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University of Shendi

Faculty of Graduate Studies and Scientific Research

Evaluation of Vitamin D Level in Diabetic Patients in Shendi locality

A thesis Submitted for partial fulfillment for the Rrquirement of Msc Degree in Medical Laboratory Sciences in (Clinical Chemistry)

By

Suha Elrayah Eltieb Alaraky

BS.c in Medical Laboratory Sciences. (Shendi University –2007)

Supervisor

Dr: Mosab Omer Khalid Mohammed Zeen

Assistant Professor of Clinical Chemistry

Faculty of Medical Laboratory Science - Shendi University

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الآيـــــة

بسم الله الرحمن الرحيم

قال تعالي :

﴿ لَا يُكَلِّفُ اللَّهُ نَفْسًا إِلَّا وُسْعَهَا لَهَا مَا كَسَبَتْ وَعَلَيْهَا مَا اكْتَسَبَتْ رَبَّنَا لَا تُؤَاخِذْنَا إِنْ نَسِينَا أَوْ أَخْطَأْنَا رَبَّنَا وَلَا تَحْمِلْ عَلَيْنَا إِصْرًا كَمَا حَمَلْتَهُ عَلَى الَّذِينَ مِنْ قَبْلِنَا رَبَّنَا وَلَا تُحَمِّلْنَا مَا لَا طَاقَةَ لَنَا بِهِ وَاعْفُ عَنَّا وَاغْفِرْ لَنَا وَارْحَمْنَا أَنْتَ مَوْلَانَا فَانْصُرْنَا عَلَى الْقَوْمِ الْكَافِرِينَ ﴾

{سورة: البقرة-الآية: (٢٨٦) }

Dedication

To the one who was there to love and care for me when the skies were grey and when I was down, she was always there to comfort me ,no one else could be what she has been to me

to the queen of my heart,

To my mother .

To my father.

It's insipid without you to anyone who one day drew a smile on my face, the person who perfume my life with happiness

My husband,,,

To the driving forces in my life,

Sisters and brothers

To my favorite friends,

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First of all I thank Allah for giving me the strength and
Thanking you is not just enough to express the gratitude that should be bestowed upon you, but my love and respect which is there for you ever since u accepted me as your student is the least I can give for my whole life. Very grateful to you
Dr: Mosab Omer Khalid Mohamed Zeen
thanks my all friends who always like my post and support and help me and stand with me thanks from the bottom of my heart to my all true helping and lovely friends
My thank also extend to my college and my teacher
lastly, I offer my regards to all of those who supported me in any aspect during the completion of this research.

List of abbreviations

1,25 (OH)2D	1,25-dihydroxyvitamin D
25(OH)D	25-dihydroxyvitamin D
7DHC	7 – dehydrocholesterol
β	Beta
DBP	Vitamin D Binding Protein
DM	Diabetes Mellitus
FNB	Food National Board
GDM	Gestational Diabetes Mellitus
IL	Interleukin
IU	International Unit
NOD	Non-Obese Diabetic
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Th1	Type 1 helper
Th2	Type 2 helper
TNF	Tumor Necrosis Factor
UVB	Ultraviolet B
Unites	
nm	Nanometer
μ g	Microgram
IU	International Unit
nmol/ l	Nano mol per liter
Mg/dl	Mali gram per deciliter

Abstract

This study was conducted in Shendi locality to evaluated vitamin D level in diabetic patients during the period from April to August 2018.

Vitamin D helps to prevent bone fractures , prevent falls in older people and osteoporosis , reduce the risk of cancer especially colon cancer , prostate cancer and breast cancer , reduce the risk of diabetes especially in young people and in those living in high altitude protect against heart disease including high blood pressure and heart failure , reduce your risk for multiple sclerosis . improve you mood and improve your lung function .

Material and method: Total of 60 blood samples were collected from Sudanese male and female (40 with Diabetic mellitus as case group and 20 healthy non – diabetic as control group). The level of vitamin D

Result: of study showed significant decrease in vitamin D level in diabetic patients , when compared with the corresponding control group with the means of case and control group (73.4 ± 22.9 – 19.9 ± 9.7 nmol/L) respectively with p.value (0.000). The study recorded significant inverse between vitamin D level with age, duration of diabetes, expotuer to sun light in diabetic group with p.value(0.001 – 0.030 – 0.001) respectively.

Conclusion: this study concluded that vitamin D level decrease in diabetic patients, statistical analysis shows significant variation between vitamin D level and age, duration of diabetes and exposed to sun light, and insignificant variation between vitamin D level with hypertention and glycemic status of diabetic patients.

ملخص البحث

أجريت هذه الدراسة في محلية شندي لتقيم مستوى فيتامين د عند مرضى السكري خلال الفترة من ابريل إلى أغسطس ٢٠١٨م.

الطريقة:

تم جمع ٦٠ عينة دم من ذكور و إناث السودانيين (٤٠ عينة يعانون من مرض السكري و ٢٠عينه أصحاء غير مصابين) لقياس معدلات فيتامين د وتم الحصول على النتائج بواسطة جهاز Ichroma™ .

النتيجة:

وقد أظهرت هذه الدراسة انخفاض معدلات فيتامين د عند مرضى السكري مقارنة مع المجموعة الضابطة بوسط حسابي (١٩,٩– ٧٣,٤)nmol/L على التوالي و بقيمة مطلقة (٠،٠٠٠)

وقد سجلت الدراسة أيضاً أن هناك علاقة بين فيتامين د والعمر ,فترة الإصابة بمرض السكري و فترة التعرض لأشعة الشمس للمصابين بمرض السكري بقيمة مطلقة على التوالي (٠،٠٠١ – ٠،٠٣٠ – ٠،٠٠١) .

الخاتمة:

من هذه الدراسة نستنتج أن هناك علاقة بين مرض السكري ونقصان معدلات فيتامين د. كما سجلت الدراسات الإحصائية نقصان في معدلات فيتامين د تبعا للعمر و فترة الإصابة بمرض السكري و فترة التعرض لأشعة الشمس. ولم تسجل الدراسة الإحصائية علاقة بين معدلات فيتامين د و الإصابة بارتفاع ضغط الدم للمصابين بمرض السكري و المعدل التراكمي لمستوى السكر في الدم.

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Chapter One

Introduction

Rationale

Objectives

1.1 Introduction

Diabetes is a chronic condition that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Hyperglycemia and other related disturbances in the body's metabolism can lead to serious damage to many of the body's systems, especially the nerves and blood vessels. It has become one of the major causes of premature illness and death in most countries, mainly through the increased risk of cardiovascular disease. Cardiovascular disease is responsible for between 50% and 80% of deaths in people with diabetes. Diabetes is a leading cause of blindness, amputation and kidney failure. These complications account for much of the social and financial burden of diabetes. [(1)]

There are two basic forms of diabetes, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). A third type of diabetes, gestational diabetes mellitus (GDM), develops during some cases of pregnancy but disappears after pregnancy. [(1)]

Recent evidence has reported that Vitamin D deficiency predisposes individuals to T1DM and T2DM. This was suggested by several studies that show that vitamin D has several effects on the immune system and is also involved in the Insulin synthesis and secretion therefore suggesting a role in the development of both T1DM and T2DM. Studies have shown that diabetic individuals are more vitamin D deficient than non-diabetic individual.[(2)]

Vitamin D is a fat-soluble vitamin that is traditionally recognized as a potent regulator of calcium and phosphorus metabolism. Prolonged and severe vitamin D deficiency is known to cause rickets in children and osteomalacia /osteoporosis in adults. [(3)] However, rickets has made an unfortunate comeback and several studies have shown vitamin D deficiency is becoming a global public health problem although it is largely unrecognized.

It has been estimated that 1 billion people worldwide have vitamin deficiency or insufficiency. Vitamin D deficiency is common in the elderly due to the decreased capacity to produce vitamin D from the skin. [4]

Vitamin D deficiency seems to discriminate between races where dark-skinned individual are likely to be more at risk than fair-skinned individuals. This is due to the melanin that is present in greater quantities in dark-skinned individuals, which absorbs the Ultraviolet B (UVB) radiation and thus reducing the vitamin D synthesis. [3]

As we can see a number of studies show that vitamin D deficiency in common and strategic measures should be taken to prevent this from happening . Studies have shown that vitamin D may play a role in the prevention of serious chronic diseases, including diabetes mellitus, cardiovascular disease; some inflammatory and autoimmune disorders; as well as some types of cancer. Therefore, showing that vitamin D does not only play a role in calcium homeostasis and bone metabolism but it also has other functions in many parts of the body. [5]

1.2 Objectives

1.2.1 General objective

To evaluate vitamin D levels in diabetic patients in Shendi locality.

1.2.2 Specific objectives

1. To evaluate the association of vitamin D level with duration of diabetes.
2. To correlate the Vitamin D levels with glycemic status of diabetic patients.
3. To assess the association of vitamin D levels according to age and gender of diabetic patients.

1.3 Rationale

Diabetic patients are the most important group in community The World Health Organization (WHO) predicts that Diabetes Mellitus (DM) would be the seventh leading cause of mortality by the year 2030 .

This study made to evaluate Vitamin D in diabetic patients and determine the effect of many factor (age – gender – duration of diabetes – exposure to sun light – glycmic status).

Chapter Two

Literature Review

2. Literature review

2.1 Diabetes Mellitus

Diabetes is a chronic condition that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Hyperglycemia and other related disturbances in the body's metabolism can lead to serious damage to many of the body's systems, especially the nerves and blood vessels. It has become one of the major causes of premature illness and death in most countries, mainly through the increased risk of cardiovascular disease. Cardiovascular disease is responsible for between 50% and 80% of deaths in people with diabetes. Diabetes is a leading cause of blindness, amputation and kidney failure. These complications account for much of the social and financial burden of diabetes. [(1)]

There are two basic forms of diabetes, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). A third type of diabetes, gestational diabetes mellitus GDM develops during some cases of pregnancy but disappears after pregnancy. [(1)]

2.1.1 Type of Diabetes Mellitus

2.1.1.1 Type1 Diabetes Mellitus

T1DM accounts for only 5–10% of those with diabetes the other 90-95% mainly have T2DM . Type 1 diabetes mellitus (T1DM) is a T-cell mediated autoimmune disease that results in the destruction of insulin-producing beta (β)- cells in the pancreas, requiring exogenous insulin for survival. [(6)] The β-cell destruction often begins during infancy and continues over many months or years. By the time, that T1DM is diagnosed, about 80% of the β-cells have been destroyed.[(7)] Although it is acknowledged to be an autoimmune disease, the causes are still considered to be unknown.[(4)] Epidemiologic studies of T1DMhave directed the search for possible genetic predispositions and related environmental factors that are still poorly defined [(8)]. Some identified

environmental risk factors operating early in life include enter viral infections in pregnant women, older maternal age (39-42 years), preeclampsia, cesarean section delivery, increased birth weight, early introduction of cow's milk proteins and an increased rate of postnatal growth (weight and height). [4] There is a great necessity to find the cause of this chronic disease because there has been an increase in incidence from one year to the next. It is estimated that currently the incidence is increasing by 3% per year and it is predicted that by 2010 the incidence of T1DM will be 40% higher than it was a decade earlier. This increase can not only be explained by genetic factors something else must be behind this. [7] Several approaches have been tried to prevent T1DM but none of them have been shown to work, and the prevention of T1DM remains an objective for the future. [1]

2.1.1.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a non-insulin dependent diabetes or adult onset diabetes. These individuals have insulin resistance and usually have a relative insulin deficiency .There are probably many different causes of this form of diabetes. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur. Most patients with this form of diabetes are obese and obesity itself causes some degree of insulin resistance. [4] At the same time, physical inactivity, both a cause and consequence of weight gain, also contributes to insulin resistance. The problem of obesity and overweight is present in developed countries but is also extending to developing countries, especially in urban areas. [1] T2DM may also occur in individuals who are not obese but that have an increased percentage of body fat distributed predominantly in the abdominal region, which increases the insulin resistance . Ethnicity is also another risk factor with, higher rates of T2DM reported in people of Asian and African origin, and in indigenous peoples of the Americas and Australasia. T2DM is often shown to be associated with a strong genetic predisposition, more than the autoimmune

form T1DM. [4] Therefore, individuals with a family history of diabetes have more probability of developing T2DM. Women who developed diabetes during pregnancy are also more at greater risk of T2DM later in life. Simple lifestyle measures have been shown to be effective in preventing or delaying the onset of T2DM (weight loss, increasing physical activity and having a healthy diet). However, more studies should be done in order to find more preventive measures because the world is facing a growing diabetes epidemic of potentially devastating proportion. At least 171 million people worldwide have diabetes (type 1 and type) and this figure is likely to increase to 366 million by 2030.

In order to stop these numbers from increasing more must be done to see all possible causes of both T1DM and T2DM . [1]

2.1.1.3 Gestationl Diabetes Mellitus

Gestationl diabetes mellitus (GDM) is defined as any degree of carbohydrate intolerance with onset or first recognition during second or third trimester of pregnancy. [8]

2.1.1.4 Secondary Diabetes Mellitus:

Uncommon diabetes can occur secondarily to other disease (e.g. chronic pencreatititis) following pancreatic surgery and in condition where there is increased secretion of hormones antagonistic to insulin (e.g. chushing□s syndrome and acromegaly. [9]

2.1.2 Complications of Diabetes Mellitus

2.1.2.1 Acute Complications

Acute symptoms of diabetes are due to sever hyperglycemia and include polyuria polydipsia, polyphagia, weiht loss and blurr vision.patients may exhibit impaired and diabetic ketoacidosis in type 1 diabetes or to the hyperglycemic hyperosmolar nonketotic syndrome in type 2 diabetes.[10]

- **Diabetic ketoacidosis**: is a potentially life-threatening complication in patients with DM. It happens predominantly in those with type 1 diabetes,

but can occur in those with type 2 under certain circumstances. Diabetic ketoacidosis result from shortage of insulin in response the body switches to burning fatty acids and producing acidc keton bodies that cause most of symptoms and complications.[11]

- **Hyperosmolar hyperglycemic state:** is a complication of DM predominantly type 2 in which high blood sugars cause sever dehydration, increases in osmolarity, and high risk of complication, coma and death. [12]

2.1.2.2 Chronic Complication

- **Diabetic Nephropathy**: is characterized at the later by increased proteinuria , arterial cardiovascular disease risk . Increased lipotoxicity, including increased synthesis of fatty acids , sphingolipid and phospholipids, has been hypothesized to directly impact on the development of diabetic nephropathy. [13]
- **Diabetic Retinopathy**: is a serious microvascular complication of diabetes and is the primary cause of blindness in 30-70 year olds worldwide. Diabetic retinopathy is a progressive disease that can characterized by a preclinical nonproliferative stage and a proliferative stage , wgich manifests as revascularization and vitreous gemorrhages. [14]
- **Atherosclerosis**: T2DM patients are at greater risk of developing atherosclerosis and coronary heart disease chiefly mediated by perturbations in lipid and glucose metabolism . while metabolomic and lipidomic studies have been conducted on patients with coronary heart disease , there are limited metabolomic studies that focus exclusively on atherosclerosis and coronary heart disease in T2D patients at present . Readers are referred to recent reviews with a focus on metabolomics and lipidomics , and coronary heart disease. [15]
- **Diabetic neuropathy:** is a common complication that is characterized by progressive loss and damage of peripheral nerve axons resulting in

decreased sensation and a reciprocal increase in pain. The primary cause is associated with decreased blood flow due to damaged microvasculature coupled to a high glucose environment. Approximately 60 % - 70 % of T2D patients have some form of neuropathy, with risk increasing with age and duration of T2d. Current treatments are limited and are focused on managing and minimizing pain: however, the symptoms often become progressing worse. Because nerve damage precedes the onset of symptoms, clinical diagnosis of diabetic neuropathy is usually associated with significant and often irreversible nerve damage . Adequate biomarkers and risk assessment of diabetic neuropathy progression is currently lacking. [16]

2.2 Vitamin D

2 . 2. 1 Vitamin D Metabolism

Vitamin D (calciferol) is a secosteroid compound that can be obtained from food, but most people achieve their vitamin D needs (85-95%) by endogenous synthesis through direct ultraviolet B-mediated synthesis in the skin. [4]

However if there is insufficient endogenous synthesis, generally caused by limited sun exposure of skin to sunlight, then a dietary supply becomes essential. By the action of UVB light (290-315nm) the B ring of 7-dehydrocholesterol (pro-vitamin D) can be broken to form precholecalciferol (pre-vitamin D), which is rapidly isomerised to vitamin D in a thermo sensitive process . The conformationa changes due to the isomerisation can deliver vitamin D into the circulation, where it is transported by vitamin D-binding protein (DBP) to the liver for further metabolization. In food and dietary supplements, vitamin D exists in the form of either ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) .Both are fatsoluble and once ingested is incorporated into the chylomicron fraction, absorbed through the lymphatic system and transported to the liver where it will also be further metabolized. Vitamin D occurs naturally in a limited number of foods, the

highest amounts appear in fatty fish (salmon, sardines, mackerel, and herring, tuna), oils from fish (including codfish liver oil) and in the lowest amounts in red meats, egg yolk, and other animal food products. It is also available in fortified foods (including milk and milk products, juice, bread, margarines and breakfast cereals). [4]

Vitamin D itself is biologically inert and requires two successive hydroxylation reactions, in order to be activated. The first hydroxylation takes in the liver and is carried out by 25-hydroxylases (mitochondrial CYP27A1 and microtonal CYP2R1) which convert vitamin D into 25-hydroxyvitamin D (25(OH)D), also known as calcidiol. The second takes place mainly in the kidneys and is carried out by the e 1α-hydroxylase (mitochondrial CYP27B1) which converts 25(OH)D to 1,25-dihydroxyvitamin D (1,25(OH)2D), also known as calcitriol, the active form of vitamin D[2] . The production of 1,25(OH)2D in the kidney is strictly regulated by several factors. The main regulatory factors are 1,25(OH)2D itself, which down regulates its own production; PTH, which stimulates the renal production of the 1,25(OH)2D; fetal growth factor 23 and serum concentration of calcium and phosphate . [17]

Many extrarenal tissues also express the 1α-hydroxylase, including osteoclasts, pancreatic islets, antigen presenting cells (macrophages/dendrite cells), monocytes, placenta, colon, skin (keratinocytes), mammary(breast), prostate, human brain, endothelium and parathyroid glands. [17] These cells have the ability to produce local concentrations of 1,25(OH)2D which have autocrine and paracrine actions. This extrarenal production plays an important role in modulating immune responses, regulation of cell differentiation, cell proliferation, and apoptosis and thus shows that vitamin D may be involved in other physiological processes, independently of calcium metabolism. [4] This extrarenal production of 1,25(OH)2D is dependent on the circulating precursor levels of 25(OH)D therefore when there is a low serum level of 25(OH)D there will be less extrarenal production. [5] The 1α-hydroxylase

present in extrarenal tissues is identical to the renal 1α-hydroxylase, but regulation of its expression and activity is different. The renal 1α-hydroxylase is principally under the control of calcemia and bone signals (such as parathyroid hormone and 1,25(OH)2D itself), the extrarenal 1α-hydroxylase is primarily regulated by immune signals, with interferon-□ and Toll-like receptor agonists being powerful stimulators. [2]

Another hydroxylation enzyme which is involved in the vitamin D metabolism is called 24-hydroxylase, which is responsible for the degradation of 25(OH)D and 1, 25(OH)2D. [2]

As shown earlier DBP binds and transports vitamin D and its metabolites in plasma. This DBP is synthesized in the liver and circulates at a concentration that is in excess of normal circulating vitamin D metabolite concentration [4]. DBP has a higher affinity for 25(OH)D than 1,25(OH)2D . Plasma levels of DBP are 20 times higher than the total amount of vitamin D metabolites, and 99% of vitamin D compounds are protein bound, mostly to DBP, although albumin and lipoproteins contribute to lesser degrees .[5] The complex 25(OH)D and DBP enters the cell via megalin-mediated endocytosis, the DBP is degraded by the lysozomes, and 25 (OH)D is release and delivered to the mitochondria by a intracellular vitamin D binding protein 3 for hydroxylation, that converts 25(OH)D to 1,25(OH)2D or reenters the circulation bound to DBP. The 1,25(OH)2D produced is transported by the DBP to nuclear vitamin D receptor (VDR) .[15] Only 1,25(OH)2D is metabolically active and exerts its effects mainly by activating the VDR. This VDR is a member of the nuclear receptor super-family of ligand-activated transcription factors which also comprises the thyroid hormone receptors, estrogen receptors, retinoic acid receptors and peroxisome proliferator-activated receptor. [2]

Binding of 1,25(OH)2D to the VDR leads to the transcription of genes regulated (over 200 genes) by the 1,25(OH)2D. The mechanism is very

complex and is only just being unraveled. These VDR's are not only expressed in classical 1,25(OH)2D-responsive target tissues (bone, kidney, intestine, parathyroid glands) but are also expressed in a broad range of other tissues . The discovery that VDR's are widely expressed in the immune system (activated T cells, macrophages, dendritic cells and the B cells) led to the recognition of the central immunomodulatory role for 1,25(OH)2D and the discovery of VDR in the pancreatic cells led to the recognition of the role of vitamin D in insulin production and secretion . [2]

2 . 2 . 2 Vitamin D recommendations

The first recommended dietary allowance (RDA) for vitamin D for Americans in 1941 was 400 international unit (IU). This value derived from an observation that this amount, which was found in a teaspoon of codfish liver oil, was sufficient to prevent rickets. In 1997, the recommended intake level by the Food and Nutrition Board (FNB) was set as an adequate intake (AI) value rather than an RDA. [18]

Table (2-1) DRI: dietary reference intakes [19]

Current Vitamin D recommendation (DRI) for Canada and the United States for 1997

Age group	Level recommended (μg)
0–6 months	5 (200 IU)
7–12 months	5 (200 IU)
1–3 years	5 (200 IU)
4–8 years	5 (200 IU)
9–18 years	5 (200 IU)
19–30 years	5 (200 IU)
31–50 years	5 (200 IU)
51–70 years	10 (400 IU)
>70 years	15 (600 IU)

The reason why it changed from RDA to AI was because of the lack of scientific data necessary to set an RDA. In order to set an RDA, one must first determine from the published literature an estimated average requirement (EAR), which is a measure of the intake needs of 50% of a specified group. Therefore, since there was not sufficient data to draw an EAR, the RDA could not be drawn . [20] Since publication of the AI, much has been learned regarding the metabolism of vitamin D allowing an EAR to be set so that an RDA may be derived . however, it is very difficult to determine an accurate value for an EAR due to the fact that sunlight exposure is difficult to quantify and that foods have variable quantities of vitamin D. However, it is crucial to set EAR and RDA values in order to facilitateplanning and nutrition initiatives . [18]

The aim of the 1997 AI is based on the need to maintain serum 25(OH)D levels, in the absence of sunlight, at or above 27.5 nmol/L for most age groups in order to prevent rickets or osteomalacia. However, it has been shown that higher serum concentrations (75-80 nmol/L) are needed in order for vitamin D to support paracrine and autocrine functions . If an adult consumes the AI of 200 IU/day the circulating 25(OH)D levels usually remain unchanged, especially during winter months therefore showing that this quantity is not enough. In a study adult submariners where supplemented with 600 IU/day for 6 months with no sunlight exposure. The results showed that not even 600 IU/day of vitamin D managed to maintain circulating 25(OH)D in a sufficient level .[21]

2 . 2 . 3 Defining vitamin D status

serum 25(OH)D is considered to be the best biochemical marker of vitamin D status as it reflects the amount ingested in the diet (including that from supplements and vitamin D fortified food products) and the amount produced in the skin in response to UVB radiation exposure . Although 1,25(OH)2D represents the active form of the vitamin, it is not a good indicator of the

vitamin D status due to the tight regulation of its production as well as a relatively short halflife(4-6hours). [4]

Controversy exists, due to insufficient evidence, on the cut-off points used to define vitamin D status.[20] Most agree that a 25(OH)D concentration <50nmol/L is an indication of vitamin D deficiency, whereas a 25(OH) D concentration of 51- 74nmol/L, is considered to indicate insufficiency and concentration of 75nmol/L is considered to be sufficiency. Vitamin D intoxication typically does not occur until 25(OH)D concentrations are >375nmol/L.[3] Hipervitaminosis D (hypercalcemia and hyperphosphatemia) has been reported with serum 25(OH)D concentration ranging from 700 1600nmol/L). The lack of consensus as to the serum vitamin D serum status is due to the variability in assays for 25(OH)D. [22] The first assays used to measure 25(OH)D was the competitive protein binding format with the DBP as the binder. The advantage of this assay was that DBP recognized 25(OH)D2 equally as well as 25(OH)D3 but it also recognized other vitamin D metabolites, including 24,25 dihydroxyvitamin, 25,26-dihydroxyvitamin D, and the 25,26-dihydroxyvitamin D-26, 23-lactone. In 1985, a radioimmunoassay (RIA) was developed for 25(OH)D. This assay (Diasorin essay) also recognized 25(OH) D2 as well as 25(OH)D3 but it also recognized 24,25(OH)2D and other polar metabolites. Thus, both the DBP and the RIA assays typically overestimated 25(OH)D levels by approximately 10–20%. In the mid 1970s high-performance liquid chromatography (HPLC) was applied to the 25(OH)D assays. HPLC was able to remove interfering vitamin D metabolites and therefore was considered to be the golden standard but the limitation was that it was not a very manageable assay, and thus, was not routinely used by reference laboratories. Later a liquid chromatography tandem mass spectroscopy was used for the direct measurement of 25(OH)D in human serum. This assay quantitatively measured both 25(OH)D2 and 25(OH)D3. [4]

Using different methods, we may find different results even if we analyze the same sample. Thus, the cutoff value for 25(OH)D used to define the vitamin D status must be defined in terms of appropriate assay methods. [18] Membership in the international Vitamin D Quality Assessment Scheme (DEQAS), an international quality-control program, and the availability of a standard serum from the National Institute of Standards and Technology should help investigators to reduce the variability among laboratories. [4]

So after knowing what serum levels are considered to be sufficient (>75nmol/L) the question is, what is the intake needed in order to reach this sufficiency? A 6 months prospective study was conducted in order to answer this question. In order to know the intake needed they took into consideration the wide dose-response curve and basal 25(OH)D concentration and found that a dose of 3800 IU/day for those with serum 25(OH)D above 55nmol/L and a dose of 5000 IU/day for those below 55nmol/L was need [22]. Other studies show that an intake of 500- 1000IU/day is needed to maintain serum level of 75nmol/L. Although in sunlight deprived subjects a daily intake of 4000IU/day may be needed. [23]

Other authors defend that healthy adults and children can obtain enough vitamin D by being exposed to sunlight in the face and hands for 2 hours per week However, they say that there are certain individuals that are more at risk of becoming vitamin D deficient (elderly, dark skinned, pregnant women, breastfeeding women and early childhood) therefore, may need extra food supplementation in order to keep normal serum levels of 25(OH)D. [4]

The lack of sun exposure is known to be the primary cause of low serum 25(OH)D. However, as stated earlier even with adequate sun exposure low serum 25(OH)D levels can be found. Table 2 (Appendix 2) shows the various factors that affect the vitamin D status in an individual. [24]

2.2.4 Role of vitamin D on the pathogenesis of T1DM

The existence of VDR in activated T lymphocytes, antigen-presenting cells (APCs) (macrophages and dendritic cells), and thymus tissue raised the idea that 1,25(OH)2D might function as an immunomodulator.[4] The fact that immuncells (activated macrophages and dendritic cells in particular) also contain the enzyme 1α-hydroxylase, which is necessary for the final activating step in the conversion of 25(OH)D to the metabolically active molecule, shows that these cells are able to synthesize and secrete 1,25(OH)2D which is able to target the immune system. [2]

In order to provide evidence that vitamin D affects the risk of developing T1DM..

Vitamin D is considered to be a potential environmental and genetic risk factor for T1DM. [4]

2.2.4.1 Vitamin D as a Environmental risk factor

Seasonal and geographical factors are both known, as risk factors, for T1DM and vitamin D deficiency and several epidemiological studies have reported that. The probability of developing T1DM has been shown to be about 400 times more likely in a child living in Finland (latitude 61ºN) than a child living in Venezuela (latitude 8ºN) .[7] An ecological study analyzed the relationship between UVB radiation, and age-standardized (<14years) incidence rate of T1DM in children, according to 51 regions of the world. The results showed that the incidence rate was higher in regions that were more distant from the equator (higher latitude), where UVB radiation is lower, than in those closer to the equator (lower latitudes), where UVB radiation is much higher. [4]

A seasonal variation of T1DM diagnosed cases is also usually observed with the largest proportion of T1DM cases diagnosed during autumn-winter and the lowest during the summer. Could this be due to the sunlight exposure and vitamin D status that are highest in the summer and lowest during autumn and

winter? Some studies explain this increase in incidence during winter months due to a diminished exposure to UVB radiation which leads to a decreased vitamin D production. [25]

Similar seasonal and geographic variations have also been suggested for the variation of serum 25(OH)D levels. The 25(OH)D levels seem to be highest in the summer and lowest during autumn and winter. [4] Several observational studies have been done throughout recent years which show that vitamin D supplementation during pregnancy and early childhood may offer protection against the development of T1DM because it prevents this vitamin D deficiency. [24]

In certain countries, such as Finland, Sweden and Norway, the exposure to sunlight is below optimal for infants and pregnant women, therefore supplementation is recommended. However, this recommendation is not done in many other countries .[4] Breast-milk is a poor source of vitamin D and so the vitamin D status of the newborn is dependent on the stored vitamin D acquired from the mother during pregnancy or dietary supplements before weaning. [26]

Codfish liver oil is a rich source of n-3 fatty acids (EPA and DHA) that have anti-inflammatory effects, which could be potentially relevant in the etiology of T1DM. However, it is also an important source of vitamin D so the question to be answered is whether it is vitamin D the one that provides this protective effect or is it the n-3 fatty acids. [4]

2.2.4.2 Vitamin D as a genetic risk factor

In addition to the environmental role of vitamin D on T1DM risk, certain allelic variations in the VDR may also be of genetic risk for T1DM. However, published findings have been conflicting. Vitamin D deficiency often runs in families, suggesting that genetic variation might account for differences in vitamin D concentrations; however, the genes regulating vitamin D concentrations remain to be identified. Genetic variation occurs in nearly all

genes of the vitamin D system, but most investigations have studied more the polymorphisms of the VDR gene. VDR polymorphisms have been associated with increase the susceptibility to T1DM in Caucasians, in Bangladeshi Indians, and in Japanese, although such association was not found in a combined scale analyses from the UK, Romania and Finland and a study conducted in Portugal. [4]

2.2.4.3 Possible mechanisms of vitamin D action on T1DM

As mentioned earlier T1DM is a chronic progressive autoimmune disease that affects genetically prone individuals. The autoimmune process is an inflammation response targeted specifically at the B-cells in the islets of Langerhans, causing their mass reduction and dysfunction. [27]

Progression of T1DM has been shown to involve infiltration into pancreatic islet cells by several types of immune cells including antigen-presenting cells (APCs - such as macrophages and dendritic cells), CD4+, and CD8+ T cells, and B cells. In this autoimmune process, the helper T cells (Th0) that derive from the CD4+ T cells have a central role. The microenvironment in which Th0 cells develop determine which of 2 subtypes predominates (Th1 or Th2).The Th1 and Th2 cell responses regulate each other and, during "normal" immune responses, the organism responds with a balance of the 2 subtypes. Th1 cells secrete interferon (IFN-□), interleukin-2 (IL-2) and tumor necrosis factor α (TNF-α) which activate cell-mediated immunity, that is, cytotoxic and inflammatory responses mediated by T cells, natural killer cells, and macrophages. Th2 cells secrete cytokines (IL 4, IL- 5, IL-6, IL-9, IL-10, IL-13) which activate humoral immunity, that is, antibody production. In autoimmune diseases like T1DM, Th1 cells are misdirected against self-proteins, which result in β-cell destruction of the pancreatic islets of Langerhans [25]. Prevention of this autoimmune destruction requires early intervention in order to prevent the β-cells destruction from happening.

Therefore, more studies should be done in order to figure out all possible factors that initiate this process. [27]

A unique feature of 1,25(OH)2D as a immunomodulator is the fact that it not only interacts with T cells, but it also target the APCs . T cells (particularly of the Th1 type) are affected by 1,25(OH)2D which suppresses their proliferation and cytokine production. It has been shown that 1,25(OH)2D decreases the secretion of interleukin IL-2 (a important T-cell differentiation factor), TNF-α and IFN-□ by Th1 and promote the Th2 cytokine production of IL- 5, IL-4 and IL-10 production, therefore tilting the T cell response towards Th2 dominance and inhibits the Th1 response. [19] In VDR knockout mice there was more Th1 cytokine secretion and less of Th2 cytokines. Therefore, in the absence of 1,25(OH)2D or its receptors the T cells compartment has a potentially stronger Th1 phenotype. [28] Therefore, 1,25(OH)2D by binding to VDR may act as a transcriptional regulator of Th cell cytokines synthesis and Th cell differentiation and therefore it is involved in the pathogenesis of T1DM. The APC also plays an important role in the immune function of 1,25(OH)2D.[25] The cytokines produced by the APCs for the recruitment and activation of T cells are directly influenced by 1,25(OH)2D. [2] The dentritic cells (DCs) for example only have antigen presenting capacity when they are in its mature state. The differentiation and maturation of DCs into a potent APC's is inhibited by 1,25(OH)2D and its analogs. [4] The mature DCs have cell-surface Major Histocompatibility Class II molecules and accessory signals for T cell activation and are able to produce higher levels of IL-12. When helper T cells (Th0) are exposed to mature DCs and the IL-12, the Th1 cells predominate over Th2 cells activating macrophages and cytotoxic T cells, which in turn can directly destroy the β-cells of the pancreas. When 1,25(OH)2D is increased it has been shown to inhibit DCs from maturing and prevents this process from happening. [4]

1,25(OH)2D seems to also suppresses the antigen presenting capacity of the macrophages but in vitro, it is known to stimulate the phagocytosis and killing of bacteria by macrophages. [2]

Taken together, these observations suggest a physiological role for 1,25(OH)2D in the immune system, with a tightly regulated secretion of 1,25(OH)2D by macrophages and dendritic cells upon immune stimulation on the one hand and a direct inhibitory effect of the molecule on antigen presentation and T cell proliferation and cytokine secretion on the other hand. These immune effects are typically mediated through the binding of 1,25(OH)2D to VDR since these receptors are present in all of these immune cells. [2]

2.2.5 Role of vitamin D on the pathogenesis of T2DM

The prevention and treatment of T2DM is a public health concern in many health systems. T2DM is usually due to resistance to insulin action in the setting of inadequate compensatory insulin secretary response. It does not emerge in all individuals with insulin resistance but rather only in those with a defect in insulin secretory capacity (presumably genetic) in which pancreatic insulin secretion fails to compensate for the insulin resistance and hyperglycemia occurs. This hyperglycemia is first exhibited as an elevated postprandial blood glucose caused by insulin resistance at the cellular level and then is followed by a elevation in fasting glucose concentration. In T2DM, there may be a progressive loss of pancreatic islet β-cells resulting in insulin deficiency and the need to administrate insulin to compensate this loss.

Both environmental and genetic factors seem to contribute to the development of the disease. Potential modifiable environmental risk factors for T2DM have been identified, the major one being obesity, particularly central obesity. [29]

Recent evidence has shown that vitamin D could also be an environmental or genetic factor that may play a role in the pathogenesis of T2DM. [4]

2.2. 5. 1 Vitamin D as a environmental risk factor

The association that vitamin D has with T2DM is suggested by a seasonal variation in glycemic control, which has been reported in patients with T2DM as being worse in winter. This may, at least in part, be due to prevalent hypovitaminosis D in the winter. In a population-based study done Hungary, managed to report seasonal pattern in the onset of T2DM, more cases were diagnosed in winter than in the summer months .[30] It has been suggested that vitamin D decreases the risk of developing T2DM and therefore individuals with hypovitaminosis are more at risk of developing the disease. A study reported that a London Bangladeshi population (with higher risk of developing T2DM) have lower serum vitamin D levels compared with the British Caucasian population (with a lower risk of developing T2DM) suggesting that vitamin D status might contribute to the pathogenesis of the disease. Short-term vitamin D replenishment in the Bangladeshi Asian population increased insulin secretion without any glycemic alterations, but with longer vitamin D treatment the glycaemias were also improved. Hypovitaminosis D is also common in obese individual and obesity as already stated is a risk factor of T2DM. This hypovitaminosis D observed in obese individuals is due to the storage of vitamin D (liposoluble vitamin) in the adipose tissue where it is no longer bioavailable.In subjects with obesity, elevated PTH levels have also been reported. This hyperparathyroidism may contribute to the production of insulin intolerance since PTH inhibits the synthesis of vitamin D thus contributes to insufficient circulating 25(OH)D levels . Therefore treating the vitamin deficiency in obese individuals we may be able to improve glucose tolerance and decrease the chance of T2DM to develop (36Some other studies have shown that vitamin D combined with calcium supplementation is also known to decrease the risk of T2DM .[4] One

of these studies, the Nurses Health Study, reported that a daily intake of >800IU of vitamin D and >1,200mg of calcium was associated with a 33% lower risk of T2DM compared to a daily intake of <400IU of vitamin D and 600mg of calcium .[31]

Another study done in older individuals with impaired fasting glucose, shows that combined calcium and vitamin D supplementation attenuated the increase in glycaemia and insulin resistance that occur with aging. This study provides a preliminary support for the important role of vitamin D and calcium supplementation in lowering the risk of progression of diabetes in individuals with glucose intolerance .[32]

2.2. 5. 2 Vitamin D as a genetic risk factor

The VDR seem to be present in the pancreatic tissue and mutations in the VDR genes have been shown to contribute to the genetic preposition of T2DM. Studies in mice expressing functionally inactive mutant VDR showed a pronounced impairment in oral glucose tolerance and insulin secretory capacity, together with a reduction in pancreatic insulin mRNA levels in normally fed animals. Studies done on humans also reported that allelic variations in the VDR gene modulated β-cell function.

Other polymorphisms beside those on VDR genes have also been studied. These polymorphisms include those on DBP (transports 25(OH)D) and 1α-hidroxylase (converts 25(OH)D to its active metabolite 1,25(OH)2D) genes. Few studies have been done on the polimormisms on the 1α-hidroxylase gene. However, polimormisms on DBP genes have been suggested to affect the availability of active vitamin D forms in β-cells and insulin secretion. Nonetheless, this effect has only been found in certain ethnic groups. [33]

2 .2.5 . 3 Potential mechanisms of vitamin D on T2DM

For glucose intolerance and T2DM to develop, defects in pancreatic B-cells function, insulin sensitivity, and systematic inflammation are often present. Evidence has been found that vitamin D has a role in all these mechanisms. [4]

2 .2. 6 Effects of vitamin D on pancreatic β-cell function

In the recent years, is has been suggested that vitamin D is essential for normal insulin release in response to glucose. Vitamin D deficiency present in both humans and animal models is shown to alter insulin synthesis and secretion . [33]

There is evidence that vitamin D may stimulate pancreatic insulin secretion directlyand indirectly. The direct effect takes place when 1,25(OH)2D binds to the nuclear VDR; which is found in a variety of tissues, including the pancreatic islet β-cells. The VDR is considered to be the master regulator of transcription and is shown to activate the protein biosynthesis in pancreatic islets therefore increasing the insulin secretion. In order to understand the role of vitamin D in β-cell function, transgenic VDR knockout mice have been generated. In these mice the circulating insulin concentrations is reported to be lower and blood glucose concentrations higher. However, data from VDR-knockout mice are conflicting because the genetic backgrounds of these knockout mice appear to be crucial, because VDR-knockout mice with a different genetic background have shown to have normal β-cell function. [4]

The indirect effects of vitamin D may be mediated via regulating extracellular calcium and calcium flux through the β-cell. Insulin secretion is a calcium dependent process; therefore, alterations in calcium flux can have adverse effects on β-cell secretory function. Vitamin D insufficiency has been shown to alter thebalance between the extracellular and intracellular β-cell calcium pools, which may interfere with normal insulin release, mainly in response to glucose. [4]

2.2.7. Effects of vitamin D on insulin resistance

Vitamin D may have an effect on insulin sensitivity either directly, by stimulating the expression of insulin receptor and thereby enhancing insulin responsiveness for glucose transport, or indirectly via its role in regulating extracellular calcium and ensuring normal calcium influx through cell

membranes and adequate intracellular cytosolic calcium pool. Intracellular cytosolic calcium is essential for optimal insulin-mediated functions in insulin-responsive tissues such as skeletal muscle and adipose tissue. Changes in intracellular cytosolic calcium in primary insulin target tissues may contribute to peripheral insulin resistance. [4]

2.2.8 Effects of vitamin D on systemic inflammation

Inflammatory factors have often been associated with insulin resistance and β-cell failure, both of which are key features of T2DM. An increase in acute-phase proteins (C-reactive protein), cytokines (TNF-α, TNF-β, IL-6) and mediators associated with endothelial dysfunction has been reported in T2DM. [33]

Vitamin D may improve insulin sensitivity and promote β-cell survival by directly modulating the generation and effects of cytokines.,[4] Vitamin D is reported todownregulate the production of several cytokines: IL2, IL6, IL12, interferon-□, TNF- α, TNF-β. To date, however, it remains to be elucidated whether the systemic inflammation observed in T2DM might be influenced by the immune properties of vitamin D. [33]

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Chapter Three

Materials and Method

3. Material and Methods

3. 1 Study design

This is observational, analytical, case control study, conducted in Shendi Locality.

3 . 2 Study area and Study period

This study was conducted in Nile Rvier Stat (Shendi Teaching Hospital) from April to August 2018 .

3 . 3 Study populations Sample size

The study included 60 individuals in both gender,40 Diabetic patients at any age

(subject) and 20 healthy people (control) match in age.

3. 4 Study Criteria

3 . 4 . 1 Inclusion criteria

For case samples patients diabetic .

For control samples healthy non diabetic.

3.4 .2 Exclusion criteria

any diabetic patient having disease affected vitamin D level (eg: Renal disease – Liver disease)

*patients on vitamin D supplementation .

3. 5 Data collection

Data were collected by using a questionnaire (appendix I) filled by the investigator after blood samples collection including the forming (age, Gender, Duration of diabetes, exposure to sun light …) and observation of the laboratory results (vi t D , FBG, HbA1c).

3 .6 Data analysis

Data were analyzed using statistical package for social sciences (SPSS) program, version 21.

3.7 Sampling

Sixty sample, volume of 6 ml blood were collected from each patient through venipuncture technique than 2ml displaced into fluoride oxalate container for glucose estimation, and 2 ml in plain container for vit D measured by ichroma and 2 ml in EDTA container for HbA1c.

3.8 Material and instruments

Cotton, alcohol, Syringe, Container (fluoride oxalate, plain container, EDTA) Centrifuge, Tubes, Stop watch, Automatic Piped ,Spectrophotometer, I chroma. see appendix II .

3.9 Ethical consideration

Informed consent was attached to each questionnaire to be obtained from the patient verbally. There was full commitment precaution sample taken and privacy and confidentiality. Shendi college of medical laboratory science ethical committee.

3 . 10 Methodology

3.10.1 Sample processing

Each blood specimen was centrifuged at 3000g for 5 minutes to obtain the (plasma, serum) the later was gently collected into plain container and stored at – 20 c until the analysis.

3.10.2 Blood glucose measurement

Glucose estimation of samples was done using Reagent kit glucose hexokinase (COD 11656) from Bio system reagents & industries (C/ Costa Brava 30 08030 Barcelona (Spain)

3.10.2.1 Principle of the method

Glucose in the sample produces , by means of the coupled reactions described below , NADPH that can be measured by Spectrophotometry.

Glucose + ATP Glucose-6-phosphate+ADP

Glucose -6-phosphate +NADP +Glaciate-6-phospate+NADPH+H

See appendix no III.

3.10.3 vit D measurement

Ichroma™ vitamin D is a fluorescence immunoassay (FIA) for the quantitative determination of total 25(OH) D2\D3 level in human serum\plasma.

3.10.3.1 principle of the method

The test uses a competitive immunodetection method . In this method , the target material in the sample binds to the fluorescence (FL) –labeled detection antibody in detection buffer, to form the complex as sample mixture . This complex is loaded to migrate onto the nitrocellulose matrix , where the covalent couple of 25(OH) D3 and bovine serum albumin (BSA) is immobilized on a test strip, and interferes with the binding of target material and F L – labeled antibody if the more target material exists in blood , the less detection antibody is accumulated, resulting the less fluorescence See appendix no IV.

3.10.4 HbA1c measurement

Ichroma ™ HbA1c is a fluorescence immunoassay (FIA) for the quantitative determination of HbA1c (HemoglobinA1c) in human whol blood . it is useful as an aid in management and monitoring of the long-term glycemic status in patients with diabetes mellitus.

3.10.4.1principle of method

The test uses a sandwich immunodetection method; the detector antibody in buffer binds to antigen in sample forming antigen-antibody complexes and migrates on to nitrocellulose matrix to be captured by the other immobilized-antibody on test strip.

The more antigen in sample froms the more antigen-antibody complex and leads to stronger intensity of fluorescence signal on detector antibody . instrument for ichroma ™ tests displays the content of glycated hemohlobin in terms of percent of the total hemoglobin in blood . See appendix V.

Chapter Four

Results

4. Results

4.1Table (4-2): the Mean and standard deviation of (Vitamin D) levels among case and control group:

	NO	Mean (nmol/L)	P.value
Control	20	73.4 ± 22.9	**0.000**
Case	40	19.9 ± 7.9	

Vitamin D level decreased in Diabetic patients.

* The p.value is significant.

Independent Samples T. Test

4.2 Table (4-3): The Mean and standard deviation of (Vitamin D) levels according to age of case group:

Age groups	NO	Mean	P.value
Under 35 years	14	24.8 ± 10.3	**0.010**
(35 - 45) years	3	17.9 ± 4.9	
More than 45 years	23	17.2 ± 4.7	

STATISTICS: Means comparing, ANOVA.

Vitamin D level decrease with increasing age in diabetic patients.

* The p.value is significant

4.3 Table (4-4): the Mean and standard deviation of (Vitamin D) levels with

Take medication of diabetes:

Take medication	NO	Mean	P.value
Regular	28	19.6 ± 5.9	**0.600**
Irregular	12	20.7 ± 11.7	

STATISTICS: Means comparing, ANOVA.

* Vitamin D level No effect of regularity to take medication of diabetes patients p.value is Not significant.

4.4 Table (4-5): the Mean and standard deviation of (Vitamin D) levels with Hypertension in case group:

Hypertension	NO	Mean	P.value
Yes	10	15.9 ± 3.5	**0.060**
No	30	21.3 ± 5.8	

STATISTICS: Means comparing, ANOVA.

Vitamin D level NO effect in diabetic hypertension patients.

* The p.value is Not Significant.

4.5 Table (4-6): the Mean and standard deviation of (Vitamin D) levels with exposed sunlight:

Exposed sunlight	NO	Mean	P.value
Rarely	4	13.5 ± 4.2	**0.001**
Sometime	8	13.3 ± 3.3	
Long	28	22.8 ± 7.6	

STATISTICS: Means comparing, ANOVA.

Vitamin D level in diabetic patients exposed long time to sun light higher than patient low exposure.

* The p.value is Significant.

4.6 Table(4-7): the Mean and standard deviation of (Vitamin D) levels with Duration of diabetes:

Duration of diabetes	NO	Mean	P.value
8> Year	16	23.7 ± 10.0	**0.030**
(8 – 16) year	16	17.9 ± 5.0	
16< year	8	16.4 ± 4.7	

STATISTICS: Means comparing, ANOVA.

Vitamin D level decrease with increasing duration of diabetes.

* The p.value is Significant.

4.7 Table (4-8): the Mean and standard deviation of (Vitamin D) levels according to Gender:

Gender	NO	Mean	P.value
Male	19	16.753 ± 10.0	**0.010**
Female	21	22.819 ± 5.0	

STATISTICS: Means comparing, ANOVA.

Vitamin D level in female higher than male in diabetic patient.

* The p.value is Significant.

4.8 Table (4 - 9): the Mean and standard deviation of (Vitamin D) levels with $HbA1_C$:

$HbA1_C$	NO	Mean	P.value
7 – 10 %	19	19.7 ± 6.3	**0.870**
11 – 13%	21	20.1 ± 9.3	

STATISTICS: Means comparing, ANOVA.

Vitamin D level No effect with glycemic status in diabetic patient.

* The p.value is Not Significant.

Chapter Five

Discussion
Conclusion
Recommendations

5.1 Discussion

This study was conducted in Shendi locality during period from April to August 2018. The study was include (60) individual both sex (40) as case group study diabetic patient and (20) as control group (healthy) non diabetic, the age (11 - 70) of this study groups match together. The blood samples were obtained to measured vitamin D levels using Ichroma™.

The study shows that Vitamin D level decrease in diabetic patients when compared with non diabetic group. Normal range (sufficient vitamin D 75-250 nmol/L).

The mean vitamin D level in case and control groups was (73.4 ± 22.9 – 19.9 ±7.9) respectively which was statistically significant (p .value 0.000). This result was agree with study done in Bahrain by Dalal in 2017 who found :low vitamin D level (mean 38.5) in 101 of diabetic patients, and also agree with the result by Arindam, 2017 who found low vitamin D (mean 16.9) in 48 cases of diabetic patient.

The study showed vitamin D level in diabetic patients were exposure long time to sun light higher comparing with diabetic patient were exposued short time the mean (13.5 – 22.8) respectively which was statistically significant (p. value 0.001) .

In this study the relationship between vitamin D levels in diabetic patients and duration of diabetes vitamin D decrease with increasing duration of diabetes, was statistically significant, the duration was divided to groups which are (> 8 year), (8 – 16 year) and (< 16 year), and the mean with these groups of duration was (23.7 – 17.9 – 16.4) respectively , with (p. value 0.030).

In this study according to age, the mean ages of the study group (under 35 year, 35-45, more than 45year) were calculated (24.8–17.9 – 17.2)respectively, which have significant relationship, Vitamin D level decrease with increasing age, with p.value (0.010).

In this study according to gender, statistically significant difference found between level of Vitamin D and gender, Vitamin D in female higher than male, with mean (22.819 ±5.0 – 16.753±10.0) respectively, with P. value (0.010).

The study showed no statistically significant difference between Vitamin D level with glycemic status, hypertensive and regularity to take medication of diabetes.

5.2 Conclusion

It can be concluded that:

1. Vitamin D level decrease in diabetic patient than non diabetic control (healthy).
2. Vitamin D level in diabetic patient exposed long time to sun light higher than patient low exposure to sun light.
3. Vitamin D level decrease with increasing duration of diabetes in diabetic patient.
4. Vitamin D level in female higher than male in diabetic patient.
5. According age Vitamin D level decrease with increasing age in diabetic patient.
6. The study showed Vitamin D level no effect with glycemic status, hypertensive and regularity to take medication of diabetic in diabetic patient.

5.3 Recommendations

1. Perform intensive study in this topic in different study area with increase sample size.
2. Further studies using advanced techniques are important to validate these result.
3. Vitamin D must be taken as supplement of all diabetic patients.

Chapter Six

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Appendix

6.1 References

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Appendix

Appendix I

بسم الله الرحمن الرحيم

Shendi University

Faculty of Post- Graduate Studies

Estimation of Vitamin D level in Diabetic Patients

Questionnaire

Personal information:

Name: …….. No ( )

Age: ……

Gender: Male ( ) Female ( )

Exposure to sun light: long time ( ) some time ( ) Rare ( )

Education: Primary ( ) Secondary ( ) University ( )
Post Graduate ( )

Patient History:

Duration of disease: …….

Which type of DM: Type one ( ) Type two ( )

Do other members of your family have diabetes ? Yes ( ) No ( )

Are you taking your medications? Regular ( ) Irregular ( )

Other chronic disease:

- Asthma: Yes ( ) No ( )
- Hypertension: Yes ( ) No ( )
- Bone pain: Yes ( ) No ( )
- Muscle weakness: Yes ( ) No ( )
- Memory status: Good ( ) Bad ( )

Dietary Habits:

How many meals per day do you eat ?

Two meals ( ) three meals ( ) more than three meals ( )

Type of food:

Do you take ?

- Milk: every day ( ) weakly ( ) monthly ( )
- Meat: every day ( ) weakly ( ) monthly ( )
- Fish: every day ( ) weakly ( ) monthly ( )

Chemical analysis:

Vitamin D: …….

FBG: ……..

AIC: ……

Appendix II

Ichroma ™ Reader
Operation Manual

Principle

ichroma™ Reader is a fluorescence scanning instrument to be used in conjunction with various ichroma™ Immunoassay Tests which are based on antigen-antibody reaction and fluorescence technology.

ichroma™ Reader uses a semiconductor diode laser as the excitation light source for illuminating the test cartridge membrane (pre-loaded with the clinical specimen duly processed as per the standard test procedure prescribed by Boditech Med Inc.) thereby triggering fluorescence from the fluorochrome molecules present on the membrane.

The fluorescent light is collected together with the scattered laser light. Pure fluorescence is filtered from the mixture of the scattered and fluoresced light. Intensity of the fluorescence is scanned and converted into an electric signal which is proportional to the intensity of fluorescence produced on the test cartridge membrane.

The on-board microprocessor computes concentration of the analyte in the clinical specimen based on a pre-programmed calibration. The computed and converted result is displayed on the display screen of the ichroma™ Reader.

Package Contents

ichroma™ Reader consists of several items included in the system. For proper operation of the system, the user must know the technical name and use of each item.

The user must ensure that following items are part of the commercial package. If any item is missing, please contact your sales distributor or Boditech Med Inc.'s customer representative at +82 (33) 243-1400 in Korea.

1. ichroma™ Reader.
2. Cover.
3. Operation Manual.
4. Power Cable and Connection Cable.
5. Power Cable and Connection Cable Box.
6. System Check Chip Set.

Appendix III

COD 11803	COD 11503	COD 11504	COD 11538
1 x 50 mL	1 x 200 mL	1 x 500 mL	1 x 1 L
STORE AT 2-8°C			
Reagents for measurement of glucose concentration Only for *in vitro* use in the clinical laboratory			

GLUCOSE

BioSystems REAGENTS & INSTRUMENTS

CE

GLUCOSE
GLUCOSE OXIDASE/PEROXIDASE

PRINCIPLE OF THE METHOD

Glucose in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry[1].

$$\text{Glucose} + \tfrac{1}{2}\,O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2\,H_2O_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

CONTENTS

	COD 11803	COD 11503	COD 11504	COD 11538
A. Reagent	1 x 50 mL	1 x 200 mL	1 x 500 mL	1 x 1 L
S. Standard	1 x 5 mL	1 x 5 mL	1 x 5 mL	1 x 5 mL

COMPOSITION

A. Reagent: Phosphate 100 mmol/L, phenol 5 mmol/L, glucose oxidase > 10 U/mL, peroxidase > 1 U/mL, 4-aminoantipyrine 0.4 mmol/L, pH 7.5

S. Glucose/Urea/Creatinine Standard. Glucose 100 mg/dL (5.55 mmol/L), urea 50 mg/dL, creatinine 2 mg/dL. Aqueous primary standard.

STORAGE

Store at 2-8°C.

Reagent and Standard are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

- Reagent: Presence of particulate material, turbidity, absorbance of the blank over 0.150 at 500 nm (1 cm cuvette).
- Standard: Presence of particulate material, turbidity.

REAGENT PREPARATION

Reagent and Standard are provided ready to use.

ADDITIONAL EQUIPMENT

- Thermostatic water bath at 37°C
- Analyzer, spectrophotometer or photometer able to read at 500 ± 20 nm

SAMPLES

Serum or plasma collected by standard procedures. Serum or plasma must be separated from the red cells promptly to prevent glycolysis. The addition of sodium fluoride to the blood sample prevent glycolysis.

Glucose in serum or plasma is stable for 5 days at 2-8°C. Heparin, EDTA, oxalate and fluoride may be used as anticoagulants.

Cerebrospinal fluid collected by standard procedures. Cerebrospinal fluid may be contaminated with bacteria or other cells and should therefore be analyzed for glucose immediately.

PROCEDURE

1. Bring the Reagent to room temperature.
2. Pipette into labelled test tubes: (Note 1)

	Blank	Standard	Sample
Glucose Standard (S)	—	10 µL	—
Sample	—	—	10 µL
Reagent (A)	1.0 mL	1.0 mL	1.0 mL

3. Mix thoroughly and incubate the tubes for 10 minutes at room temperature (15-25°C) or for 5 minutes at 37°C.
4. Measure the absorbance (A) of the Standard and the Sample at 500 nm against the Blank. The colour is stable for at least 2 hours.

CALCULATIONS

The glucose concentration in the sample is calculated using the following general formula:

$$\frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times C_{\text{Standard}} - C_{\text{Sample}}$$

If the Glucose Standard provided has been used to calibrate (Note 2):

$\frac{A_{\text{Sample}}}{A_{\text{Standard}}}$	x 100 = mg/dL glucose x 5.55 = mmol/L glucose

REFERENCE VALUES

Serum and plasma[2]:

Newborn, premature	25-80 mg/dL = 1.39-4.44 mmol/L
Newborn, term	30-90 mg/dL = 1.67-5.00 mmol/L
Children, adult	70-105 mg/dL = 3.89-5.83 mmol/L

Cerebrospinal fluid[2]:

Children	60-80 mg/dL = 3.33-4.44 mmol/L
Adult	40-70 mg/dL = 2.22-3.89 mmol/L

These ranges are given for orientation only; each laboratory should establish its own reference ranges.

According to the National Diabetes Data Group (US)[3], elevation of fasting plasma glucose values over 140 mg/dL (7.77 mmol/L) on more than one occasion is diagnostic of diabetes mellitus.

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, 18009 and 18042) and II (cod. 18007, 18010 and 18043) to verify the performance of the measurement procedure.

Each laboratory should establish its own internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

- Detection limit: 0.23 mg/dL = 0.0126 mmol/L
- Linearity limit: 500 mg/dL = 27.5 mmol/L. For higher values dilute sample 1/4 with distilled water and repeat measurement.
- Repeatibility (within run):

Mean Concentration	CV	n
88 mg/dL = 4.84 mmol/L	1.2 %	20
326 mg/dL = 17.93 mmol/L	0.9 %	20

- Reproducibility (run to run):

Mean Concentration	CV	n
88 mg/dL = 4.84 mmol/L	2.7 %	25
326 mg/dL = 17.93 mmol/L	1.9 %	25

- Sensitivity: 4 mA dL/mg = 0.22 mA L/mmol
- Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 2). Details of the comparison experiments are available on request.
- Interferences: Hemoglobin (> 3 g/L), lipemia (triglycerides > 1.25 g/L) and bilirubin (10 mg/dL) may interfere. Other drugs and substances may interfere[4].

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

Glucose is the major source of energy in the body. Insulin, produced by islet cells in the pancreas, facilitates glucose entry into the tissue cells. A deficiency of insulin or a decrease of its effectiveness increases blood glucose.

Elevated serum or plasma glucose concentration is found in diabetes mellitus (insulin dependent, non-insulin dependent) and in other conditions and syndromes[2,3].

Hypoglycemia can occur in response to fasting, or it may be due to drugs, poisons, inborn errors of metabolism or previous gastrectomy[2,5].

Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. These reagents may be used in several automatic analysers. Specific instructions for application in many of them are available on request.
2. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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BioSystems S.A. Costa Brava 30, 08030 Barcelona (Spain)
Quality System certified according to EN ISO 13485 and EN ISO 9001 standards

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Appendix IV

Document No. : INS-VD-EN (Rev. 06)
Revision date : January 6, 2017

ichroma™ Vitamin D

bodiTech

INTENDED USE

ichroma™ Vitamin D is a fluorescence immunoassay (FIA) for the quantitative determination of total 25(OH)D2/D3 level in human serum/plasma. It is useful as an aid in management and monitoring of regulating the concentration of calcium and phosphate in the bloodstream and promoting the healthy growth and remodeling of bone.

For *in vitro* diagnostic use only.

INTRODUCTION

Vitamin D from the diet or dermal synthesis from sunlight is biologically inactive and is a fat-soluble steroid hormone involved in the active intestinal absorption of calcium and in the regulation of its homeostasis. In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D2 (ergocalciferol).[1] In the liver, cholecalciferol (vitamin D3) is converted to calcidiol, 25-hydroxycholecalciferol (abbreviated 25(OH)D3); Ergocalciferol (vitamin D2) is converted in the liver to 25-hydroxyergocalciferol (25(OH)D2). It is widely known that circulating 25(OH)D is the best indicator of vitamin D status.[2,3] 25(OH)D3 is then converted in the kidneys (by the enzyme 25(OH)D-1α-hydroxylase) into 1,25-(OH)$_2$D3, a steroid hormone that is the active form of vitamin D. It can also be converted to 24,25-(OH)$_2$D3 by 24-hydroxylation.[4,5] 1,25-(OH)$_2$D3 circulates as a hormone in the blood, regulating the concentration of calcium and phosphate in the bloodstream and promoting the healthy growth and remodeling of bone. 1,25-(OH)$_2$D3 also affects neuromuscular and immune function.[6] Vitamin D has a significant role in calcium homeostasis and metabolism. Its discovery was due to effort to find the dietary substance lacking in rickets (the childhood form of osteomalacia).[7]

This test can be used to diagnose vitamin D deficiency and it is indicated in patients with high risk for vitamin D deficiency and when the results of the test would be used as supporting evidence for beginning aggressive therapies.[8] Patients with osteoporosis, chronic kidney disease, malabsorption, obesity, and some other infections may be high risk and thus have greater indication for this test.[9,10]

PRINCIPLE

The test uses a competitive immunodetection method: in this method, the target material in the sample binds to the fluorescence (FL)-labeled detection antibody in detection buffer, to form the complex as sample mixture. This complex is loaded to migrate onto the nitrocellulose matrix where the complex of 25(OH)D3 and bovine serum albumin (BSA) is immobilized on a test strip, and interferes with the binding of target material and FL-labeled antibody. If the more target material exists in blood, the less detection antibody is accumulated, resulting in the less fluorescence signal.

COMPONENTS

ichroma™ Vitamin D consists of 'Cartridges', 'Detection Buffer Vial', 'Releasing Buffer Vial', 'Sample Mixing Tubes' and an 'ID chip'.

- The cartridge contains a test strip, the membrane which has 25(OH)D3-BSA conjugate at the test line, while rabbit IgG at the control line.
- Each cartridge is individually sealed in an aluminum foil pouch containing a desiccant. 25 sealed cartridges are packed in a box which also contains an ID chip.
- The 'detection buffer' contains NaOH and DMSO.
- The 'detection buffer vial' contains anti-25(OH)D2/3 fluorescence conjugate, anti-rabbit IgG-fluorescence conjugate, gelatin as a stabilizer and sodium azide in Tris-HCl buffer as a preservative.
- The releasing buffer and detection buffer are dispensed in a vial. Releasing buffer vial and detection buffer vial are packed in a Styrofoam box with ice-pack for the shipment.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- Carefully follow the instructions and procedures described in this 'Instructions for use'.
- Use only fresh samples and avoid direct sunlight.
- Lot numbers of all the test components (cartridge, ID chip, releasing buffer and detection buffer) must match each other. Do not interchange the test components between different lots or use the test components after the expiration date, either of which might yield misleading of test result(s).
- Do not reuse. A sample mixing tube should be used for processing one sample only. So should a cartridge.
- The cartridge should remain sealed in its original pouch before use. Do not use the cartridge, if is damaged or already opened.
- Frozen sample should be thawed only once. For shipping, samples must be packed in accordance with the regulations.
- Sample with severe hemolytic and hyperlipidemia cannot be used and should be recollected.
- Just before use, allow the cartridge, detection buffer, releasing buffer and sample to be at room temperature for approximately 30 minutes.
- ichroma™ Vitamin D as well as the instrument for ichroma™ tests should be used away from vibration and/or magnetic field. During normal usage, it can be noted that instrument for ichroma™ tests may produce minor vibration.
- Used detection buffer vial, releasing buffer vial, pipette tips and cartridges should be handled carefully and discarded by an appropriate method in accordance with relevant local regulations.
- As releasing buffer is basic and contain organic solvent, please avoid contact with eyes, skin or clothing.
- An exposure to larger quantities of sodium azide may cause certain health issues like convulsions, low blood pressure and heart rate, loss of consciousness, lung injury and respiratory failure.
- ichroma™ Vitamin D will provide accurate and reliable results subject to the following conditions.
  - Use ichroma™ Vitamin D should be used only in conjunction with instrument for ichroma™ tests.
  - Any anticoagulants other than EDTA, heparin, sodium citrate should be avoided.

STORAGE AND STABILITY

- The cartridge is stable for 20 months (while sealed in an aluminum foil pouch) if stored at 4-30 °C.
- The detection buffer dispensed in a vial is stable for 20 month if stored at 2-8 °C.
- The releasing buffer dispensed in a vial is stable for 20 months if stored at 2-8 °C.
- Opened detection buffer and releasing buffer are stable for 12 months at 2-8 °C if kept capped in original container and free from contaminations.
- After the cartridge pouch is opened, the test should be performed immediately.

LIMITATION OF THE TEST SYSTEM

- The test may yield false positive result(s) due to the cross-reactions and/or non-specific adhesion of certain sample components to the capture/detector antibodies.
- The test may yield false negative result. The non-responsiveness of the antigen to the antibodies is most common where the epitope is masked by some unknown components, so as not to be detected or captured by the antibodies. The instability or degradation of the antigen with time and/or temperature may cause the false negative as it makes antigen unrecognizable by the antibodies.
- Other factors may interfere with the test and cause erroneous results, such as technical/procedural errors, degradation of the

test components/reagents or presence of interfering substances in the test samples.
- Any clinical diagnosis based on the test result must be supported by a comprehensive judgment of the concerned physician including clinical symptoms and other relevant test results.

MATERIALS SUPPLIED

REF CFPC-47

Components of ichroma™ Vitamin D
- Cartridge Box:
  - Cartridges 25
  - Sample Mixing Tubes 25
  - ID Chip 1
  - Instruction For Use 1
- Detection Buffer Vial (3 mL) 1
- Releasing Buffer Vial (2 mL) 1

MATERIALS REQUIRED BUT SUPPLIED ON DEMAND

Following items can be purchased separately from ichroma™ Vitamin D. Please contact our sales division for more information.
- Instrument for ichroma™ tests
  - ichroma™ Reader REF FR203
  - ichroma™ II REF FPRR021
- ichroma™ Printer REF FPRR007
- i-chamber REF FPRR009
- ichroma™ Vitamin D Control REF CFPO-79
- Inserting tube block (It could be displaced with heating block.)

SAMPLE COLLECTION AND PROCESSING

The sample type for ichroma™ Vitamin D is human serum/plasma.
- It is recommended to test the sample within 24 hours after collection.
- Samples may be stored for up to a week at 2-8 °C prior to being tested. If testing will be delayed more than a week, samples should be frozen at -20 °C.
- Samples stored frozen at -20 °C for 6 months showed no performance difference.
- Once the sample was frozen, it should be used one time only for test, because repeated freezing and thawing can result in the change of test values.

TEST SET UP

- Check the contents of ichroma™ Vitamin D: Sealed Cartridge, Detection Buffer Vial, Releasing Buffer Vial, Sample Mixing Tubes and ID Chip.
- Ensure that the lot number of the cartridge matches that of the ID chip as well as the detection buffer & releasing buffer.
- Keep the sealed cartridge (if stored in refrigerator), Detection buffer vial and Releasing buffer vial at room temperature for at least 30 minutes just prior to the test. Place the cartridge on a clean, dust-free and flat surface.
- Turn on the instrument for ichroma™ tests.
- Insert the ID Chip into the ID chip port of the instrument for ichroma™ tests.
- Press the 'Select' button on the instrument for ichroma™ tests.
- Turn on the i-Chamber and set the temperature at 35 °C.
- Insert 'inserting tube block' into the i-Chamber slot at least 10 min before the test.
  (Please refer to the 'instrument for ichroma™ tests Operation Manual' for complete information and operating instructions.)

TEST PROCEDURE

1) Put the test cartridge into the i-Chamber slot.
2) Transfer 50 µL of releasing buffer using a transfer pipette to a sample mixing tube.
3) Add 50 µL sample (Human serum/plasma/control) using a transfer pipette to the sample mixing tube containing releasing buffer and mix well by pipetting 10 times.
4) Insert the sample mixing tube into the inserting tube block and leave the tube in the inserting tube block at 35 °C for 5 min.
5) Add 100 µL of detection buffer using a transfer pipette with new tip to the sample mixing tube containing the releasing buffer and sample mixture.
6) Mix well by pipetting 10 times and leave it in the inserting tube block again at 35 °C for 15 min.
7) Take out the half of test cartridge from the i-Chamber, pipette out 75 µL of incubated mixture and load it into the sample well on the test cartridge. Then push the test cartridge into the i-Chamber slot fully.
8) Leave the sample-loaded test cartridge in i-Chamber for 8 minutes.
   ⚠ *Scan the sample-loaded cartridge immediately when the incubation time is over. If not, it will cause inexact test result.*
9) To scan the sample-loaded cartridge, insert it into the cartridge holder of the instrument for ichroma™ tests. Ensure proper orientation of the cartridge before pushing it all the way inside the cartridge holder. An arrow has been marked on the cartridge especially for this purpose.
10) Press 'Select' button on the instrument for ichroma™ tests to start the scanning process.
11) Instrument for ichroma™ tests will start scanning the sample-loaded cartridge immediately.
12) Read the test result on the display screen of the instrument for ichroma™ tests.

INTERPRETATION OF TEST RESULT

- Instrument for ichroma™ tests calculates the test result automatically and displays total 25(OH)D2/D3 concentration of the test sample in terms of ng/mL.
- The cut-off (reference range)

25(OH)D		Status
<10 ng/mL	<25 nmol/L	Deficiency
10-30 ng/mL	25-75 nmol/L	Insufficiency
30-100 ng/mL	75-250 nmol/L	Sufficiency

- Working range: 8.0-70 ng/mL
- Conversion factor: 2.5 x ng/mL = nmol/L

QUALITY CONTROL

- Quality control tests are a part of the good testing practice to confirm the expected results and validity of the assay and should be performed at regular intervals.
- The control tests should be performed immediately after opening a new test lot to ensure the test performance is not altered.
- Quality control tests should also be performed whenever there is any question concerning the validity of the test results.
- Control materials are provided with ichroma™ Vitamin D. ichroma™ Vitamin D Control can be used as a calibration as well as quality control test. For more information regarding obtaining the control materials, contact Boditech Med Inc.'s Sales Division for assistance.
  (Please refer to the instruction for use of control material.)

PERFORMANCE CHARACTERISTICS

- **Analytical sensitivity**
  - Limit of Blank (LoB) 6.50 ng/mL (16.25 nmol/L)
  - Limit of Detection (LoD) 7.40 ng/mL (18.50 nmol/L)
  - Limit of Quantification (LoQ) 7.99 ng/mL (19.98 nmol/L)

- **Analytical specificity**
  - Cross-reactivity
    There was no significant cross-reactivity from these materials with the ichroma™ Vitamin D test measurements.

Cross-reactivity material	Standard material conc. (ng/mL)		
	9.59	23.76	64.78
	Bias (%)		
Vitamin D2 (300 ng/ml)	9.37	8.32	2.97
Vitamin D3 (300 ng/ml)	5.98	6.65	-2.30

  - Interference
    There was no significant interference from these materials with the ichroma™ Vitamin D test measurements.

Interference material	Standard material conc. (ng/mL)		
	9.59	23.76	64.78
	Bias (%)		
EDTA (2 mg/ml)	0.17	5.44	-2.51
Heparin (200 U/ml)	-1.40	-0.93	-6.03
Sodium citrate (38 mg/ml)	-1.87	6.77	-3.61
Urea (2.6 mg/ml)	7.68	-3.74	-3.64
Ascorbic acid (300 µg/ml)	-2.25	4.14	1.09

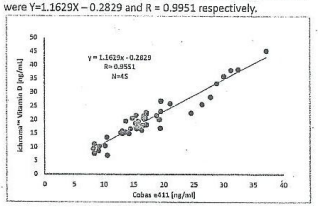
- **Precision**
  - Between lot/person/day/site
    The precision was confirmed by 3 different evaluators with 3 different lots, during 5 days, testing five times each different concentrations.

conc. (ng/mL)	Between-lot		Between-person		Between-day		Between-site	
	AVG	CV (%)	AVG	CV (%)	AVG	CV (%)	AVG	CV (%)
9.59	9.63	9.12	9.37	8.80	9.40	11.26	9.53	10.03
23.76	23.08	5.87	23.03	5.87	24.26	6.11	23.02	6.68
64.78	64.29	5.30	64.21	4.65	64.58	4.25	64.64	3.56

- **Accuracy**
  The accuracy was confirmed by 3 different lots testing ten times each different concentrations.

conc. (ng/ml)	Lot1	Lot2	Lot3	AVG	SD	CV (%)	Recovery (%)
9.59	9.56	9.74	9.61	9.63	0.88	9.12	100.46
23.76	22.71	22.84	23.68	23.08	1.35	5.87	97.13
64.78	63.21	65.05	64.62	64.29	3.41	5.30	99.25

- **Comparability:** 25(OH)D concentrations of 45 serum samples were quantified independently with ichroma™ Vitamin D and Roche Cobas e411 as per prescribed test procedures. Test results were compared and their comparability was investigated with linear regression and coefficient of correlation (R). Linear regression and coefficient of correlation between the two tests were Y=1.1629X − 0.2829 and R = 0.9951 respectively.



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Note: Please refer to the table below to identify various symbols

	Sufficient for <n> tests
	Read instruction for use
	Use by Date
LOT	Batch code
REF	Catalog number
	Caution
	Manufacturer
EC REP	Authorized representative of the European Community
IVD	In vitro diagnostic medical device
	Temperature limit
	Do not reuse
CE	This product fulfills the requirements of the Directive 98/79/EC on in vitro diagnostic medical devices

For technical assistance; please contact:
Boditech Med Inc.'s Technical Services
Tel: +82 33 243-1400
E-mail: sales@boditech.co.kr

Boditech Med Incorporated
43, Geodudanji 1-gil, Dongnae-myeon,
Chuncheon-si, Gang-won-do, 24398
Republic of Korea
Tel: +(82) -33-243-1400
Fax: +(82) -33-243-9373
www.boditech.co.kr

EC REP Obelis s.a
Bd. Général Wahis 53,
1030 Brussels, BELGIUM
Tel: +(32) -2-732-59-54
Fax: +(32) -2-732-60-03
E-Mail: mail@obelis.net

CE

Appendix V

Document No. : INS-AA-EN (Rev. 14)
Revision date : February 8, 2017

Diabetes

ichroma™

HbA1c

INTENDED USE

ichroma™ HbA1c is a fluorescence Immunoassay (FIA) for the quantitative determination of HbA1c(Hemoglobin A1c) in human whole blood. It is useful as an aid in management and monitoring of the long-term glycemic status in patients with diabetes mellitus.
For *in vitro* diagnostic use only.

INTRODUCTION

Glycated protein is formed post-translationally through the slow, nonenzymatic reaction between glucose and amino groups on proteins. HbA1c is a clinically useful index of mean glycemia during the preceding 120 days, the average life span of erythrocytes. Carefully controlled studies have documented a close relationship between the concentrations of HbA1c and mean glycemia. HbA1c is considered as a more reliable parameter in monitoring glycemia over the glycemic reading with the conventional glucometer.

PRINCIPLE

The test uses a sandwich immunodetection method; the detector antibody in buffer binds to antigen in sample, forming antigen-antibody complexes, and migrates onto nitrocellulose matrix to be captured by the other immobilized-antibody on test strip.
The more antigen in sample forms the more antigen-antibody complex and leads to stronger intensity of fluorescence signal on detector antibody. Instrument for ichroma™ tests displays the content of glycated hemoglobin in terms of percent of the total hemoglobin in blood.

COMPONENTS

ichroma™ HbA1c consists of 'Cartridges', 'Detection Buffer Tubes', 'Hemolysis Buffer Vial' and an 'ID chip'.

- The cartridge contains a test strip, the membrane which has anti human HbA1c at the test line, while rabbit IgG at the control line.
- Each cartridge is individually sealed in an aluminum foil pouch containing a desiccant. 25 sealed cartridges are packed in a box which also contains an ID chip.
- The detection buffer contains anti human HbA1c-fluorescence conjugate, anti rabbit IgG-fluorescence conjugate, bovine serum albumin (BSA) as a stabilizer and sodium azide in phosphate buffered saline (PBS) as a preservative.
- The detection buffer is pre-dispensed in a separate tube.
- The hemolysis Buffer contains nonionic detergent and sodium azide as preservative in PBS.
- 25 detection buffer tubes and hemolysis buffer vial are packaged in a box and further packed in a Styrofoam box with ice-pack for the shipment.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- Carefully follow the instructions and procedures described in this 'Instruction for use'.
- It is recommended to use fresh samples.
- It is possible to use frozen samples. Please refer to "SAMPLE COLLECTION AND PROCESSING".
- Do not expose ichroma™ HbA1c test kit to direct sunlight.
- Lot numbers of all the test components (cartridge, ID chip, detection buffer and hemolysis buffer) must match each other.
- Do not interchange the test components between different lots or use the test components after the expiration date, either of which might yield misleading of test result(s).
- Do not reuse. A detection buffer tube should be used for processing one sample only. So should a cartridge.
- The cartridge should remain sealed in its original pouch before use. Do not use the cartridge, if is damaged or already opened.
- Frozen sample should be thawed only once. For shipping, samples must be packed in accordance with the regulations. HbA1c sample with severe hemolytic and hyperlipidemia cannot be used and should be recollected.
- Just before use, allow the cartridge, detection buffer and sample to be at room temperature about 30 minutes.
- **ichroma™ HbA1c** as well as the instrument for ichroma™ tests should be used away from vibration and/or magnetic field. During normal usage, it can be noted that instrument for ichroma™ tests may produce minor vibration.
- Used detection buffer tubes, pipette tips and cartridges should be handled carefully and discarded by an appropriate method in accordance with relevant local regulations.
- The mixture of Detection Buffer and Hemolysis buffer must be used within 1 hour after mixing.
- An exposure to larger quantities of sodium azide may cause certain health issues like convulsions, low blood pressure and heart rate, loss of consciousness, lung injury and respiratory failure.
- **ichroma™ HbA1c** will provide accurate and reliable results subject to the following conditions.
  - **ichroma™ HbA1c** should be used only in conjunction with instrument for ichroma™ tests.
  - Any anticoagulants other than **EDTA, sodium heparin, sodium citrate** should be avoided.

STORAGE AND STABILITY

- The cartridge is stable for 20 months (while sealed in an aluminum foil pouch) if stored at 4-30 °C.
- The detection buffer pre-dispensed in a tube is stable for 20 months if stored at 2-8 °C.
- The hemolysis buffer dispensed in a vial is stable for 20 months if stored at 4-30 °C.
- After the cartridge pouch is opened, the test should be performed immediately.

LIMITATIONS OF THE TEST SYSTEM

- The test may yield false positive result(s) due to the cross-reactions and/or non-specific adhesion of certain sample components to the capture/detector antibodies.
- The test may yield false negative result. The non-responsiveness of the antigen to the antibodies is most common where the epitope is masked by some unknown components, so as not to be detected or captured by the antibodies. The instability or degradation of the antigen with time and/or temperature may cause the false negative as it makes antigen unrecognizable by the antibodies.
- Other factors may interfere with the test and cause erroneous results, such as technical/procedural errors, degradation of the test components/reagents or presence of interfering substances in the test samples.
- Any clinical diagnosis based on the test result must be supported by a comprehensive judgment of the concerned physician including clinical symptoms and other relevant test results.
- The test environment conditions for **ichroma™ HbA1c** are as follow.
  - Temperature: 20-30 °C
  - Humidity: 10-70 %
  - i-chamber target temperature: 30 °C

MATERIALS SUPPLIED

REF CFPC-38

Components of **ichroma™ HbA1c**

▪ Cartridge Box:	
- Cartridges	25
- ID Chip	1
- Instruction For Use	1
▪ Detection Buffer Box	
- Detection Buffer Tubes	25
- Hemolysis Buffer Vial (3 mL)	1

MATERIALS REQUIRED BUT SUPPLIED ON DEMAND

Following items can be purchased separately from **ichroma™ HbA1c**. Please contact our sales division for more information.

▪ Instrument for ichroma™ tests	
- **ichroma™ Reader**	REF FR203
- **ichroma™ II**	REF FPRR021
- **ichroma™ D**	REF 13303
▪ **i-Chamber**	REF FPRR009

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- **ichroma™ Printer** REF FPRR007
- **Boditech HbA1c Control** REF CFPO-96
- **Boditech HbA1c Calibrator** REF CFPO-108
- **5 µL Capillary tube** REF CFPO-19

SAMPLE COLLECTION AND PROCESSING

The sample type for **ichroma™ HbA1c** is human whole blood.

- It is recommended to test the sample within 12 hours after collection.
- Samples may be stored for up to a week at 2-8 °C prior to being tested.
- If testing will be delayed more than a week, samples should be frozen at -70 °C or below. Samples stored frozen at -70 °C or below for 3 months showed no performance difference.
- Once the sample was frozen, it should be used one time only for test, because repeated freezing and thawing can result in erroneous results.

TEST SETUP

- Check the components of the **ichroma™ HbA1c** as described below. : Cartridge, ID chip, instruction for use, detection buffer tube and hemolysis buffer vial.
- Ensure that the lot number of the test cartridge matches that of ID chip, detection buffer as well as hemolysis buffer.
- Keep the sealed cartridge (if stored in refrigerator), detection buffer and hemolysis buffer at room temperature for at least 30 minutes just prior to the test. Place the cartridge on a clean, dust-free and flat surface.
- Turn on the instrument for ichroma™ test.
- Insert the ID chip into the "ID chip port".
- Press the 'Select' button on the instrument for ichroma™ test. (Please refer to the 'Instrument for ichroma™ tests Operation manual' for complete information and operating instructions.)
- Insert a cartridge into i-Chamber slot. Temperature of i-chamber should be 30 °C.

TEST PROCEDURE

1) Draw 100 µL of hemolysis buffer and transfer it into detection buffer tube.
2) Draw 5 µL of fingertip blood or tube blood using 5 µL capillary tube and put the capillary tube into the detection buffer tube.
3) Close the lid of the detection buffer tube and mix the sample thoroughly by shaking it about 15 times.
4) Take out the cartridge half from i-Chamber slot.
5) Pipette out 75 µL of the sample mixture and load it into a sample well in the test cartridge.
6) Wait till the sample mixture flow appears in the windows. (about 10 seconds)
7) Insert the cartridge into i-Chamber slot (30 °C).
8) Leave the cartridge in i-Chamber for 12 minutes before removing.
   ⚠ *Scan the sample-loaded cartridge immediately when the incubation time is over. If not, it will cause inexact test result.*
9) To scan the sample-loaded cartridge, insert it into the cartridge holder of the instrument for ichroma™ tests. Ensure proper orientation of the cartridge before pushing it all the way inside the cartridge holder. An arrow has been marked on the cartridge especially for this purpose.
10) Press 'Select' button on the instrument for ichroma™ tests to start the scanning process.
11) Instrument for ichroma™ tests will start scanning the sample-loaded cartridge immediately.
12) Read the test result on the display screen of the instrument for ichroma™ tests.

INTERPRETATION OF TEST RESULT

- Instrument for ichroma™ tests calculates the test result automatically and displays HbA1c concentration of the test sample in terms of % (NGSP), mmol/mol (IFCC), mg/dL (eAG).
- The cut-off (reference range)
  - NGSP (%): 4.5-6.5 %
  - IFCC (mmol/mol): 26-48 mmol/mol
- Working range
  - NGSP (%): 4-15 %
  - IFCC (mmol/mol): 20.2-140.4 mmol/mol
  - eAG (mg/dL): 68.1-383.8 mg/dL

QUALITY CONTROL

- Quality control tests are a part of the good testing practice to confirm the expected results and validity of the assay and should be performed at regular intervals.
- The control tests should be performed immediately after opening a new test lot to ensure the test performance is not altered.
- Quality control tests should also be performed whenever there is any question concerning the validity of the test results.
- Control materials are not provided with **ichroma™ HbA1c**. For more information regarding obtaining the control materials, contact Boditech Med Inc.'s Sales Division for assistance. (Please refer to the instruction for use of control material.)

PERFORMANCE CHARACTERISTICS

- **Analytical Specificity**
  - Cross-reactivity

There was no significant cross-reactivity from these materials with the **ichroma™ HbA1c** test measurements.

Cross-reactivity material	Standard material conc.		
	5.2 %	6.5 %	10.5 %
	Recovery (%)		
HbA0 (20 mg/mL)	99.9	96.1	99.0
HbA1a,A1b (20 mg/mL)	100.9	96.8	101.0
Acetylated hemoglobin (100 mg/mL)	101.0	98.4	99.7
Carbamylated hemoglobin (100 mg/mL)	100.5	97.8	100.0
Glycated h-Albumin (100 mg/mL)	100.3	97.4	100.6
HbA1d (100 mg/mL)	100.9	97.0	100.3
Acetylaldehyde hemoglobin (100 mg/mL)	100.8	95.6	99.1

- Interference

There was no significant interference from these materials with the **ichroma™ HbA1c** test measurements.

Interference material	Standard material conc.		
	5.2 %	6.5 %	10.5 %
	Recovery (%)		
Non-interference	101.0	96.2	98.7
Acetaminophen (20 mg/dL)	100.4	97.8	100.9
L-ascorbic acid (500 mg/dL)	101.0	97.8	99.8
Bilirubin (2 g/dL)	100.8	97.8	100.4
D-glucose (1,000 mg/dL)	100.9	97.6	99.8
Intralipid (800 U/L)	100.8	96.2	100.6
Triglyceride (327 M)	100.9	96.1	99.6
Urea (10 g/dL)	100.1	98.1	99.7

- **Precision**

The intra-assay precision was calculated by one evaluator, who tested different concentration of control standard five times each with three different lots of **ichroma™ HbA1c**.

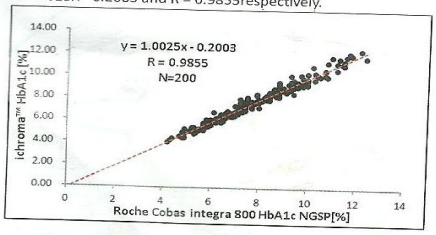
HbA1c (%)	Lot 1	Lot 2	Lot 3	AVG	SD	CV (%)	Accuracy (%)
5.2	5.28	5.18	5.24	5.23	0.12	2.36	100.6
6.5	6.46	6.48	6.34	6.43	0.13	1.99	98.9
10.5	10.4	10.56	10.58	10.51	0.19	1.83	100.1

The inter-assay precision was confirmed by 3 different evaluators with 3 different lots, testing five times each different concentration.

HbA1c (%)	Between-person			Between-lot		
	AVG	SD	CV (%)	AVG	SD	CV (%)
5.2	5.19	0.03	0.61	5.23	0.05	0.96
6.5	6.51	0.02	0.36	6.43	0.07	1.12
10.5	10.50	0.01	0.10	10.51	0.10	0.92

- **Comparability:**

HbA1c concentrations of 200 clinical samples were quantified independently with **ichroma™ HbA1c** and Roche Cobas integra800 as per prescribed test procedures. Test results were compared and their comparability was investigated with linear regression and coefficient of correlation (R). Linear regression and coefficient of correlation between the two tests were Y=1.0025X - 0.2003 and R = 0.9855respectively.



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