozygous AA (745 bp). C) Multiple sequence alignment of VDR-Apa1 gene polymorphism (presence of A instead of C).
Table 3.5 Comparison of serum Vitamin D levels in different VDR-polymorphisms genotypes (Bsm1, Fok1, Taql, Apa1) in patients

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>15.4 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>Bb</td>
<td>12.7 ± 6.7</td>
<td>0.236</td>
</tr>
<tr>
<td>bb</td>
<td>15.2 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>13.4 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>Ff</td>
<td>14.8 ± 7.6</td>
<td>0.338</td>
</tr>
<tr>
<td>ff</td>
<td>9.2 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>17.9 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Tt</td>
<td>13.5 ± 7.4</td>
<td>0.054</td>
</tr>
<tr>
<td>tt</td>
<td>13.0 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>TT vs tt+Tt</td>
<td>17.9 ± 8.5</td>
<td>13.1 ± 7.1</td>
</tr>
<tr>
<td>tt vs TT+Tt</td>
<td>13.0 ± 7.0</td>
<td>15.3 ± 8.1</td>
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<td>AA</td>
<td>14.2 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>Aa</td>
<td>12.8 ± 5.7</td>
<td>0.598</td>
</tr>
<tr>
<td>aa</td>
<td>14.2 ± 7.2</td>
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4. Discussion, Conclusions and Recommendations

4.1 Discussion:

This study has shown that Sudanese women are deficient in serum VD levels and there was no difference between patients and controls in this respect. VD deficiency was reported in 99.5% of the patients and 96.3% of the healthy controls. This fact is in agreement with a previous finding in the Sudan that, VD deficiency is common in healthy and diabetic Sudanese women (Mohammed and Amar 2014). It also agrees with a research done in a West African country that reported, VD deficiency was highly prevalent among TB patients and healthy controls, as 46% of the controls were VD deficient (Wejse, Olesen et al. 2007). A cross sectional study done among healthy female college students at Qatar University, subjects were from different countries including Sudan showed that, 97.2% of female students were VD deficient and the color of the skin significantly correlated with vitamin D levels (Sharif and Rizk 2011).

Though Sudan is a tropical country with high sun exposure, this finding can be attributed to the following VD-related factors (nutritional factors, time of exposure, darker skin, clothes, and sun-blockers) of our population. We anticipate that our subjects are likely suffering from inadequate exposure to early morning sunlight and probably to the factor of clothing.

Other studies reported that, VD deficiency was common in a young adult healthy Lebanese population, whether rural or urban during winter season and a reason was inadequate sun exposure (Gannage-Yared, Chemali et al. 2000). Conversely a study in North India noted that, significant differences in the vitamin D levels of rural and urban individuals, the rural has higher VD level duo to adequate exposure to sun light (Goswami, Kochupillai et al. 2008). In addition, VD deficiency was more common in veiled Kuwaiti women compared with non veiled women (Gannage-Yared, Chemali et al. 2000). A comparative study done by AL–Horani et al. reported
that, a significant differences was found between Jordanian western styles wearing female students and covered Jordanian female students, the western styles wearing females had higher VD levels (Al-Horani, Abu Dayyih et al. 2016).

In the present study there was a significant inverse correlation of serum VD levels with BMI among patients. There is a clear association of obesity with even low VD levels. Also an inverse correlation between serum vitamin D levels and BMI in controls was found. Obese female with BMI >30 kg/m² had lower VD level. However these findings were statistically not significant. It has been reported that body fat act as a reservoir for storage of fat soluble vitamins including VD, and that may reduce its bioavailability (Lagunova, Porojnicu et al. 2009), and it has been reported that, treatment with UV radiation or oral vitamin D does not raise circulating 25(OH)D levels in obese subjects to the same extent seen in normal-weight subjects (Wortsman, Matsuoka et al. 2000, Boonchaya-anant, Holick et al. 2014). Wortsman et al. reported that, 57% of obese subjects had lower VD levels than non obese subjects after radiation (Wortsman, Matsuoka et al. 2000). VD release from the fat is slow and is proportional to the concentration of the vitamin in the fat, hence lower levels will be released to the circulation (Lagunova, Porojnicu et al. 2009). This finding suggests that, obese and overweight patients are more predisposed to VD deficiency, thus predicting higher risk to breast cancer. This comes in line with Imtiaz and Siddiqui study that reported, VD deficiency was highly prevalent among breast cancer females and serum VD levels exhibited an inverse correlation with high BMI (Imtiaz and Siddiqui 2014). Several studies have reported an inverse correlation between serum VD levels and obesity among healthy population and patients (Parikh, Edelman et al. 2004, Konradsen, Ag et al. 2008, Garanty-Bogacka, Syrenicz et al. 2011, Tamer, Mesci et al. 2012). In a cross sectional study
conducted on obese patients, VD levels were associated with BMI > 40 kg/m² (Minambres, Sanchez-Hernandez et al. 2012).

In the present study, age revealed no significant correlation with serum VD levels in patients and controls. This finding comes in line with Ganmaa et al study that reported, no correlation was found between age and vitamin D levels (r= 0.03) (Ganmaa, Holick et al. 2014). In another study conducted by Al-Horani et al which found that, vitamin D levels were affected by age and health status, thus they recommended vitamin D supplementation for groups with low levels, particularly old and hyperlipidemic groups (Al-Horani, Abu Dayyih et al. 2016).

In the current study, parity revealed no significant correlation with vitamin D levels in patients. This finding is similar to a study done by Ganmaa et al. which reported that there was no significant correlation between parity and vitamin D levels (Ganmaa, Holick et al. 2014). Conversely a study done in Iranian women noted that, there was a significant inverse correlation between vitamin D levels and parity (Alipour, Saberi et al. 2014).

Educational level of the patients and controls in this study did not show significant differences with vitamin D levels. This finding matches two cross-sectional studies which showed that, no significant correlation was found between educational level and vitamin D levels (Alkerwi, Sauvageot et al. 2015, Bachhel, Singh et al. 2015).

Our study showed that, the majority of the patients and controls were housewives and there were nearly significant differences between the status of housewives and employed regarding vitamin D levels in patients and significant in controls; employed females had lower VD levels (Figure 3.7). In contrast to a cross-sectional study done in Kuwait demonstrated that, significant differences were found between occupation status and vitamin D levels, a higher proportion of subjects with VD deficiency were found in indoor work or were house wives when compared
with others involved in field (outdoor) (Gaafar and Bader 2013). Furthermore, in another study conducted in Korean wage workers showed that office and service workers had significantly lower vitamin D levels compared with other (Jeong, Hong et al. 2014). Shi et al reported that, education and occupation status were not significantly associated with vitamin D levels (Shi, Nechuta et al. 2014).

This study also evaluated the VDR-polymerorphisms (Taq1, Fok1, Bsm1 and Apa1) and their association with breast cancer risk. For the VDR-Taq1 polymorphism, there was a genotypic and allelic significant difference between patients and controls. VDR-Taq1 was found to be significantly associated with a lower risk of breast cancer, OR at 95% CI 0.5 (0.3- 0.7). This agrees with Reimers et al. study that noted, Taq1 polymorphism was significantly associated with decreased breast cancer risk (OR= 0.7) (Reimers, Crew et al. 2015). Several studies done by Wang et al reported that, no significant association with Taq1 polymorphism and risk for breast cancer (Hou, Tien et al. 2002, Newcomb, Kim et al. 2002, Buyru, Tezol et al. 2003, McCullough, Stevens et al. 2007, Wang, He et al. 2013).

In the present study no significant differences were found in allelic VDR-SNPs (Fok1, Bsm1 and Apa1) between study groups. VDR-SNPs (Bsm1 and Apa1) were not associated with the risk of breast cancer, OR at 95% CI [1.0 (0.6 – 1.7), 0.7 (0.5 – 1.2)] respectively. In a study done in Chinese females with breast cancer showed no association between VDR-SNPs (Bsm1 and Taq1) and breast cancer risk, while they found an association with VDR-Apa1 (Guo, Jiang et al. 2015). Although our study did not show a statistically significant association of VDR-Fok1 with the risk of breast cancer, there may be an increased risk of breast cancer among females with FF genotype when compared with ff genotype, suggesting females homozygous for minor allele, might be at higher risk than females carrying homozygous major allele (OR= 1.3). A meta-
analysis of 39 studies demonstrated that, VDR-Fok1 polymorphism was associated with an increase risk of breast cancer (Zhang and Song 2014). Furthermore individuals how were homozygous for minor allele genotype were more likely to develop breast cancer (Zhang and Song 2014). This finding was counteracted by Talaneh et al report, that VDR-Fok1 had a protective effect against breast cancer in Iranian women (OR=0.2) (Talaneh, Ghorbani et al. 2016). In contrast other studies reported that, the four polymorphisms of VDR (Fok1, Bsm1, Apa1, and Taq1) were not associated with breast cancer risk (McCullough, Stevens et al. 2007, Yang, Liu et al. 2014). In a case-control study that assessed the four VDR-polymorphisms (Fok1, Bsm1, Apa1, and Taq1) among breast cancer demonstrated that, Apa1 and Fok1 were not associated with risk of breast cancer, while Bsm1 and Taq1 may be a potential factor affecting the risk of breast cancer (Huang, Liao et al. 2014). Furthermore, two studies reported an increased risk of breast cancer in women with Fok1 polymorphism, while no association was observed between Bsm1 polymorphism and breast cancer risk (Chen, Bertone-Johnson et al. 2005, Sinotte, Rousseau et al. 2008).

It has been reported that, VDR- Bsm1, Apa1 and Taq1 polymorphisms located in the 3’UTR region, which seems not affecting VDR protein structure, however, these polymorphisms may influence the regulation and the stability of VDR and may change the expression levels of proteins. The Fok1 polymorphism located at exon 2 start codon of the VDR gene, has been associated with frame-shift in the VDR protein (Bai, Lu et al. 2012). Therefore individuals with f (T) allele have VDR proteins three amino acids less than F (C) allele. The later longer VDR proteins have been shown to be functionally less efficient (Uitterlinden, Fang et al. 2004).

In this study there were no significant differences found in the means of serum vitamin D levels and VDR-SNPs (Fok1, Bsm1 and Apa1) in patients, while significant differences were observed
with VDR-Taq1 dominant genotypes model. A study conducted in Indian population reported a significant correlation between serum vitamin D levels and Taq1 SNP, whereas no correlation was observed with Fok1 SNP (Bhanushali, Lajpal et al. 2009). Another study noted that no differences in serum 25(OH) VD concentrations by Fok1 and Taq1 VDR genotypes (Abbas, Nieters et al. 2008). A study conducted in UK Caucasian females with breast cancer reported that, low levels of VD alone and in combination with the VDR-Bsm1 homozygous genotype for minor allele have been associated with risk breast cancer (Lowe, Guy et al. 2005). Some studies have shown that VD levels in plasma are not directly correlated with genotypic frequency differences, suggesting that genotype difference could affect breast cancer cells through other pathways (Guo, Jiang et al. 2015). This study as far as we know is the first study to assess VDR polymorphisms in Sudanese females with breast cancer.
4.2 Conclusions:

- Vitamin D was deficient in both study groups (patients and controls).
- There was inverse significant correlation between serum vitamin D levels and BMI and negative but not significant in controls.
- Age and parity revealed no correlation with serum vitamin D levels.
- There was no significant difference between educational level and vitamin D levels in patients and controls.
- Occupation status revealed nearly significant difference with vitamin D levels in patients and significant in controls.
- VDR-Taq1 polymorphism was associated with low risk of breast cancer.
- No associations were found between VDR-polymorphisms (Fok1, Bsm1 and Apa1) and risk of breast cancer.
- Vitamin D levels were associated with TaqI-polymorphism genotype (dominant model), whereas there were no associations in other VDR-polymorphisms (Fok1, Bsm1, and Apa1) in patients.
4.3 Recommendations:

1. Oral supplementation is recommended for individuals with low level of vitamin D.

2. Establish health education programme to improve level of vitamin D.

3. Further studies are needed to be done to detect vitamin D enzymes genes polymorphisms (CYP27B1 and CYP24A1).
References


"Epidemiology of breast cancer: retrospective study in the Central African Republic."  
BMC Public Health **16**(1): 1230.


"Frequency of fokI and taqI polymorphism of vitamin D receptor gene in Indian population and its association with 25-hydroxyvitamin D levels."  


dihydroxyvitamin D3: physiologic and pathologic modulation of circulating hormone levels."

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