

بسم الله الرحمن الرحيم

**Sudan University of Science and Technology**

**College of Graduated Studies**

**Levels of Vitamin D and Parathyroid Hormone among  
Sudanese Patients with Rheumatoid Arthritis in  
Khartoum State**

مستويات فيتامين (د) وهرمون الغدة جار الدرقية في المرضى السودانيين  
المصابين بالتهاب المفاصل الريماتويدي بولاية الخرطوم

**A thesis submitted in partial fulfillment for the requirement of MSc  
degree in medical laboratory science (clinical chemistry)**

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*I will be grateful forever for your love.*

### **Abstract**

**Background:** Rheumatoid arthritis is a chronic inflammatory disease in which the synovial membrane of the joint becomes inflamed, resulting in a swelling, stiffness, pain, limited range of motion, joint deformity and disability.

The role of vitamin D in the pathogenesis of rheumatoid arthritis is under investigation. This study was designed to evaluate the correlation between serum values of vitamin D and rheumatoid arthritis and to correlate the relationship between vitamin D and parathyroid hormone level in this patient.

**Aim:** To detect level of serum vitamin D in patients of Rheumatoid arthritis (RA) and to establish relationship between serum vitamin D and parathyroid hormone level in RA patient.

**Methods:** Included 40RA patients admitted to hospital as case group, and 60 healthy individual as control group blood samples were obtained as 5ml venous blood in plain container, and then centrifuged to obtain serum which stored at -20°C until processing. Vitamin D and parathyroid hormone levels measured by eletrochemiluminescence immunoassay technique using cobas e 411 fully automated analyzer. The data obtained were analyzed and presented using statistical package for social science (SPSS) computer software version 16 for windows.

**Result:** The mean vitamin D serum values were  $38.165 \pm 12.57$  nmol/L in patients and  $42.63 \pm 10.56$  nmol/L in controls. This result showed no significant difference in vitamin D level (P.V=.066).

The mean PTH serum values were  $45.29 \pm 29.19$  nmol/L in patients and  $44.10 \pm 26.17$  nmol/L in controls. There was no significant difference in PTH level (P=0.835). Vitamin D level correlated with anti cyclic citrullinated peptide (ACCP) (p=0.029, R2=-0.22).

**Conclusion:** The vit D was negatively correlated with ACCP ( R2= -0.22, P.v=0.029), the study concluded that RA have no effect on vit D and PTH level.

## المستخلص

**خلفيه:** التهاب المفاصل الروماتويدي هو مرض مزمن، من الأمراض الانضدادية التي تؤدي بالجهاز المناعي لمهاجمة المفاصل، مسببة التهابات وتدمير لها. وفي بعض الحالات، يسبب المرض إعاقة، مؤدية إلى فقدان القدرة على الحركة والإنتاجية.

السبب الرئيسي لالتهاب المفاصل -الناشيء عن الداء الرثياني- هو التهاب الغشاء الزلالي (السينوفيلي) المبطن للمفصل وأغشية الأربطة العضلية. حيث يصيب المفاصل ويحدث حرارة وألم وانتفاخ مع الحركة أو اللمس، وتصلب مما يعوقها عن الحركة. هذه الدراسة صممت لتقييم العلاقة بين فيتامين د والتهاب المفاصل الروماتزمي ، ولربط العلاقة بين فيتامين د ومستوي هرمون الغده فوق الدرقية بالنسبة للمرضى.

**الهدف:** تهدف إلى الكشف عن مستوى فيتامين د (د) في المرضى الذين يعانون من التهاب المفاصل الروماتزمي وربط العلاقة بين فيتامين د وهرمونات الغدة فوقالدرقية بنشاط المرض .

**الطرق المستخدمة في الدراسة:** تم قياس مستوى فيتامين د وهرمون الغدة الدرقية باستخدام التقنية المناعية ECL (electrochemilumenscence) عن طريق استخدام جهاز التحليل كوباس e 411 , وقد تم تحليل البيانات التي تم الحصول عليها وعرضها باستخدام الحزمة الإحصائية للعلوم الاجتماعية (SPSS)برامج الكمبيوتر إصدار 16.

**النتائج:** وجد ان متوسط مستوى فيتامين د (د) في الاصحاء اعلي من المرضى بالتهاب المفاصل الروماتزمي, متوسط فيتامين د في المرضى  $12.57 \pm 38,165$  نانومول / لتر و في الاصحاء  $10.56 \pm 42,63$  نانومول / لتر. وأظهرت نتائج التحليل الإحصائي بأنه لا يوجد فرق احصائي واضح بين مستويات فيتامين د في مرضى التهاب المفاصل الروماتزمي و الاصحاء (PV = 0.066) .

كما ان متوسط قيم هرمونات الغدة الدرقية في المرضى  $29.19 \pm 45.29$  نانومول / لتر في المرضى و  $26.17 \pm 44,10$  نانومول / لتر في الاصحاء . لم يكن هناك ارتباط بين مرضى التهاب المفاصل الروماتزمي و مستوى قيم هرمونات الغدة الدرقية (P = 0.835). مستويات فيتامين د (د) قد ارتبط سلبياً مع قيم ACCP (P = 0.029) (RS = -0.22) .

**الخلاصة:** خلصت هذه الدراسة بأنه لا يوجد فرق احصائي واضح بين مستويات فيتامين د (د) في مرضى التهاب المفاصل الروماتزمي و الاصحاء , و انه لا يوجد ارتباط بين هرمونات الغدة الدرقية و مجموعة المرضى المصابين بالتهاب المفاصل الروماتزمي. وايضا خلصت الي ان مستويات فيتامين د (د) قد ارتبط سلبياً مع قيم ACCP .

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### List of Abbreviations

<b>1,25(OH)2D3</b>	1,25-dihydroxy vitamin D3
<b>25(OH)D3</b>	25-hydroxy vitamin D3
<b>ACPA</b>	Anti-Citrullinated Protein Antibody



<b>ACR</b>	AmericanCollege of Rheumatology
<b>CCP</b>	Cyclic CitrullinatedPeptide
<b>CRP</b>	C-Reactive Protein
<b>ECL</b>	ElectrochemilumescenceTechnique
<b>PTH</b>	Parathyroid Hormone
<b>RA</b>	Rheumatoid Arthritis
<b>RF</b>	Rheumatoid Factor
<b>SLE</b>	Systemic Lupus Erythematosus
<b>TNF-<math>\alpha</math></b>	Tumor Necrosis Factor alpha
<b>TNF-<math>\beta</math></b>	TumorNecrosisFactor beta

# CHAPTER ONE

# CHAPTER ONE

## 1.1. Introduction:

Rheumatoid arthritis is a chronic inflammatory disease in which the synovial membrane of the joint becomes inflamed, resulting in a swelling, stiffness, pain, limited range of motion, joint deformity and disability.

Vitamin D is the "sunshine vitamin" which is converted in the body to a hormone 1, 25-dihydroxyvitamin D<sub>3</sub> by the photolytic action of ultraviolet light on the skin.

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid glands as a polypeptide containing 84 amino acids, PTH acts to increase the concentration of ionic calcium (Ca<sup>2+</sup>) in the blood, calcitonin, a hormone produced by the parafollicular cells (C cells) of the thyroid gland, acts to decrease ionic calcium concentration.

Emerging evidence suggests that vitamin D plays an important role in immune regulation. Vitamin D receptors are found on several immune cells and *in vitro* studies have shown that vitamin D metabolites modulate T cell proliferation and dendritic cell function (Jones G et al., 2007). Epidemiological data also imply that vitamin D deficiency may be a risk for development of autoimmune and other chronic diseases (Holick MF 2004, 2007).

## 1.2 Rationale:

Rheumatoid arthritis is a chronic inflammatory disease in which the synovial membrane of the joint becomes inflamed, resulting in a swelling, stiffness, pain, limited range of motion, joint deformity and disability.

Lower level vitamin D may be associated with increased incidence of Rheumatoid Arthritis (RA). Vitamin D may also have a role in modulating RA disease activity and is already known to be important in osteoporosis and falls, which are common in RA.

### **1.3 Objectives:**

**General objective:** To study the level of vitamin D and parathyroid hormone level in Sudanese patient with rheumatoid arthritis.

**Specific objective:**

1. To estimate vitamin D level in rheumatoid arthritis Patients and normal healthy individuals.
2. To estimate parathyroid hormone level in rheumatoid arthritis Patients and normal healthy individuals.
3. To compare the relation between vitamin D and parathyroid hormone
4. To find correlation between vit D and anti-cyclic citrullinated peptide (ACCP) in RA.

# CHAPTER TWO

## CHAPTER TWO

### 2. Literature Review

#### 2.1 Rheumatoid arthritis:

RA is an autoimmune disease, in which a person's immune system attacks his or her own healthy tissues (Lee, R., 2007).

RA is the most common inflammatory arthritis across the world. Although the etiology of RA remains a mystery, a variety of studies suggest that a blend of environmental and genetic factors are responsible and both affecting the prevalence of autoimmune disease (Turhanoglu et al., 2010). Arthritic conditions encompass more than 100 different diseases and conditions affecting the joints, the tissues surrounding the joints and the connective tissue.

Arthritic conditions are among the most common diseases in the world and include osteoarthritis, Rheumatoid Arthritis (RA) and gout (Rizzo, D., 2005).

Common misconceptions about arthritic conditions are they only affect older persons, that they are an inevitable consequence of aging; they are diagnosed in people of all ages, including children and teens (Lee, R., 2007).

There are several factors known to increase the risk of arthritic conditions, three of which are modifiable: overweight, joint injuries and infections. Non-modifiable risk factors include female sex, age and family history (Rizzo, D., 2005).

The name (RA) is based on the term "rheumatic fever", an illness which includes joint pain and is derived from the Greek word rheumatosis ("flowing"). The suffix-oid ("resembling") gives the translation as joint inflammation that resembles rheumatic fever.

The first recognized description of rheumatoid arthritis was made in 1800 by Dr. Augustin Jacob Landré-Beauvais (1772-1840) of Paris (Landre-Beauvais, A., 2001).

Rheumatoid arthritis is a chronic inflammatory disease in which the synovial membrane of the joint becomes inflamed, resulting in a swelling, stiffness, pain, limited range of motion, joint deformity and disability (Lipsky, P., 2005).

RA affects approximately 0.8% of the population, is more common in older persons and affects female three times more often than males (Pattison et al., 2004).

Although RA primarily affects the joints, it also can affect other tissues, resulting in anorexia, weight loss, fatigue, general itching and stiffness (Lee, R., 2007).

Extra-articular ("outside the joints") manifestations occur in about 15% of individuals with rheumatoid arthritis. It can be difficult to determine whether disease manifestations are directly caused by the rheumatoid process itself, or from side effects of the medications commonly used to treat it-for example, lung fibrosis from methotrexate, or osteoporosis from corticosteroids (Turesson et al., 2003).

## **2.2 Pathophysiology:**

Rheumatoid arthritis is an autoimmune disease, the cause for which is still unknown. It is a systemic (whole body) disorder principally affecting synovial joints.

As with most autoimmune diseases, it is important to distinguish between the causes that trigger the inflammatory process and those that permit it to persist and progress.

Chemical mediators (Cytokines) give rise to inflammation of joint synovium.

Constitutional symptoms such as fever, malaise, loss of appetite and weight loss are also due to cytokines released in to the blood stream.

Blood vessel inflammation (vasculitis) affecting many other organ systems can give rise to systemic complications (Choy, E. and G. Panayi, 2001).

It has long been suspected that certain infections could be triggers for this disease.

The "mistaken identity" theory suggests that an infection triggers an immune response, leaving behind antibodies that should be specific to that organism.

The antibodies are not sufficiently specific, though and set off an immune attack against part of the host.

Because the normal host molecule "looks like" a molecule on the offending organism that triggered the initial immune reaction-this phenomenon is called molecular mimicry.

Some infectious organisms suspected of triggering rheumatoid arthritis include *Mycoplasma*, *Erysipelothrix*, parvovirus B19 and rubella, but these associations have never been supported in epidemiological studies. Nor has convincing evidence been presented for other types of triggers such as food allergies. There is also no clear evidence that physical and emotional effects, stress and improper diet could be a trigger for the disease.

The many negative findings suggest that either the trigger varies, or that it might in fact be a chance event (Edwards, J. et al., 1999).

Epidemiological studies have confirmed a potential association between RA and two herpesvirus infections: Epstein-Barr Virus (EBV) and Human Herpes Virus 6 (HHV-6) (Alvarez-Lafuente et al., 2005).

Individuals with RA are more likely to exhibit an abnormal immune response to the Epstein-Barr virus (Ferrell et al., 1981 and Catalano et al. 1979).

The allele HLA-DRB1\*0404 is associated with low frequencies of T cells specific for the EBV glycoprotein 110 and predisposes one to develop RA (Balandraud, N. and R. Roudier, 2004).

The factors that allow the inflammation, once initiated, to become permanent and chronic, are much more clearly understood. The genetic association with HLA-DR4 is believed to play a major role in this, as well as the newly discovered associations with the gene PTPN22 and with two additional genes, all involved in regulating immune responses (Plenge et al., 2007).

It has also become clear from recent studies that these genetic factors may interact with the most clearly defined environmental risk factor for rheumatoid arthritis, namely cigarette smoking (Padyukov et al., 2004).

Both T and B lymphocytes are involved in the pathogenesis of the disease (Choy, E. 2012).

The role of T lymphocytes as well as that of B lymphocytes in the pathogenesis of RA has been further proved by the therapeutic efficacy of methods affecting both T and B lymphocytes, namely the biological agents (Keystone et al. 2012).

### **2.3 Diagnosis:**

Diagnosis of RA depends on the symptoms and results of a physical exam, such as warmth, swelling and pain in the joints. Some blood tests also can help confirm RA.

There is no single test that confirms an RA diagnosis for most patients with this disease.

Telltale signs include: Anemia (a low red blood cell count), Rheumatoid factor (an antibody, or blood protein, found in about 80% of patients with RA in time, but in as few as 30% at the start of arthritis), Antibodies to cyclic citrullinated peptides (pieces of proteins), or anti-CCP for short (found in 60–70% of patients with RA) and Elevated erythrocyte sedimentation rate (a blood test that, in most patients with RA, confirms the amount of inflammation in the joints)



When RA is being clinically suspected, immunological studies are required, such as Rheumatoid Factor (RF, a specific antibody). A negative RF does not rule out RA; rather, the arthritis is called *seronegative*.

During the first year of illness, RF is frequently negative. 80% of individuals eventually convert to seropositive status.

RF is also seen in other illnesses, like Sjogren's syndrome and in approximately 10% of the healthy population, therefore the test is not very specific (American Association for Clinical Chemistry, 2006).

Because of this low specificity, a new serological test has been developed in recent years, which tests for the presence of so called Anti-Citrullinated Protein Antibodies (ACPA) (American Association for Clinical Chemistry, 2006).

Like RF, this test can detect approximately 80% of all RA cases, but is rarely positive if RA is not present, giving it a specificity of around 98%.

In addition, ACP antibodies sometimes can be detected in early stages of the disease, or even before onset of clinical disease.

Currently, the most common test for ACP antibodies is the anti-CCP (cyclic citrullinated peptide) test (American Association for Clinical Chemistry, 2005).

X-rays can help in detecting RA, but may not show anything abnormal in early arthritis.

Even so, these first X-rays may be useful later to show if the disease is progressing.

Often, MRI and ultrasound scanning are done to help judge the severity of RA (American College of Rheumatology 2012).

## **2.4 Vitamin D**

Vitamin D is the "sunshine vitamin" which is converted in the body to a hormone 1, 25-dihydroxyvitamin D<sub>3</sub> by the photolytic action of ultraviolet light on the skin.

Vitamin D plays an important role, along with the essential minerals calcium and phosphorus, in the maintenance of healthy bones and teeth (Combs, G.F., 1988).

Moreover vitamin D sufficiency, especially during the childhood and adolescent years, is critically important not only for bone health, but also for the prevention of many serious chronic diseases, including cancer, cardiovascular heart disease and autoimmune diseases.

It has been suggested that vitamin D deficiency during infancy and childhood may imprint an increased risk of these chronic diseases for the rest of one's life (Combs, G.F., 1988).

Vitamin D (calciferol) comprises a group of fat soluble seco-sterols found naturally only in a few foods, such as fish-liver oils, fatty fish, mushrooms, egg yolks, and liver. The two major physiologically relevant forms of vitamin D are  $D_2$  (ergocalciferol) and  $D_3$  (cholecalciferol).

Vitamin  $D_3$  is photosynthesized in the skin of vertebrates by the action of solar ultraviolet (UV) B radiation on 7-dehydrocholesterol (Fieser LF, Fieser M. 1959).

Vitamin  $D_2$  is produced by UV irradiation of ergosterol, which occurs in molds, yeast, and higher-order plants.

Under conditions of regular sun exposure, dietary vitamin D intake is of minor importance.

However, latitude, season, aging, sunscreen use, and skin pigmentation influence the production of vitamin  $D_3$  by the skin (Institute of Medicine 1997).

Most of the dietary intake of vitamin D comes from fortified milk products and other fortified foods such as breakfast cereals and orange juice (Institute of Medicine 1997).

Both vitamin  $D_2$  and  $D_3$  are used in nonprescription vitamin D supplements, but vitamin  $D_2$  is the form available by prescription in the United States (Holick, M.F., 2007).

Vitamin D without a subscript represents either  $D_2$  or  $D_3$  or both and is biologically inert.

Vitamin D from the skin or diet is only short-lived in circulation (with a half-life of 1–2 days), as it is either stored in fat cells or metabolized in the liver (Mawer EB et al., 1971).

In circulation, vitamin D is bound to vitamin D-binding protein and transported to the liver, where it is converted to 25-hydroxyvitamin D [25(OH)D] (DeLuca HF. 1984).

This major circulating form of vitamin D is a good reflection of cumulative effects of exposure to sunlight and dietary intake of vitamin D and is therefore used by clinicians to determine vitamin D status.

To be biologically activated at physiologic concentrations, 25(OH)D must be converted in the kidneys to 1,25-dihydroxyvitamin D [1,25(OH) $_2$ D], which is thought to be responsible for most, if not all, of the biologic functions of vitamin D (DeLuca HF 1988 and Reichel H et al., 1989).

The production of 25(OH)D in the liver and of 1,25(OH) $_2$ D in the kidney is tightly regulated.

In the liver, vitamin D-25-hydroxylase is down-regulated by vitamin D and its metabolites, thereby limiting any increase in the circulating concentration of 25(OH)D following intakes or following production of vitamin D after exposure to sunlight.

In the kidney, in response to serum calcium and phosphorus concentrations, the production of 1,25(OH)<sub>2</sub>D is regulated through the action of parathyroid hormone (PTH) (DeLuca HF 1988 and Reichel H et al., 1989).

Active vitamin D functions as a hormone, and its main biologic function in people is to maintain serum calcium and phosphorus concentrations within the normal range by enhancing the efficiency of the small intestine to absorb these minerals from the diet (DeLuca HF 1988 and Reichel H et al., 1989).

When dietary calcium intake is inadequate to satisfy the body's calcium requirement, 1,25(OH)<sub>2</sub>D, along with PTH, mobilizes calcium stores from the bone. In the kidney, 1,25(OH)<sub>2</sub>D increases calcium reabsorption by the distal renal tubules.

Apart from these traditional calcium-related actions, 1,25(OH)<sub>2</sub>D and its synthetic analogs are increasingly recognized for their potent anti-proliferative, prodifferentiative, and immunomodulatory activities (Nagpal 2005).

## **2.5 Parathyroid hormone (PTH)**

Parathyroid hormone (PTH), an 84 amino acid peptide produced by the parathyroid gland, is used for clinical diagnosis and monitoring of hyper- and hypoparathyroidism.

The primary role of PTH is maintenance of calcium homeostasis; making PTH quantification an important diagnostic aid for calcium metabolism disorders (Ashby JP et al., 1997).

Normally, serum PTH and calcium concentrations are inversely correlated; for example, when calcium is decreased, PTH is increased (Al Zahrani A et al., 1997).

Serum PTH concentrations are coupled with serum ionized calcium concentrations for the differential diagnosis of primary hyper- and hypoparathyroidism, hypercalcemia of malignancy and renal failure.

**Intact PTH (I-PTH) assays** (Capillary Electrophoresis Immunoassay (CEIA)) are commonly used for the differential diagnosis of calcium homeostasis disorders (Ashby JP et al., 1997) .

Generally less than 5 to 25% of total immunoreactive PTH is intact hormone.

The remaining 75 to 95% is inactive midregion/carboxyl fragments.

Due to the major shortcomings of the first generation single-site C- or N-terminal assays, which reacted with a variety of inactive PTH fragments, two-site PTH assays were developed and termed intact PTH.

Two site immunometric assays for I-PTH were once the gold standard (Ashby JP et al., 1997) .

However, some PTH molecules that are considered to be "intact" in the majority of two-site immunoassays have no bioactivity due to loss of key amino acids at the N-terminal (Martin KJ et al., 2004).

In hypercalcemia,secretion of these inactive forms persists, while secretion of intact hormone is greatly reduced or absent. In fact, ~50% of the "intact" PTH detected in the serum of patients with chronic renal disease is biologically inactive,(Nagpal 2005), or even may be an antagonist of PTH, such as the PTH(7-84) amino acid fragment.

Many I-PTH assays react with circulating molecular forms of PTH other than PTH (1-84), (Brossard JH et al., 2000) , (*Inaba M. etla., 2004*) quantifying inactive fragments, and overestimating PTH secretion by 80- 120%(Lepage R et al., 1998) .

**Bio-intact PTH (BI-PTH)** by chemiluminescence eliminates interference from inactive PTH fragments, specifically the 7-84 PTH fragments,(*Inaba M. etla., 2004*),(*Martin KJ et al., 2004*) and offers improved sensitivity and specificity to diagnose secondary hyperparathyroid disease in individuals with early and end-stage renal disease (*Guthrie E et al., 2001*).

## **2.6 Rheumatoid arthritis and vitamin D:**

The discovery of VDR in the cells of the immune system and the fact that activated dendritic cells produce vitamin D hormone suggested that vitamin D could have immunoregulatory properties (*Fritsche et al., 2003*).

VDR, a member of the nuclear hormone receptor superfamily, was identified in mononuclear cells, dendritic cells, antigen-presenting cells and activated T-B lymphocytes (*Arnson et al., 2007*).

A physiological role for vitamin D in the immune system is supported by the presence of the VDR in primary lymphoid organs.

The primary lymphoid organs (bone marrow and thymus) are the centers where the immune system develops and differentiates (*Deluca, H.F. and M.T. Cantorna, 2001*).

As a matter of fact, both genetic and environmental factors contribute to the etiology of autoimmune diseases.

T cells (lymphocytes that differentiate in the thymus) have been shown to play fundamental roles in autoimmune diseases.

Quiescent CD4 + T cells express VDRs at low concentrations, which increases five-fold after their activation (Mahon, B.D. et al., 2003).

The effects of vitamin D on the acquired, antigen-specific immune response, are characterized by inhibition of T-lymphocyte proliferation (Lemire, J.M., 1992) , particularly of the Th1 arm (Th 1 is a subset of the T helper cells that secretes cytokines) (Mattner, F. et al., 2000).

Treatment of CD4 T cells with vitamin D inhibits Th1 cell proliferation and cytokine production (Boonstra, A. et al., 2000).

Addition of vitamin D was shown also to inhibit the expression of the Interleukin-6 (IL-6).

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine (Stockinger, B., 2007).

Interestingly, in B cells vitamin D has been shown to inhibit antibody secretion and autoantibody production (Linker-Israeli et al 2001).

*In vitro*, vitamin D inhibits the differentiation of monocytes into dendritic cells and interferes with the stimulatory activity that T cells exert on them (Griffin, M.D. et al., 2001).

It has been shown that vitamin D is one of the most efficient blockers of dendritic cell differentiation and of interleukin secretion.

*In vitro* vitamin D stimulates phagocytosis and killing of bacteria by macrophages but suppresses the antigen presenting capacity of these cells and of dendritic cells (Griffin, M.D. et al., 2000).

Vitamin D has been found to promote the induction of monocytic differentiation to macrophages and modulate macrophage responses, preventing them from releasing inflammatory cytokines and chemokines(Helming, L. et al., 2005).

It has been observed that greater intake of vitamin D was associated with a lower risk of RA, as well as lower vitamin D was found associated with higher disease activity (Merlino, L.A. et al., 2004).

Since lower vitamin D serum levels have been also associated with higher RA disease activity, in a recent study were evaluated serum 25(OH)D3 levels in 64 female RA patients from north

Europe (Estonia) and 54 RA patients from south Europe (Italy) during winter and summer and were correlated with the disease activity score (DAS28) (Cutolo, M. et al., 2006).

In addition, there may be a higher vitamin D requirement for patients at risk for developing autoimmunity and for those that already have an autoimmune disease such as systemic lupus erythematosus (Kamen, D.L. et al., 2006).

In fact, the optimal amount of vitamin D to support the immune response may be different from the amount required to prevent vitamin D deficiency or to maintain calcium homeostasis (Rejnmark, L. et al., 2004).

One review suggest that the optimal plasma 25(OH)D3 concentration lies between 50-80 nmoles/L, other experts suggesting between 75-125 nmol/L (Chatfield, S.M. et al., 2007).

# **CHAPTER THREE**

## **CHAPTER THREE**

### **3. Materials and methods:**

#### **3.1. Study approach and design:**

This is a quantitative, analytic, case-control hospital-based study.

##### **3.1.1. Study area and period:**

This study carried out in Khartoum state in 2016.

##### **3.1.2 Target population:**

Sudanese patients with rheumatoid arthritis.

##### **3.1.3 Sample size:**

This study included 40 patients with rheumatoid arthritis and 60 healthy participants.

##### **3.1.4 Selecting criteria:**

###### **3.1.4.1 Inclusion criteria:**

Patients diagnosed with rheumatoid arthritis( diagnosis depends on ACCP positive).

###### **3.1.4.2 Exclusion criteria:**

Patients of RA having chronic renal failure, systemic lupus erythematosus, diabetes mellitus, any systemic illness and patients on enzyme inducer drugs or on calcium and vitamin D supplements were excluded from study.

###### **3.1.5 Ethical consideration:**

All participants have informed of the aim of this study and their approval gated by informed concept.



## **3.2 Methodology:**

### **3.2.1 Sample preparation:**

Blood samples were collected in plain containers, and then subjected to centrifugation to obtain serum. The obtained serum stored at -20°C until processing.

### **3.2.2 Detection of Vit D:**

The Roche Diagnostics Vitamin D total assay is a competitive electrochemiluminescence protein binding assay intended for the quantitative determination of total 25-OH vitamin D in human serum and plasma.

The assay employs a vitamin D binding protein (VDBP) as capture protein, which binds to both 25-OH D3 and 25-OH D2

The assay utilizes a 3-step incubation process, which has duration of 27 minutes.

In step 1, the sample is incubated with pretreatment reagent, which releases bound 25-OH vitamin D from the VDBP.

In step 2, the pretreated sample is incubated with ruthenium labeled VDBP creating a complex between the 25-OH vitamin D and the ruthenylated VDBP.

The third incubation step sees the addition of streptavidin-coated microparticles and 25-OH vitamin D labeled with biotin.

The free sites of the ruthenium labeled VDBP become occupied, forming a complex consisting of the ruthenium labeled vitamin D binding protein and the biotinylated 25-OH vitamin D.

The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The entire complex becomes bound to the solid phase (by the interaction of biotin and streptavidin-coated microparticles which are captured on the surface of the electrode). Unbound substances are removed. Applying voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via an instrument-

specific calibration curve which is generated by 2-point calibration and a calibration master curve provided via the reagent barcode.

### **3.2.2.1 Detection limit of method:**

The limit of detection of vit D by this method 3.00 ng/mL (7.50 nmol/L).

### **3.2.2.2 Sensitivity:**

4.01 ng/mL (10.0 nmol/L) (CV 18.5 %)

### **3.2.2.3 Repeatability:**

Within-run precision: <15 ng/mL: SD ≤ 1 ng/mL  
>15 ng/mL: ≤ 6.5 %

### **3.2.2.4 Reproducibility:**

Intermediate precision: <15 ng/mL: SD ≤ 1.7 ng/mL  
>15 ng/mL: ≤ 11.5 %

### **3.2.2.5 Expected values:**

Most experts agree that vitamin D deficiency should be defined as vitamin D (25-OH) of ≤ 20 ng/mL.<sup>4</sup> Vitamin D insufficiency is recognized as 21-29 ng/mL.<sup>4</sup> The preferred level for vitamin D (25-OH) is recommended to be ≥ 30 ng/mL

## **3.2.3 Detection of parathyroid hormone:**

The Roche Diagnosticscobas e 411 is a fully automatic run-oriented analyzer system for the determination of immunological tests using the ECL/Origen electrochemiluminescent process.

All components and reagents for routine analysis are integrated in or on the analyzer.

Parathyroid hormone is measured on the Elecsys 1010 using a sandwich principle.

In the first incubation: 50 µl sample, a biotinylated monoclonal PTH-specific antibody and monoclonal PTH-specific antibody labeled with a ruthenium complex form a sandwich complex.

In the second incubation: After addition of streptavidin-labeled microparticles, the complex produced is bound to the solid phase via biotin-streptavidin interaction.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode.

Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined via a calibration curve. This curve is instrument-specifically generated by a 2-point calibration and a master curve provided via the reagent barcode.

Total duration of assay is 9 minutes on the cobas e411 and the reference values are 10–65 pg/mL.

### **3.2.3.1 Detection limit of method:**

The detection limit of PTH by this method 3,50pg/ml

### **3.2.3.2 Sensitivity:**

Sensitivity of this method 5,5pg/ml

### **3.2.3.3 Expected values:**

The normal range was in a clinical study with 596 samples of apparently healthy persons determined 14.9- 56.9 pg / ml (mean value 31.3 pg/ml).

### **3.2.4Data analysis:**

The data obtained were analyzed and presented using statistical package for social science (SPSS) computer software version 16 for windows.

### **3.2.5 Quality control:**

The precision and accuracy of all methods used in the study has been checked each time a batch was analyzed by including commercially prepared control sera.

# CHAPTER FOUR

The study included Case subjects (n=40) ; n=60) were randomly selected (table 4.1).

Most cases 40% aged from 39 to 59 and most of controls 26% aged from 18 to 38.

Females have higher percentage in both case 48% and control 33% in compare with male's percentage in case 12% and control 7% .( Table 4.1)

There was no correlation between Rheumatoid arthritis patients and vitamin D (P.V=.066) (Table 4.2)

There was no correlation between Rheumatoid arthritis patients and PTH (P.V=.776) (Table 4.2)

Vitamin D level negatively correlated with ACCP (P.V=.030) as shown in (Figure 4.2). There was no correlation between PTH and ACCP (P=.007) as shown in (Figure 4.3)

There was correlation between Rheumatoid arthritis patients and age (P.V<.001), CRP (P.V=.019), RF (P.V<.001), ACCP (P.V<.001) .table (4.3)

#### **Table 4.1 Baseline characteristics of study population**

criteria	study population				p.value
	Control N=60		Case N=40		
	count	Percentage	count	Percentage	
Age groups					<.001
18-38	20	26%	26	26%	
39-59	40	40%	12	12%	
60-80	0	0%	2	2%	
Gender					.604
Male	12	12%	7	7%	
Female	48	48%	33	33%	

Chi-square test

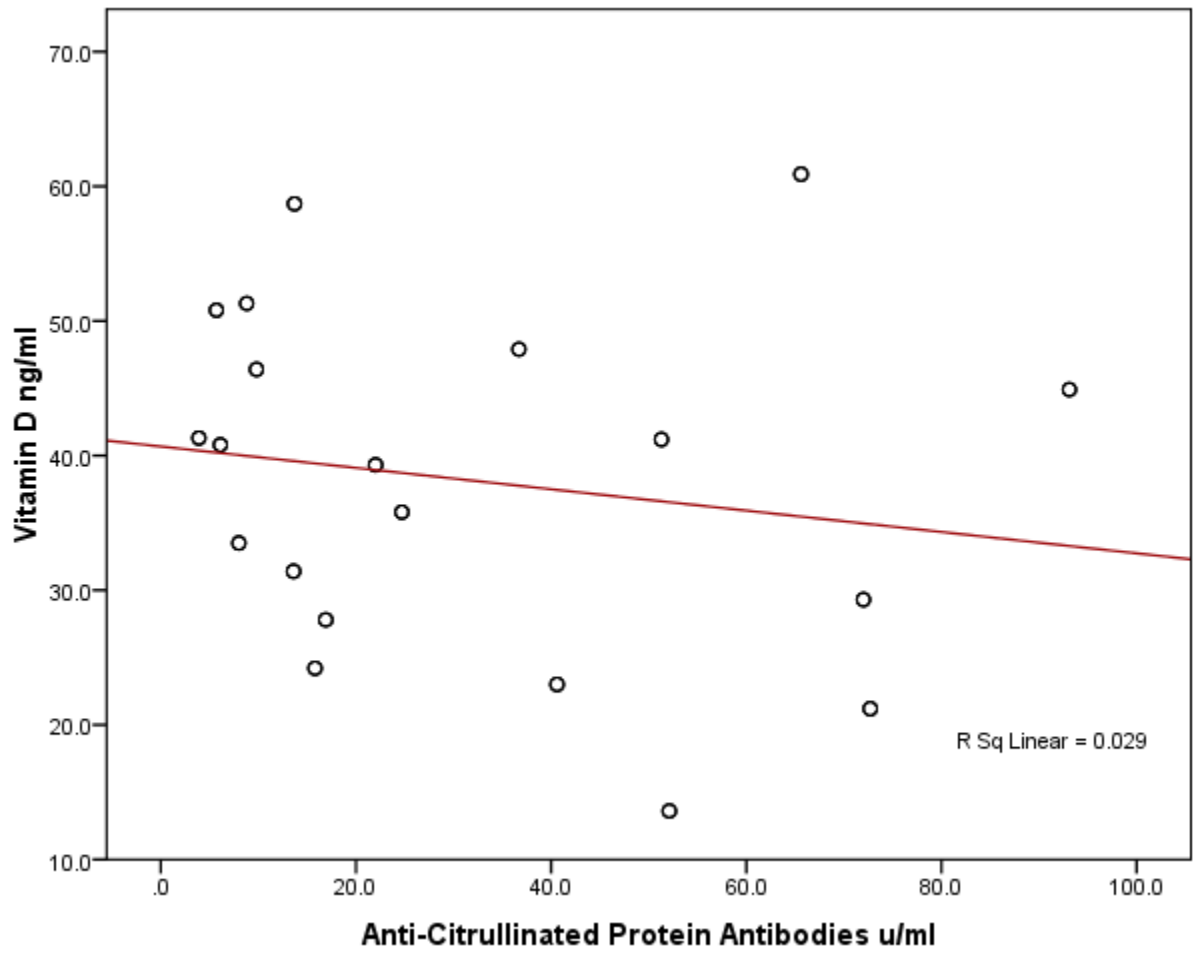
≤0.05 considered significant

**Table 4.2 Comparison between vitamin D, RF, ACCP, CRP and PTH among patients and healthy individuals**

Criteria	study population		p.value
	Control N=60	case N=40	
	Mean±SD	Mean±SD	
RF(IU\ml)	13.34± 4.36	10 ±.000	<.001
ACCP(U\ml)	31.65± 26.74	2.79 ±6.19	<.001
CRP(mg\L)	6.43± 5.96	11.13 ±13.47	.019
PTH(nmol\L)	45.29 ±29.67	43.652± 26.60	.776
Vit D(nmol\L)	38.16± 12.51	41.491± 10.9	.066

Independent T .test

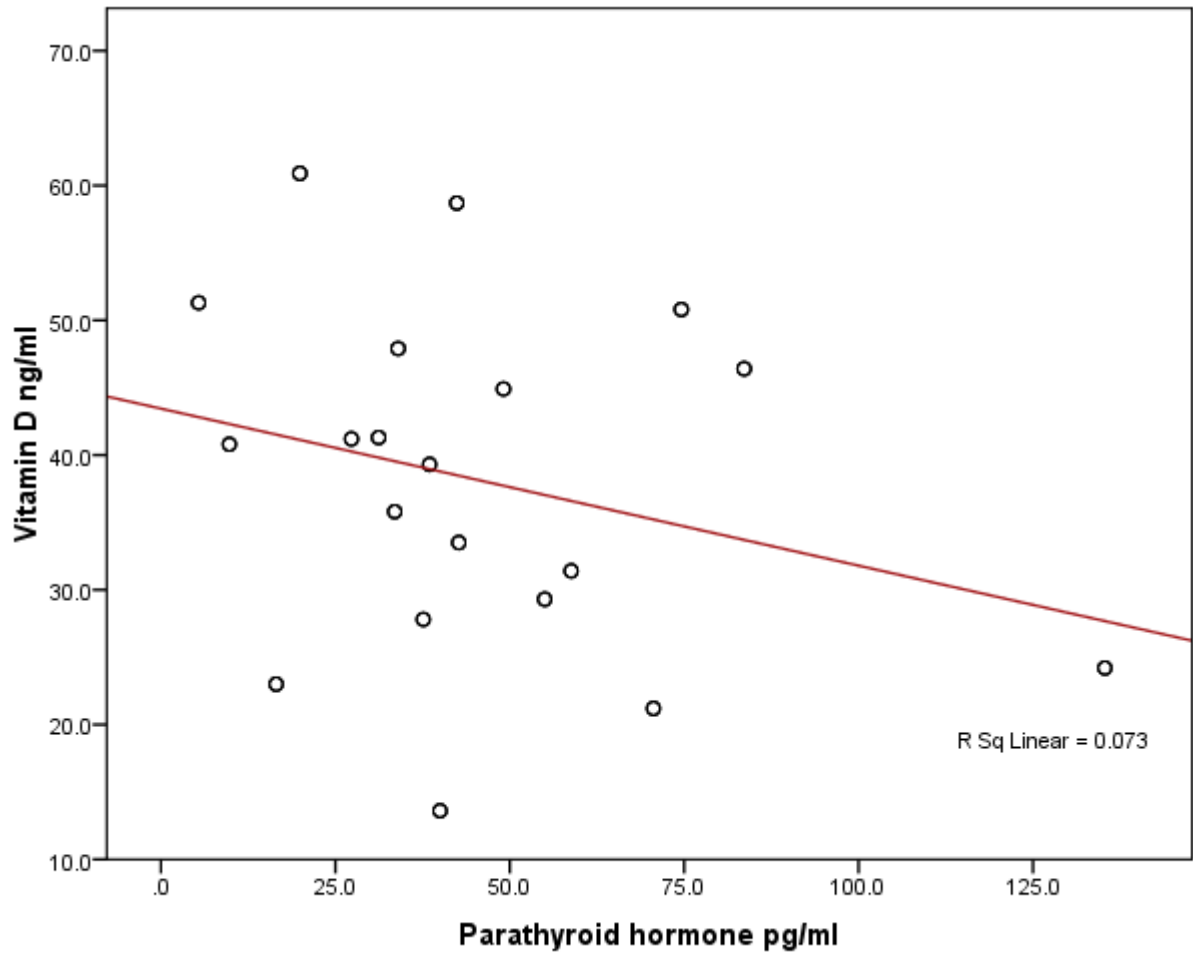
≤0.05 considered significant



( $r^2=.029$ , P.V=.030).

**Figure (4.1) correlation of vitamin D with ACCP in RA patients**





**Figure( 4.2) correlation of vitamin D and PTH in RA patients**

# **CHAPTER FIVE**

## 5.1 Discussion:

The study was conducted with the objective to evaluate the status of serum vitamin D levels in patients of rheumatoid arthritis and to compare it with healthy volunteers.

In this study, the level of vitamin D was almost equal in both groups and both groups were having comparable serum levels of vitamin D.

We confirmed that the serum values of vitamin D are not a good indicator of disease activity. However, the newly diagnosed RA patients showed lower serum values of vitamin D compared with those with chronic disease. Another important outcome of the present study was that there is a negative correlation between serum values of vitamin D and anti-CCP in early RA. It should be mentioned that future studies considering new protocols of vitamin D measurements.

There are some explanations for these findings slightly lower levels of serum vitamin D in case group might be attributed to less sunlight exposure. Also the most patients were under treatment with physiological doses of vitamin D according to the recommendations for the osteoporosis prevention (800U/d).

Etiology of RA is still unknown and many environmental and genetic factors play a role in the development of RA. As mentioned earlier, vitamin D might play a role in modulation of immune function and inflammatory responses has been suggested because vitamin D is known to be synthesized normally by antigen presenting cells like macrophages and dendritic cells while activated lymphocytes synthesize it during inflammatory process only. It is believed that vitamin D suppresses the production of IFN- $\gamma$  and IL-2 while increase the production of IL-4, IL-5 and IL-10 cytokines. Because of this, an association of vitamin D deficiency and autoimmunity has been suggested (Hewison M 2012, Heidari B et al., 2012, Kamen DL et al., 2010).

As noted the documents in agreement with the regulatory role of vitamin D on the specific facets of human immunity have been shown to be on the rise in recent years and it seems that ,in the future, correction of vitamin D deficiency will be a part of immunomodulation strategies in several autoimmune diseases (Kamen DL et al., 2010, Bansal AS et al., 2012, Merlino LA et al., 2004, Nielen MMJ et al., 2006, Dorr J et al., 2012, Oelzner P et al., 1998) .

Several studies have been conducted on the vitamin D serum values in RA patients compared with healthy controls.

However, the direct influence of vitamin D on some of innate immune cells should not be ignored. The results of our study are in line with most of the previous studies on the correlation between RF and vitamin D serum levels (Heidari B. et al.2012).

Most of the studies have proposed that vitamin D deficiency is a predisposing factor for the initiation and progression of autoimmune process. For instance, several studies have shown that vitamin D serum levels are lower in the newly diagnosed RA patients compared with healthy controls (Hewison M 2012,Heidari B et al., 2012,Kamen DL et al., 2010).

In the current study, we observed a negative correlation between serum levels of 25(OH)D and anti-CCP in recently

(Heidari B. et al.2012) conducted a study in which the levels of vitamin D in 108 established rheumatoid arthritis patients and 39 undifferentiated inflammatory arthritis patients were compared with 239 healthy controls. They observed no significant differences in mean serum vitamin D between rheumatoid arthritis and healthy controls ( $37 \pm 37.7$  vs.  $33.2 \pm 28.6$  ng/ml,  $P = 0.96$ ) however the mean serum vitamin D levels in patients with undifferentiated inflammatory arthritis were significantly lower than in the controls ( $25.1 \pm 23.9$  vs.  $33.2 \pm 28.6$  ng/ml,  $P = 0.04$ ) (Maryam Sahebari (MD), Zahra Mirfeizi (MD), Zahra Rezaieyazdi (MD)) in Iran conducted a study in which found no correlation between vitamin D serum values and DAS over a short duration of disease course.

## **5.2 Limitation:**

The limitations of this study include small sample size for RA group which was not adequate to reach statistical significance.

As all patients were on their routine treatment with disease modifying anti-rheumatic drugs, we did not assess the disease activity scores using suitable disease activity score questionnaire (e.g. DAS-28) so association between serum vitamin D and disease activity could be established.

### **5.3 Conclusion:**

We found no statistical difference between the serum levels of vitamin D of the rheumatoid arthritis and healthy controls.

RA have no effect on vit D and PTH levels.

In the early diagnosed patients, vitamin D and anti-CCP serum values was negatively correlated. There was no correlation between PTH serum levels and patient group with RA.

### **5.4 Recommendation:**

1. A study with a good sample size should be conduct to confirm the result of this study
2. Establish the association between serum vitamin D and disease activity scores using suitable disease activity score questionnaire (e.g. DAS-28).

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# Appendix

Questionnaire

Informed concept

Figures

# Questionnaire

Sudan University of Science and Technology

College of Graduated Studies

Questionnaire on Sudanese with rheumatoid factor e to investigating their vitamin D and PTH levels

NO ( )

Name .....

Age.....year.

Gender

Male ( )- female ( ).

Having history of any inflammatory disease?

Yes ( ), NO ( ).

Do you have Hypertension?

Yes ( ), NO ( ).

Do you have diabetes mellitus?

Yes ( ), NO ( ).

Serology result

Rheumatoid factor	IU/ml
Anti CCP	U/ml
C reactive protein	Mg /dl

Date

.....

signature

.....

## Informed concept

Rheumatoid arthritis is a chronic inflammatory disease in which the synovial membrane of the joint becomes inflamed, resulting in a swelling, stiffness, pain, limited range of motion, joint deformity and disability.

The aim of this study to detect level of serum vitamin D in patients of Rheumatoid arthritis (RA) and to establish relationship between serum vitamin D and parathyroid hormone level in RA patient. And this may help in prevention and treatment of this disease , and by sinning this you approve to participate on it.

التهاب المفاصل الرثياني و الالتهاب المفصلي الروماتزمي هو مرض مزمن، من الأمراض الانتزادية التي تؤدي بالجهاز المناعي لمهاجمة المفاصل، مسببة التهابات وتدميرا لها. ومن الممكن أيضا أن يدمر جهاز المناعة وأعضاء أخرى في الجسم مثل الرئو والجلد. وفي بعض الحالات، يسبب المرض والإعاقة، مؤدية إلى فقدان القدرة على الحركة والإنتاجية.

هذا الدارسة تهدف إلى الكشف عن مستوى فيتامين د(د) في المرضى الذين يعانون من التهاب المفاصل الروماتويدي وربط العلاقة بين فيتامين د وهرمونات الغدة الدرقية بنشاط المرض. مما يساعد في الوقايه والمساهمه في علاج هذا المرض . وبتوقيعك توافق علي المشاركة في البحث بملي الاستبيان وتقديم عينه دم لاجراء القياس ولكم جزيل الشكر والتقدير

Name.....

Phone NO.....

Date

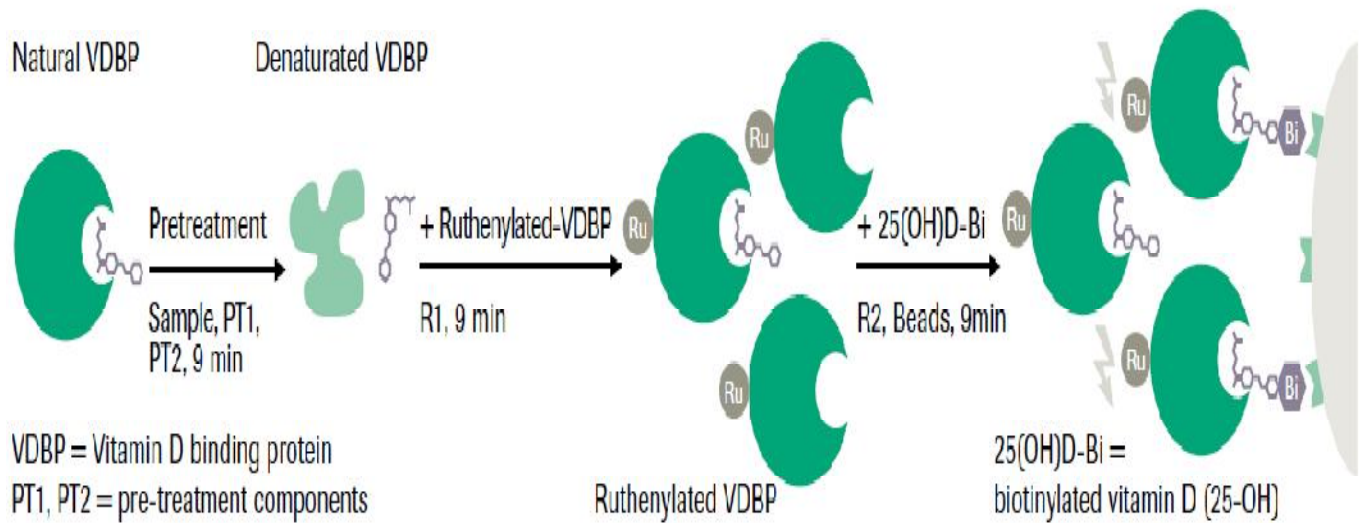
signature

.....

.....

## Figures

### Test principle: Competitive protein binding assay



### Electrochemiluminescence protein binding assay principle for the quantitative determination of total 25-OH vitamin D