Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Anbar /College of Science
Department Of Chemistry

Association of Vitamin D_3 Receptor Gene and Biochemical Parameters in Iraqi Gestational Diabetic Patients

A Dissertation Submitted to

The Council of the College of Science /University of Anbar in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Chemistry

BY

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2019 A.D 1440 A.H

Dedication

- To the utmost knowledge lighthouse , to our greatest and most honoured…. Prophet Mohammed – may peace and grace from Allah be upon him .

- To the spring that never stops giving , who weaves my happiness with strings from her merciful heart …. My beloved mother .

- To whom he strives to bless comfort and welfare and never stints what he owns to push me in the success way who taught me to promote life stairs wisely and patiently ….. My dearest father .

- To whose love flows in my veins , and my heart , always remembers them …. My brothers and my sisters .

- To who leads me through the valley of darkness with light of hope and support ….My husband .

- To whom I cannot force myself to stop loving …. My beloved kids .

RAYA

Acknowledgment

In the name of Allah , the most merciful , the most compassionate all praise be to Allah , the lord of the world ; and prayers and peace be upon Mohammed his servant and messenger .

First and foremost , I must acknowledge my limitless thanks to Allah, for his favor upon me in accomplishing this thesis . I am totally sure that this work would have never become truth without his guidance .

I am grateful to some people , who worked hard with me from the beginning till the completion of the present research particularly my supervisors Assistant Prof . Dr . Hameed Hussein Ali and Prof . Dr . Wajeeh Younis Al– Ani , who introduced me to the extensive field of research on vitamin D .Thank you for having faith in me , Your combination of enthusiasm and go – ahead spirit together with a scientific mind and long – term experience have helped me through the obstacles on the road , and your door have always been open for immediate response to my questions and concerns .

I wish to express my deepest thanks to Dr . Omar Mohammed Hasan to help me in statistical analysis .

It has been a great experience and I am truly grateful for all those who have helped me get to where I am today .

Summary

Gestational diabetes mellitus (GDM) is defined as glucose intolerance of variable severity that occurs during pregnancy . Women with GDM represent a heterogeneous group .GDM is characterized by pancreatic β cell function that is insufficient to meet body insulin needs , probably due to autoimmune diseases , insulin resistance or genetic abnormalities .

The prevalence of GDM may range from 1 to 14% of all pregnancies. Diagnosis of GDM is by using a two - hour OGTT with a 75 g dose of glucose .

This study aims to examine concentrations of 25 (OH) vitamin D in Iraqi women with normal pregnancies and pregnancies complicated by GDM , and with two single nucleotide polymorphism (SNP A/G rs # 1544410 and SNP C/T rs # 10735810 in vitamin D receptor (VDR) to the susceptibility to gestational diabetes in the Iraqi women , The second aim of this study is to investigate the association of VDR together with 25-hydroxy vitamin D serum levels, and biochemical parameters (Fasting Blood Glucose, Glycated Hemoglobin, Insulin hormone, Insulin Resistance, Parathyroid Hormone, Insulin Like Growth Factor Hormone, Calcium ion , Phosphorus ion and lipid profile .

This cross-sectional study was carried out at Ramadi Teaching Hospital for gynecology and pediatrics ,office Wahj Al-DNA scientific and Al-Mawla laboratory for media lab during the period from 1st September 2017 to the end of April 2018. It involved 40 cases pregnant with GDM, 30 cases pregnant without GDM and 30 cases with normal vitamin D and non pregnant to represent the control group .

A venous blood samples were collected at morning after fasting for 10-12 hours. The blood samples were divided into two aliquots, 2 ml were transferred into EDTA for HbA1c and DNA extraction, and 3 ml used for laboratory investigation .

The mean of serum vitamin D concentrations was lower in both groups: pregnant with GDM (6.83 ± 3.547ng/ml) and pregnant without GDM (8.73±4.158 ng/ml) and there was no significant differences between these groups but there was significant differences in concentrations of FBG, HbA1c, insulin hormone, HOMA-IR, lipid profile except of high density lipoprotein cholesterol, parathyroid hormone and calcium ion between normal pregnant and gestational pregenant. There was no significant differences in concentrations in insulin – like growth factor hormone and phosphorus ion .

Vitamin D had a significant negative correlation with HOMA-IR ($p < 0.014$) in pregnant with GDM

The results showed a relationship between age and GDM ($p < 0.001$) and also between body mass index and GDM ($p < 0.001$) .

Vitamin D receptor rs (10735810) and rs (1544410) polymorphisms were not associated with Iraqi gestational dibetes mellitus. Homozygous CC and homozygous GG turned out to be as a preventive genotype of gestational diabetes mellitus (OR < 1) while CT ,TT and GA turned out to be related with risk of GDM (OR ≥1).The allele C and allele G represent as a preventive allele (preventive factor) , while the allele T and allele A act as etiological factor.

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BMI	Body Mass Index
bp	Base pair
Ca^{+2}	Calcium Ion
CI	Confidence Intervalse
DBP	Vitamin D Binding Protein
DM	Diabetes Mellitus
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Di amine Tetra Acetic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FBG	Fasting Blood Glucose
GDM	Gestational Diabetes Mellitus
HbA1c	Glycated Hemoglobin
HDL	High Density Lipoprotein
HOMA-IR	Home Ostatic Model Assessment Insulin Hormone
IGF	Insulin- Like Growth Factor
IR	Insulin Resistance
IU	International Unit
LDL	Low Density Lipoprotein
LSD	Least Significant Difference
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
$)PO_4)^{-2}$	Phosphorus Ion
PTH	Parathyroid Hormone
P value	Probability value
RFLP	Restriction Fragment Length Polymorphism

RXR	Retinoid X Receptor
VDR	Vitamin D Receptor
VLDL	Very Low Density Lipoprotein

Chapter One

Introduction and Literature Review

Introduction

Vitamin D is a fat soluble steroid hormone precursor that is mainly produced in the skin by exposure to sunlight,Vitamin D is biologically inert and must undergo two successive hydroxylations in the liver and kidney to become biologically active 1,25 –dihydroxyvitamin D [(1)]. The two most important forms of vitamin D are vitamin D_3 (cholecalciferol) and vitamin D_2(ergocalciferol). In contrast to vitamin D_3 ,the human body can not produce vitamin D_2 which is taken up with fortified food or given by supplements [(2)] . In human plasma vitamin D_3 and D_2 are bound to the vitamin D binding protein and transported to the liver where both are hydroxlated to form 25-hydoxyvitamin D, It is commonly agreed that 25-hydroxyvitamin D is the metabolite to determine the overall vitamin D status as it is the major storage form of vitamin D in the human body [(3)]. This primary circulation form of vitamin D is biologically inactive with levels approximately 1000 –fold greater than the circulating 1,25-dihydroxyvitamin D, The half- life of circulating vitamin D (25-OH) is 2-3 weeks, Most of the vitamin D (25-OH) measurable in serum , is vitamin D_3 (25-OH) whereas vitamin D_2 (25-OH) reaches measurable levels only in patients taking vitaminD_2 supplements.[(4)] Vitamin D_2 is considered to be less effective .[(5)] Vitamin D is essential for bone health, In children ,severe deficiency leads to bone –malformation , known as rickets , Milder degrees of insufficiency are believed to cause reduced efficiency in the utilization of dietary [(6)].

Vitamin D deficiency causes muscle weakness , In elderly the risk of falling has been attributed to the effect of vitamin D on muscle function [(7)].

Vitamin D has been shown to affect the expression of over 200 different genes. Insufficiency has been linked to diabetes [8], different forms of cancer ,cardiovascular disease , autoimmune diseases and innate immunity [2].

Relationship between the vitamin D receptor (VDR) gene and diabetes has been noted in several population. The VDR is encoded by a gene located in chromosome 12q12 and several polymorphisms have been described for the VDR gene.[9] In addition, the VDR is present in pancreatic β-cells .Thus ,1,25$(OH)_2D_3$ may play an important role in insulin secretion and insulin sensitivity in diabetes by either increasing the intracellular calcium concentration in the β-cell to induce insulin secretion or by increasing the conversion of proinsulin to insulin [10].

Diabetes mellitus (DM) is a major challenge for global puplic health, and it comprises a heterogeneous etiology of diseases characterized by elevated blood glucose. [11]Three main types of DM are recognized :

Type 1 DM is caused by autoimmune destruction of insulin – producing pancreatic β cells ,culminating insulinopenia with resultant hyperglycemia .It is diagnosed during infancy and requiring exogenous insulin for survival [12].

Type 2 DM is a complex disorder characterized by imbalance between insulin resistance and insulin secretion that induce liver glucose output by preventing glycogen formation and stimulating glycogenolysis and gluconeogenesis [13].

Gestational diabetes mellitus (GDM) usually reveals itself in the latter half of pregnancy and it is identified by carbohydrate intolerance of variable severity [14]. Hyperglycemia develops during pregnancy because of the secretion of placental hormones , which causes insulin resistance[15].

Risk factors for GDM include :glucoseurea, age over 30 years , obesity, family history of diabetes, past history of GDM and previous macrosomic child[16]. Vitamin D deficiency may play a role in the pathogenesis of GDM [17].

Other specific types of diabetes are caused by specific genetic defect of β cell function or insulin action , the pancreas disease and drug or chemical induced diabetes mellitus [18].

1.1 Vitamin D

1.1.1 Physiology of vitamin D

Vitamin D is actually a hormone , not technically a vitamin , Vitamin is defined as a substance that is not made by the body but supplied in the diet to maintain life processes [19]. The body itself has the ability to produce vitamin D , when UV radiation (wave length 290 – 315 nm) reaches perfect 7-dehydrocholesterol in the skin [20].

A latitude, season, sunscreen , aging and skin pigmentation influence the production of vitamin D_3 by the skin [21].

Vitamin D represents a group of fat soluble compounds . It has a 27 carbon secosteroid whose molecular structure is like that of classic steroid hormones including cortisol , estradiol and aldosterone [22] . Two main forms of vitamin D are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) [23,24] .These have unlike side chains : vitamin D_2 has a methyl group at C_{24} as well as a double bond between C_{22} and C_{23} while vitamin D_3 has a single bond between C_{22} and C_{23} , and hasn't methyl group at C_{24} [24]. These structures are shown in figure 1.1.

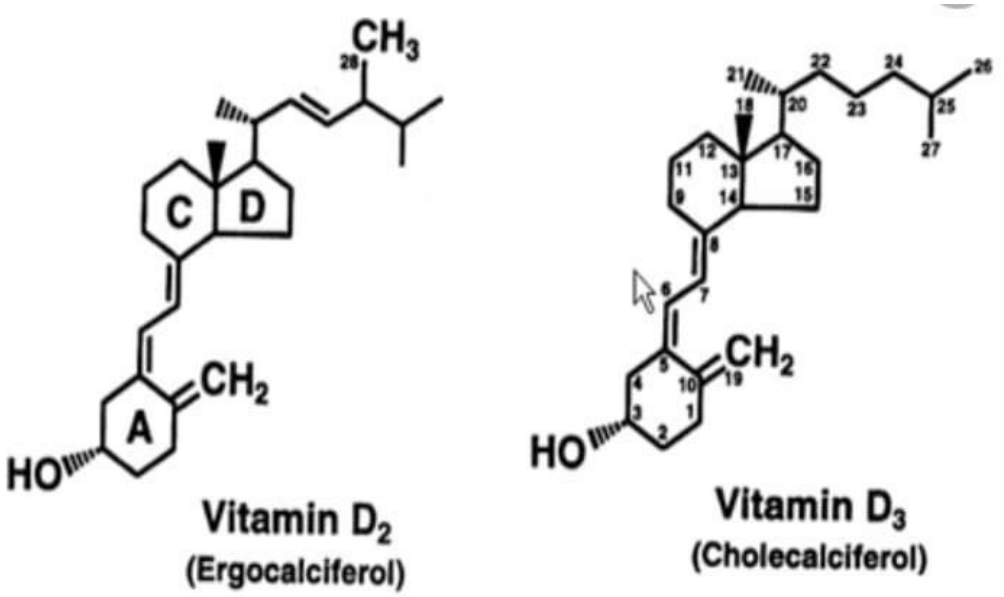

Figure 1.1: Structures of vitamin D_2 and vitamin D_3 [24] .

1.1.2 Source of vitamin D

The primary source of vitamin D is the skinny synthesis of vitamin D through sunlight exposure , specially ultraviolet radiation (290 – 315nm) [25] . In addition to endogenous production of vitamin D , humans can obtain vitamin D from the food [26] . Plants and fungi produce vitamin D_2 , while vitamin D_3 is synthesized by animals (such as liver, fish, egg yolk)[27] . Small amounts of vitamin D_3 are found in butter, cream. Human and cows` milk is poor sources of vitamin D_3. In many parts of the world , fluid and dried milk is supplemented with vitamin D[28].Vitamin D has been given by intramuscular injection or by oral [29].

A UK trial examined the use of oral vitamin D_3 100000 IU every four months for five years in retired male doctors , there was a reduction in fractures at any specific site [30] . Most studies proved that vitamin D_2 is less effective than vitamin D [31,32].

Vitamin D_2 and vitamin D_3 supplementation were evenly effective in maintaining serum 25 (OH) D levels [33]. Generally vitamin D_3 used for nutritional and clinical purpose [34].

1.1.3 Metabolism of vitamin D

Vitamin D endocrine system plays an important role in regulation of bone remodeling and mineral homeostasis [35]. Also it has important role in physiological processes , vitamin D endocrine system participates in pathological processes including autoimmune disorders , cardiovascular disease and diabetes mellitus [36,37].

During exposure to sunlight , 7-dehydrocholesterol is present in the epidermis and dermis absorbs UV photons. [38] The absorbed energy rises the double bonds to rearrange causing a break in the bond between 9 and 10 carbons leading to formation of pre-vitamin D_3 [38,39] .

Pre-vitamin D_3 exists in two forms cis , cis and cis , trans. The cis , trans pre-vitamin D_3 form is the most stable. But it cannot convert to vitamin D_3. So , in an organic solvent , it takes approximately 24 hours for converting 50% of pre-vitamin D_3 to vitamin D_3 [40].

Vitamin D , whether endogenously synthesized or ingested , should undergo two hydroxylations in the body before it becomes an active hormone. Once vitamin D is transported in circulation by binding to the vitamin D binding protein DBP and troubles to the liver, it is hydroxylated by the cytochrome P450 enzyme to 25-hydroxyvitamin D (25(OH)D) which is also known calcidiol [41,42] . The half-life of 25-OH D is 20-30 days. [43,44] 25-hydroxyvitamin D is the major circulating form of vitamin D in the body [41,42].

25(OH)D then transported to the kidney where it is hydroxylated by the enzyme 25-hydroxy vitamin D 1-alpha-hydroxylase (25(OH)D-1α OH ase) to produce the active form of vitamin D metabolite , 1,25-dihydroxyvitamin D well known calcitriol [45] .The half-life of it is only a few hours [43].

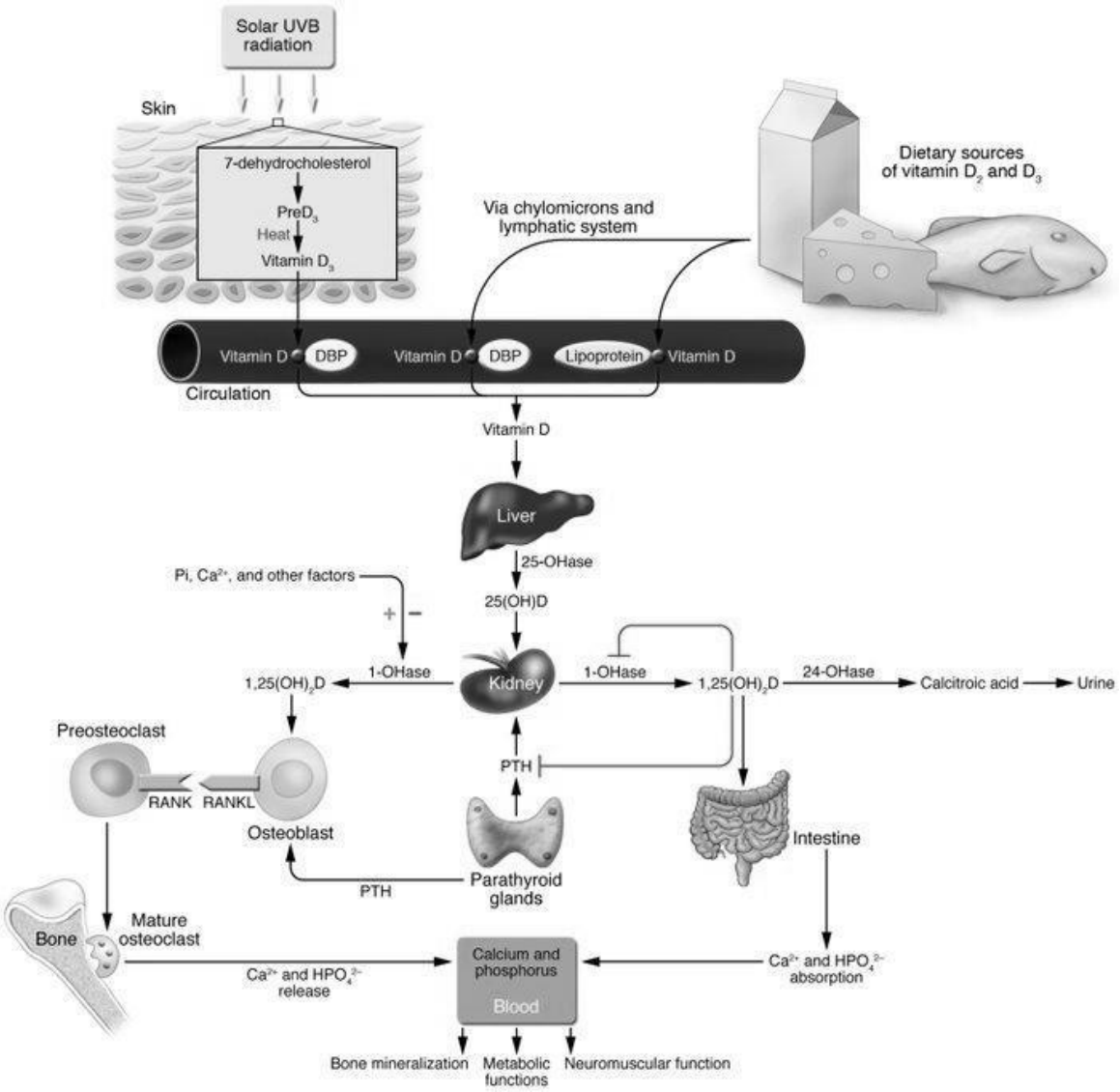

Figure1.2: Biosynthesis and metabolism of vitamin D from photoproduction or dietary intake of vitamin D_2 ,D_3 [46].

The renal production of 1,25$(OH)_2$ D is regulated by parathyroid hormone (PTH), calcium, phosphorus and 1,25 $(OH)_2$ D itself [47] . When blood calcium levels are insufficient , increase level of PTH signals an increase of calcitriol synthesis which increases intestinal absorption of calcium. [48] When phosphate levels are high , this stimulates the production of fibroblast growth factor-23 (FGF-23) in the bone , which inhibits synthesis of calcitriol in the kidney [49] , when 1,25 $(OH)_2$ D level is sufficient , this induces the catabolic enzyme 1,25-dihydroxyvitamin D-24-hydroxylase in the kidney [41,50] .

For years, calcitriol appeared to be central to every thing regarding vitamin D , common 25(OH)D was regarded an inactive precursor , and all neglected in patient with chronic renal failure , the realization that many non-renal tissues have 25(OH)D-1-hydroxylase has provided a mechanistic basis of clinical epidemiologic findings which show that 25(OH)D concentration is normally more relevant to health and disease prevention than are calcitriol concentration.[51] Circulating 25(OH)D serves as the substrate for paracrine and autocrine control systems in many body tissues , most of these are related to calcium homeostasis.[52]

The index disease for vitamin D is rickets in children and osteomalacia in adults with long term , mild deficiency. Osteoporosis is regarded as the consequence of too little vitamin D [53] . As support for this , mild degrees of vitamin D insufficient increased bone turnover markers , and increased fracture risk [51,53]. Essentially all clinical research into vitamin D supplementation has focused on early childhood and the elderly.

The consequences of vitamin D nutritional status on musculoskeletal health in the skeleton of early adolescence are unclear ,

little is known about how to treat the situation, This case is of particular relevance because of the growing recognition that serum 25(OH)D concentration is low in children and adolescents.[54]

1.1.4 Assessing of vitamin D nutritional status

Plasma or serum 25(OH)D is considered to be the best measure of vitamin D nutritional status , it is the major form of vitamin D in the body and it is reflective of both dietary intake and cutaneous synthesis.[55] As a result of its stability when bound to DBP , as well , renal production of $1,25(OH)_2D$ is tightly regulated. [56] And $1,25(OH)_2D$ level is often normal or even slightly elevated in the presence of 25(OH)D insufficiency . There has been some controversy over whether the measurement of 25(OH)D is adequate for making dietary recommendations which gives disagreement regarding the optimal serum level for this metabolite , and also It gives significant variability in the assays measuring serum 25(OH)D. [57,58] Although other potential functional markers of vitamin D status have been suggested including PTH , bone mineral density and calcium absorption [59,60] , a systematic review of these existing markers confirmed that circulating 25(OH)D was a robust and reliable marker of vitamin D status. [61]

The sufficient of vitamin D defined as 25(OH)D level > 20 ng/ml upon evidence related to bone health , on the other hand , the endocrine society guidelines define vitamin D insufficiency as 25(OH)D level of 21-29 ng/ml [41,62].

1.1.5 Vitamin D in bone health

Numerous studies have indicated a relationship between vitamin D and bone health .Vitamin D like calcium , has long been regarded as a fundamental part of the prevention and treatment of osteoporosis.[63] Findings from observational studies showed inconsistent associations between bone mineral density and vitamin D status[64],and debate continues regarding optimum concentrations of 25-hydroxyvitamin D for the best possible skeletal health [65].

Calcitriol increases levels of ionized calcium in blood by binding to the VDR. In the intestine , the Calcitriol-VDR complex acts as a transcription factor for the expression of transport proteins involved in calcium absorption [66], and an increase in intestinal absorption of calcium results. In the kidney , Calcitriol increases renal tubular reabsorption of calcium , and in bone , Calcitriol indirectly stimulates osteoclast action to increase calcium release into blood. Calcitriol also acts directly on the parathyroid gland to decrease parathyroid hormone (PTH) production and indirectly decrease PTH by increasing serum calcium concentration [46].

Vitamin D is primarily responsible for regulating absorption of calcium and phosphorus from the intestine. Low levels of vitamin D result in only 10 to 15% of dietary calcium and a low level of dietary phosphorus being absorbed. When vitamin D levels are sufficient , 30 to 40% of dietary calcium and a higher level of phosphorus are absorbed [67,2]. Low levels of vitamin D result in insufficient calcium absorption to maintain calcium homeostasis , and low serum calcium stimulates PTH release , PTH increases serum calcium by acting on bone to increase calcium release , acting on the kidney to increase renal

tubular calcium reabsorption , and increasing formation of Calcitriol.[46] In infants and adolescents , these bone stores of calcium occasionally cannot be released quickly enough to meet the demand during periods of rapid growth , and this may result in hypocalcemic seizures or tetany [68,69] . In school-aged children , chronic mobilization of calcium from bone is usually sufficient to maintain normal serum calcium , but demineralization and subsequent deformity of the bone can result in rickets [69]. Bone breakdown is also the reason that low vitamin D in pregnancy and in children may impair the attainment of peak bone mass in children [70] . The same process in adults may result in bone pain , proximal muscle weakness , osteomalacia , osteoporosis , and increase risk of fractures [71,72].

1.1.6 Non - skeletal roles of vitamin D

The presence of vitamin D receptor in a wide variety of non-skeletal organs and tissue suggests that vitamin D likely plays a role in many physiological processes. A role for vitamin D has been implicated in cardiovascular health [23], autoimmune disease [73], neurological development [74], cancer [75], and immune function. [76] In addition vitamin D levels have been associated with dermatologic disease [77], preeclampsia [78] , mental health , specifically schizophrenia[79] and obesity [80] . The mechanisms by which vitamin D exerts its non-skeletal effects are manifold. Calcitriol directly or indirectly controls more than 200 genes. It is involved in regulation of cellular differentiation , proliferation and angiogenesis , apoptosis.[81] It inhibits renin synthesis [82] increase myocardial contractility [83] and is involved in insulin production [84]. It also acts as an immune-modulator, as it dampens systemic inflammatory responses through

functional vitamin D receptors present on all major immune cell. [85] As shown in figure 1.3.

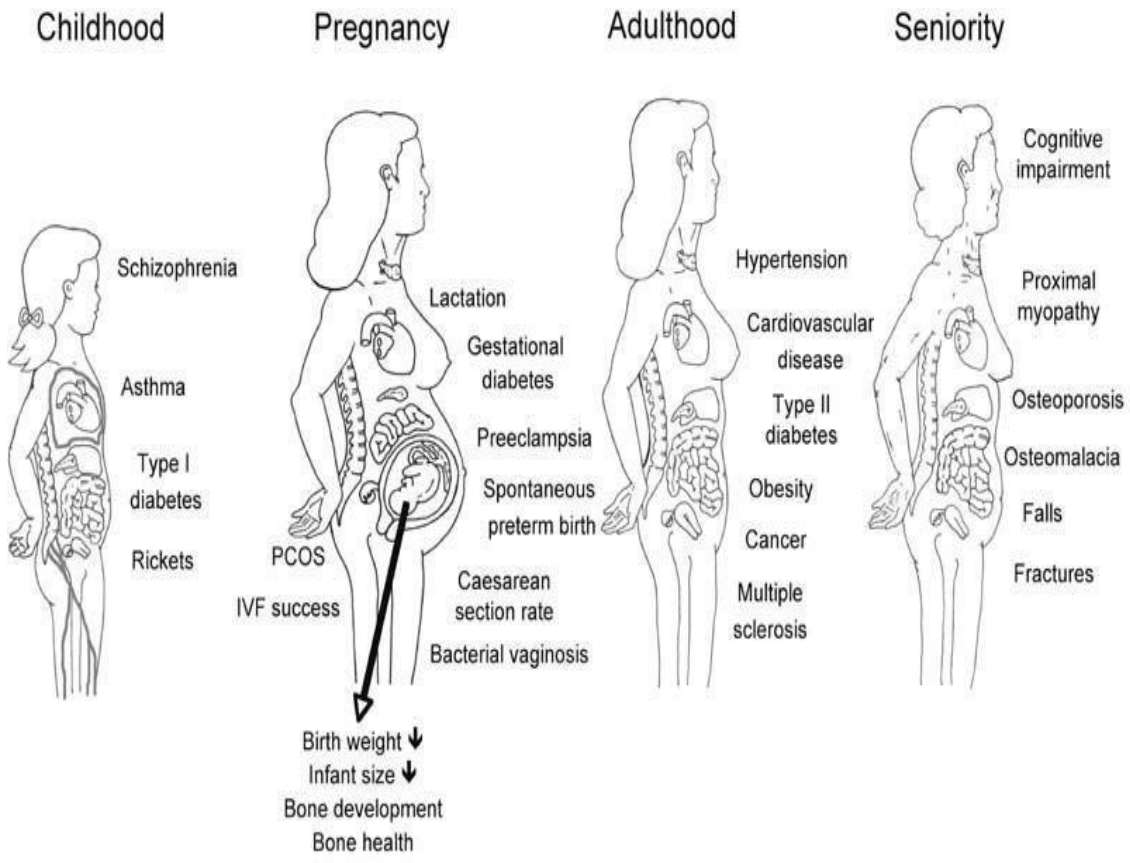

Figure 1.3: Proposed health implications of vitamin D deficiency from infancy to seniority [79]

1.2 The vitamin D receptor

VDR is a member of the steroid / thyroid hormone receptor family [86] that binds calcitriol and acts as a transcription factor during gene expression [87] , after the VDR is bound to calcitriol , the complex dimerizes with the retinoid x receptor (RXR).The Calcitriol – VDR –

RXR heterodimer translocates into the nucleus and binds to vitamin D responsive elements in the promoter regions of vitamin D responsive genes , inducing their expression [76] , VDR has been identified in most of body's tissues including pancreatic islets , myocardium [88,89] , placenta , brain , gonads , lung , prostate , skin , breast , small intestine , colon , immune system. [90,91,92]

The presence of the VDR in this wide variety of tissue types suggests that vitamin D's role in physiology is more multifaceted than previously recognized.

The function of this nuclear receptor is influenced by the presence of several genetic polymorphism. [93,94] The VDR gene , located on chromosome 12q12-q14 , includes eight protein coding exons (exons 2-9) and one un translated exon [95,96], over 200 polymorphisms have been discovered in the VDR gene in different individuals. [97,98]

The most commonly investigated VDR polymorphisms are FokI (rs10735810) located in exon 2 of VDR , BsmI (rs 1544410) and ApaI (rs 7975232) , located in intron 8 , and TaqI (rs 731236) located in exon 9 of VDR. [95]

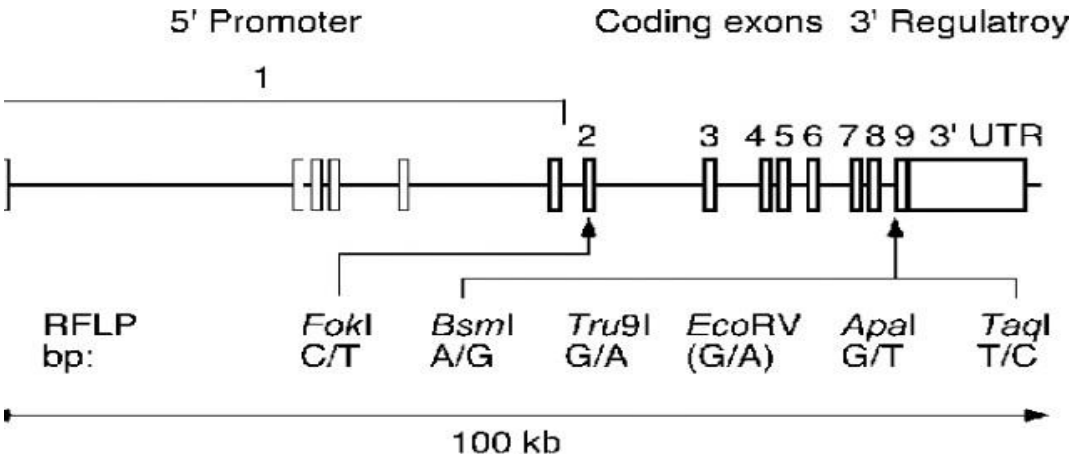

Figure 1.4: Location of the vitamin D receptor gene located on chromosome 12.[95]

Genetic alterations of the VDR gene may lead to defects in gene activation or changes in the protein structure of the VDR , both of which could affect the cellular functions of vitamin D. [99]

VDR genetic mutations can confer susceptibility to bone demineralization , particularly in the context of other risk factors such as age , suboptimal calcium intake , and physical inactivity. [100,101,102] Genetic mutations in the VDR have also been associated with increased risk of developing cancers [103,104,105]. There is a study proved that the rs 1544410 polymorphism may be a risk factor for susceptibility to type 1 diabetes mellitus in the East Asian population , and the rs10735810 polymorphism is associated with an increased risk of type 1 diabetes mellitus in the west Asian population. [106] The role of VDR polymorphisms in the pathogenesis of type 1 diabetes mellitus was not clear , large number of studies has investigated the association between the aforementioned SNPs and the risk of T1 DM , but the results were inconsistent [95,107-114].

1.3 Diabetes mellitus (DM)

Diabetes mellitus is a metabolic disorder , which is characterized by high blood sugar (glucose) levels due to insulin deficiency. People who suffer from diabetes can't produce energy from sugar in a sufficient way. Insulin plays a crucial role in this disease. Insulin is a hormone produced by the pancreas . This hormone regulates carbohydrates and fat metabolism.

Glycated hemoglobin (Hb A1c) can be measured in the blood to find out the amount of sugar in the blood [115].

General symptoms of marked hyperglycemia include weight loss , polydipsia , polyphagia , and polyuria are more frequent in type 1 than type 2 DM.[116]

Diabetes prevalence is estimated to rise in all age groups , the ratio continues to increase with the increase in the number of individuals with diabetes [117].

The prevalence is similar in men and women throughout most age ranges but is slightly greater in men > 60 years. Diabetes increased markedly with a prevalence rate 25.3 per 1000 persons in the 30-49 age group , further increases in the rates are seen after age 50 , with a prevalence rate of 143.8 per 1000 persons [118].

Diabetes is likely to be underreported as a cause of death simply because diabetes leads to many complications that ultimately cause death [119].

The complications of diabetes become one of the major causes of morbidity and mortality in most countries related to increased risk of stroke , cardiovascular disease , autoimmune disease , metabolic disease , obesity , hypertension , nephropathy , neuropathy , retinopathy and skin lesions [120].

In a year , more than 231,000 individuals in the united states and more than 3.96 million individuals worldwide die from diabetes and its complications [121].

Numerous cross – sectional studies have reported a significant inverse association between serum vitamin D and the presence of diabetes mellitus.[122,123] Where Gorham ,et al explained that most case – control studies have also found that patients with type 2 diabetes or impaired glucose tolerance are significantly more likely to have a lower serum vitamin D concentration than those without diabetes[124]. Almost all prospective studies have assessed vitamin D status at baseline and reported a significant inverse association of baseline serum vitamin D with the occurrence of diabetes [125,126].

1.4 Classification of Diabetes Mellitus

There are numerous types of diabetes mellitus , the world health organization (WHO) and the American Diabetes Association (ADA) classify these types according to the etiology of the disease , and not according to the form of treatment , into : type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM) and other specific types of diabetes mellitus [127].

1.4.1 Type 1 Diabetes Mellitus

Type 1 DM is a multifactorial disease caused by interactions between many genetic and environmental factors. It is caused by autoimmune destruction of pancreatic β-cells [128], and characterized by deficiency of insulin secretion and ketosis-prone hyperglycemia [129].

It is diagnosed during infancy requiring exogenous insulin for survival , often the loss of β-cells begins in infancy and continues for several months or years. Diagnosis of the type 1 DM may occur when 80% of the β-cells have been destroyed [12] , whereas studies suggested that 40-50% of the viability β - cells may be present at the onset of hyperglycemia , and a condition that may be related to the age of the person , body mass and physical activity [130,131].

Type 1 DM has the fourth highest hospitalization rate of all chronic disorders diagnosed in infancy after asthma , infancy cancer and epilepsy [132].

Type 1 DM usually occurs in childhood or early adulthood and also could occur late in some individuals. The percentage of this type represents about 5 to 10 of all those with diabetes [133].

1.4.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus accounts for approximately 90 percent of all diabetes cases. It is characterized by hyperglycemia , and thus its diagnosis is determined based on a fasting plasma glucose level or oral glucose tolerance test.[134,135]

Type 2 diabetes is a progressive disease , where individuals with normal glucose tolerance progress to impaired fasting glucose and impaired glucose tolerance and then to overt type 2 diabetes .Pre-diabetes encompasses either impaired fasting glucose , impaired glucose tolerance or both , as blood glucose levels are higher than normal but not high enough for a diagnosis of type 2 diabetes [136].

The development or diagnosis of type 2 DM occurs in younger adults even during childhood but usually occurs in people after the age of 40 years [121].

Type 2 DM is a multifactorial metabolic disorder which arises from two primary underlying pathophysiological disorders : insulin resistance and pancreatic β-cell dysfunction. Insulin resistance refers to decreased insulin action at the level of the liver , adipose tissue and muscle.[137] β-cell dysfunction is defined as the suboptimal secretion of Insulin from the β-cells to meet the needs of the body in maintaining glucose homeostasis [138].

Both insulin resistance and β-cell dysfunction have been shown to predict the development of incident type 2 DM independently of other risk factors for the disease.[139-142] In most cases , early in the progression to type 2 DM , insulin resistance is established but glucose tolerance remains normal due to compensatory response from pancreatic β-cells which will increase insulin secretion to maintain

glucose homeostasis. However , this compensatory response is impaired and that resulting β-cell dysfunction eventually leads to the elevation of blood glucose concentrations into the diabetes range[134,135].

1.4.3 Gestational Diabetes Mellitus (GDM)

GDM usually reveals itself in the latter half of pregnancy and it is identified by carbohydrate in tolerance of variable severity.[14] Data from western countries suggest that the prevalence of GDM is increasing , being almost 10% of pregnancies and probably reflecting the global obesity epidemic [145].

The pathophysiology of GDM is parallel to that of T2DM in many aspects including insulin resistant and relative insulin insufficiency. Women with previous GDM are of increase risk for developing T2DM in their later life [146].

Vitamin D deficiency has reappeared as an important public health problem in developed and developing countries , and vitamin D deficiency during pregnancy becomes increasingly common. In recent studies in the united states , Canada , Australia , Iran , Sweden , and Pakistan , 24 -95.8% of pregnancy women were deficient and 54-100% were insufficient for vitamin D, with a general trend towards increased prevalence of vitamin D deficiency or insufficiency in women with darker skin pigmentation, serum 25(OH)D levels were negatively correlated with fasting glucose and insulin level [17].

Risk factors for GDM include : glucose urea , age over 30 years , obesity , family history of diabetes , past history of GDM and previous macrosomic child.[16] Furthermore , females who have a history of GDM have a 40-60% chance of developing diabetes in the following 10 years. Therefore , changes in lifestyle implemented to normalize blood

glucose during gestation become essential preventive measures against the development of preventive measures against the development of type 2 diabetes [147,148].

1.4.4 Other specific types

Other etiologies for specific types are presently less common causes of diabetes mellitus but are those in which the underlying defect or disease process. Such as genetic (genetic defects of β-cell function , genetic defects in insulin action) diseases of the exocrine pancreas (pancreatitis , trauma , pancreatectomy , cystic fibrosis), endocrinopathies (acromegaly , glucagonoma , hyperthyroidism) , drug or chemical induce vacor , pentamidine , nicotinic acid , infections (congenital rubella , cytomegalovirus) [149].

1.5 Insulin

Insulin is a polypeptide hormone [150] . which is synthesized in the β-cells of the islets of Langerhans in the endocrine pancreas and consists of two dissimilar polypeptide chains, A and B. Insulin is derived from pro-insulin and consists of the A- chain (21 amino acids) and the B. chain (30 amino acids) linked by two inter disulfide bonds.[151] Pro-insulin is structurally correlated to Insulin –like growth factors which bind weakly to the Insulin receptor and make the cells in the muscle, liver and adipose tissue to assume the glucose from the bloodstream, storing as glycogen inside this tissue.[152] The end effect of these actions is a reduction in blood glucose level. With regard to protein metabolism, insulin stimulates transfer of amino acids via membranes, stimulates protein synthesis, and inhibits proteolysis. Incorporation of fatty acids from circulating triglyceride into adipose triglyceride and

lipid synthesis are stimulated by insulin, Insulin contributes to nucleic acid synthesis by stimulating the formation of ATP, DNA and RNA.[153]

Palomer et al have been found that vitamin D is necessary for normal insulin release in response to glucose [154] .

Insulin also affects other body functions like the cognition and vascular and it is considered the most important significant regulator of metabolic equilibrium. When insulin arrived the human brain it improves memory and learning and helps verbal memory in particular.[143] If the amount of available insulin is insufficient, if cells respond poorly to the effects of insulin (Insulin insensitivity or insulin resistance),and if the Insulin itself is defective, then glucose will not be absorbed properly by the body cells that require it and it will not be stored appropriately in the liver and muscles. The net effect is persistently high levels of blood glucose, low protein synthesis, and other metabolic derangements, such as acidosis.[155]

1.6 Insulin Resistance (IR)

IR is a failure in the function of insulin targets cells like fat cells , liver cells and muscle cells to react to the insulin action[156], Also it is referred to as metabolic syndrome which has been postulated to comprise hyper insulinemia , glucose intolerance , low high density lipoprotein, high triglycerides and obesity [157]. Several reasons contribute to the occurrence of insulin resistance and these reasons are involved in the functioning of insulin which can be either at the stage of the binding of insulin to the receptor of after the level of downstream signaling .[120] These defects are caused by mutations in the insulin receotor gene which may include changes in the receptor number ,structure and function in signaling resulting in insulin resistance.[158]

Vitamin D may effect on insulin sensitivity either directly by stimulating the expression of insulin receptor in bone marrow cells , or indirectly via its role in regulating extracellular and intracellular calcium through the membranes of cells.[159] In a study with healthy adults , there was a positive correlation between 25 (OH) D level with insulin sensitivity and a negative effect between vitamin D deficiency on β cell function, even after correction for confounding factors such as body composition [84].

1.7 Insulin- like growth factor-1 (IGF-1)

IGF-1 is a 70 amino acid single – chain polypeptide with a molecular weight of 7-7.5 kDa.[160] The sequence of amino acids is very similar to that of pro-insulin, The synthesis of IGF-1 is controlled by a number of endogenous (genetic and hormonal) and exogenous (nutrition and physical activity) factors. The peptide is produced by a number of tissues , including adipose tissue [161] , but its main source is the liver , where the dominating regulators of IGF-1 synthesis are insulin [162] and growth hormone (GH) [163].

Insulin shortage in diabetes mellitus type 1 is characterized by low IGF-1 levels. Further factors are involved in the control of homeostasis of the IGF-1 system, IGF-1 production is significantly stimulated by sex hormones, Serum IGF-1 levels in childhood , similarly as GH levels , are very low and they rise during puberty. Later , the IGF-1 levels decline very slowly.[164] In the osseous tissue , the production of IGF-1 is stimulated by the parathyroid hormone (PTH) [165].

The production of IGF-1 in human osteoblasts is also stimulated by $1,25(OH)_2 D_3$ and calcitonin.[166] Although IGF-1 is linked mainly to its own specific receptors , where it activated , similarly to insulin , insulin

receptors of many tissues , including the adipose tissue, can thus be activated by IGF [167].

Moreover, the distribution of receptors for insulin in the mammalian organism differs somewhat from the distribution of receptors for IGF-1, while the classical target organ of insulin is the liver and adipose tissue, receptors for IGF-1 are not present in these tissue. Conversely , muscular tissue possesses both these types of receptors [168].

1.8 Parathyroid hormone (PTH)

PTH also called para thormone or parathyrin is a hormone secreted by the chief cells of the parathyroid glands as a polypeptide containing 84 amino acids. PTH half-life is approximately 4 minutes[169], it has a molecular mass of approximately 9500 Da.[170] Data indicate that PTH is also possibly secreted in small amounts from the brain and thymus.[171] While PTH acts to increase the concentration of ionic calcium (Ca^{+2}) in the blood , calcitonin , a hormone produced by the para follicular cells of the thyroid gland , PTH essentially acts to increase the concentration of calcium in the blood by acting upon the parathyroid hormone 1 receptor , which is present at high levels in bone and kidney , and parathyroid hormone 2 receptor which is present at high levels in the central nervous system , pancreas, testis and placenta.[172]

PTH increases the activity of 25- hydroxy cholecalciferol-1-α-hydroxylase enzyme , which converts 25-hydroxy cholecalciferol, the major circulating form of inactive vitamin D , into 1,25-dihydroxy chole calciferol , the active form of vitamin D [173,174]. PTH levels are related to diabetes [161] and also to depression.[175] A number of previous studies have reported significant associations of low vitamin D and

elevated Parathyroid Hormone with increased risk of metabolic syndrome [177-179].

Aims of the study

This study investigates the association of VDR together with 25 (OH) D serum levels, and biochemical parameters (Fasting Blood Glucose, Glycated hemoglobin, Lipid profile, Para Thyroid Hormone, Insulin Hormone, Insulin Resistance, Insulin – like Growth Factor I, Calcium ion, Phosphorus ion)

An association between the level of vitamin D and the risk of pregnancy –related complications remains unclear . The aim of this study is to examine concentrations of 25 (OH) vitamin D in Iraqi women with normal pregnancies and pregnancies complicated by gestational diabetes mellitus (GDM) and with two Single Nucleotide Polymorphisms (SNP G / A rs #1544410 ,SNP C / T rs # 10735810) in vitamin D receptor to the susceptibility to gestational diabetes in the Iraqi women .

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 : Equipment

The equipment used in the present study are listed in table 2.1 with the producing company and the country .

Table 2.1 The equipment used in the present study

Equipment	Company	Origin
AURA TM PCR cabinet		Italy
Bio TDB -100 ,Dry blok thermostat built	Biosan	Germany
Centrifuge	Hettich	Germany
Clover A1c	Infopia	Korea
Cobas e 411	Roche	U.S.A
Deep freeze	Sanyo	Japan
Document system	Labnet	U.S.A
Electronic balance	Ohaus	U.S.A
ELISA device washer	Universal	U.S.A
ELISA devise Reader	Universal	U.S.A
Gel electrophoresis	Consort	U.S.A
Incubator	Memmert	Germany
Micropipettes	Gilson	France
Microspin	Lative	Biosan
Microspin 12 , High-speed Mini-centrifuge	Biosan	Germany
Microwave oven	White – Westinghouse	U.S.A
Mini –powder supply 300V ,200V		Chain

Multi Gene optim ax Gradient thermal cycler	Labnet	U.S.A
Pipette variable volume 2-20 ml		Germany
Spectrophotometer	Apple PD303	Japan
V -1 plus, personal vortex for tube	Digsystem	Germany
Water bath	Grand	England
Water distiller	GFL	Germany

2.1.2 Chemical materials and kits

The chemical materials and kits used in this study are listed in table **2.2** with the producing company and the country .

Table 2.2 Chemical material and kits used in the study

Material	**Company**	**Country**
25-OH Vitamin D total –Kit	Demeditec	Germany
6 X loading dye	Intron	Korea
Absolute ethanol	Fluka	Switzerland
Agarose powder	Promega	U.S.A
Calcium – kit	Biolabo	France
Cholesterol – kit	Spinreact	Spain
Glucose – kit	Biolabo	France
HbA1c – kit	Human	Germany
HDL- cholesterol – kit	Biosystem	Spain
Insulin – like growth factor 1 – kit	Demeditec	Germany
Insulin hormone – kit	Demeditec	U.S.A
Ladder 100 bp	Kopa	U.S.A
Para thyroid hormone – kit	Demeditec	Germany
Phosphorus – kit	Biolabo	France
Premix PCR	Intron	Korea
Primers (forward and revers of BsmI and Fok1)	Integrated DNA technologies	U.S.A
Quick – 9DNA TM blood mini prep	Zymo	U.S.A
Red safe staining solution	Intron	Korea
Triglyceride – kit	Spinreact	Spain

2.2 Subjects

2.2.1 Patients and controls

The cases samples collected from Iraqi women. This study include three groups:-

- Group I included 40 cases pregnant with gestational diabetes mellitus (GDM).

- Group II included 30 cases pregnant without gestational diabetes mellitus (GDM).

- Group III included 30 cases control who had normal vitamin D and non pregnant.

This study covered the period from September,2017 to April 2018 .

The diagnosis of GDM was made on the basis of the recommended criteria by ADA. [180]

2.2.2 Collection of blood samples

From each patient and healthy human (5ml) of blood were obtained by vein puncture using 5ml disposable syringes after 10-12 hours fasting. The blood sample was divided into two aliquots. The first aliquot (2ml) was transferred into EDTA tube for HbA1c determination and then used for DNA extraction. The second aliquot (3ml) of separated serum was used for assays of fasting blood glucose , lipid profile , calcium, phosphorus, insulin hormone, parathyroid hormone, insulin-like growth factor1 and vitamin D .

2.3 Methods

2.3.1 Calculation Of Body Mass Index (BMI)

BMI was calculated by dividing a person's weight (kg) by length squared (M^2). The weight was measured by electronic sensitive balance, while the height was measured in meters using standard metric bar [181] .

BMI = weight (kg) / length^2 (M) ^{2}

Table 2.3 : The body mass index and weight status [181]

BMI (kg /m^2)	Wight status
< 18.5	Under weight
18.5 to 24.9	Healthy weight
25.0 to 29.9	Over weight
30.0 to 39.9	Obese
> 40	Highly obese

2.3.2 Laboratory investigation

The following parameters were measured in all patients and controls.

1- Fasting blood glucose (FBG).

2- Glycated hemoglobin (HbA1c).

3- Lipid profile.

4- 25-OH total vitamin D.

5- Para thyroid hormone (PTH).

6- Insulin hormone.

7- Insulin Resistance (IR).

8- Insulin-like Growth Factor1 (IGF1).

9- Calcium Ion .

10- Phosphorus Ion .

2.3.2.1 Measurement of Fasting Blood Glucose (FBG)

The determination of glucose level in serum is based on enzymatic reaction. [182]

A. Principle of test

The serum glucose was oxidized by glucose oxidase (GOD) to gluconate with the formation of hydrogen peroxide (H2O2). In the presence of peroxidase (POD), a mixture of phenol and 4-amino antipyrin(4-AA) is oxidized by hydrogen peroxide , to form a red quinoneimine dye proportional to the concentration of glucose in the

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$\text{4-AA} + \text{phenol} \xrightarrow[\text{POD}]{\text{H2O2}} \text{Quinoneimine} + H_2O$$

B. Procedure

1- Bring reagent consisting of phosphate buffer PH 7.5 ,glucose oxidase, peroxidase , 4-aminoantipyrine and phenol with samples to room temperature .

2- Pipette into labeled tubes.

Tubes	**Blank**	**Sample**	**Standard**
Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10µl	-
Standard	-	-	10 µl

3- Mix and let the tubes stand 5 minutes at 37 ℃.

4- Read the absorbance (A) of the sample and the standard at 500nm against the reagent blank.

The concentration of glucose in sample was calculated according to the equation:-

$$\textbf{Conc. Of Glucose mg/dl} = \frac{\textbf{A sample}}{\textbf{A standard}} * \textbf{C}_{\textbf{standard}}$$

2.3.2.2 Determination of Glycated Hemoglobin (HbA1c)

HbA1c was measured by Clover A1c system

A. Principle of test

The clover A1c system is fully automated boronate affinity assay for the determination of the percentage of hemoglobin A1c (HbA1c%) in whole blood . The clover A1c test cartridge includes a cartridge and reagent pack. ″The reagent pack is pre-filled with reagent solution and rinsing solution ″. The reagent solution contains agents that lyse erythrocytes and bind hemoglobin as well as boronate resin that bind cis-diols of glycated hemoglobin. A blood sample size of 4µl is obtained with capillary tip of the reagent pack , the hemoglobin and boronate resin binding the glycated hemoglobin. The blood sample mixture is rotated to the determination zone of the cartridge ,where the amount of total hemoglobin in the blood sample is measured by the reflectance of the photo sensor which consists of light emitting diode (LED) and aphotodiode (PD). The cartridge is then rotated so that the rinsing solution washes out non-glycated hemoglobin from the blood

sample, thus the amount of glycated hemoglobin can be photometrically measured.

The ratio of glycated hemoglobin with respect to total hemoglobin is calculated [183].

$$\mathbf{HbA1c\ \%\ = A \times \{ \frac{HbA1c}{total\ hemoglobin} \times 100 \} \div B}$$

HbA1c and total hemoglobin are values obtained from the clover A1c system and B which are the slope and intercept factor to correct value for the calibration standard of National Glycol hemoglobin Standardization Program (NGSP) .

B. Procedures

1- The cover of clover A1c analyzer was opened and inserted a new test cartridge .

2- The reagent pack was mixed 5-6 times before applying sample.

3- 4µl of sample were taken into the built-in capillary tip on the reagent pack.

4- The reagent pack was put into test cartridge and the lid was closed .

5- The test started automatically when the lid was closed.

6- The measuring time was 5 minutes and the test result would be displayed in % or mmol/ mol.

7- The cartridge was removed after the test was completed.

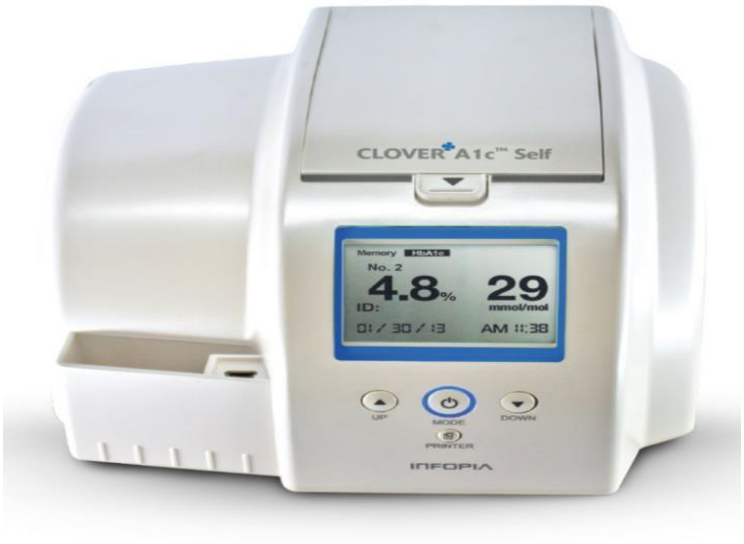

Figure 2.1: The clover A1c analyzer

2.3.2.3 Determination of serum total cholesterol

Serum total cholesterol was determined by the enzymatic colorimetric method [184].

A. Principle of test

The method for the measurement of total cholesterol in serum involves the use of three enzymes : cholesterol esterase (CE) , cholesterol Oxidase (CO) and peroxidase (POD) . In the presence of the former , the mixture of phenol and 4- amino anti pyrine (4-AA) are condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in the sample .

$$\text{Cholesterol esters} + H_2O \xrightarrow{CE} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CO} \text{Cholestenone} + H_2O_2$$

$$\text{4-AA} + \text{phenol} \xrightarrow{H2O2} \text{Quinoneimine} + 4H_2O$$

B . Procedures

1- Bring reagent consisting of PIPES pH 7.0, Sodium cholate, 4- amino antipyrine and phenol . with samples to room temperature .

2- Pipette into labeled tube :-

Tubes	**Blank**	**Sample**	**Standard**
Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 μl	-
Standard	-	-	10 µl

3- Mix and incubate the tubes 5 minutes at 37^{o} c .

4- Read the absorbance (A) of the sample and the standard at 500 nm against the reagent blank .

The concentration of cholesterol in sample calculate according to the equation

$$\textbf{Conc. of cholesterol mg/ dl} = \frac{\textbf{A sample}}{\textbf{A standard}} * \textbf{C}_{\textbf{standard}}$$

2.3.2.4 Determination of Serum Triglycerides (S.TG)

Serum triglyceride was determined by the enzymatic method [185] .

A. Principle of test

The method is based on the enzymatic hydrolysis of serum triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL) . The glycerol is phosphorylated by adenosine triphosphate (ATP) in the present of glycerol kinase (GK) to form glycerol – 3 – phosphate (G-3-p) and adenosine diphosphate (ADP) . G-3-p is oxidized by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide .

A red chromogen is produced by the peroxidase (POD) catalyzed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H_2O_2) , proportional to the concentration of triglyceride in the sample

$$\text{Triglycerides} + 3\,H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + 3\,\text{FFA}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-p} + \text{ADP}$$

$$\text{Glycerol-3-P} + O_2 \xrightarrow{\text{GPO}} \text{DHAP} + H_2O_2$$

$$4\text{-AA} + 4\,\text{phenol} \xrightarrow[POD]{H2O2} \text{Quinoneimine} + H_2O$$

B. Procedures

1- Bring reagent consisting of pipes PH 6.8 , Lipoprotein Lipase , Glycerol Kinase , Glycerol -3-phosphate oxidase , Peroxidase , 4-Aminoantipyrine , Adenosin triphosphate and phenol with samples to room temperature .

2- Pipette into labeled tube

Tubes	**Blank**	**Sample**	**Standard**
Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 µl	-
Standard	-	-	10 µl

3- Mix and let the tubes stand 5 minutes at 37^{o} C.

4- Read the absorbance (A) of the samples and the standard at 500 nm against the regent blank .

The concentration of Triglycerides in sample was calculated according to the equation

$$\textbf{Conc. of triglycerides mg/ dl} = \frac{\textbf{A sample}}{\textbf{A standard}} * \textbf{C}_{\textbf{standard}}$$

2.3.2.5 Determination of serum High Density Lipoprotein cholesterol (HDL-C)

Serum HDL-cholesterol was determined by Reflotron system .

A. Principle of test

The reflotron plus clinical chemistry analyzer is in a vitro diagnostic device designed for the quantitative determination of clinical chemistry parameter using reflotron test reagent strips . After the application of sample to the reagent carrier (strip) , LDL fraction and chylomicrons are precipitated by means of magnesium ions and dextran sulphate. [186]

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterse}} \text{cholesterol} + \text{RcooH}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + H_2O_2$$

$$H_2O_2 + \text{indicater} \xrightarrow{POD} \text{dye} + H_2O$$

B. Procedures

1- Thirty-two µl of sample material were drawn into the pipette and applied adrop to the center of the yellow application zone.

2- The flap or sliding cover of reflotron device was opened .

3- The test strip was put into the guide within 15 sec and slid forward horizontally until it locks into place .

4- The sliding cover was closed then reading the result after 85 sec .

Figure 2.2 : Reflotron analyzer

2.3.2.6 Calculation of low density lipoprotein cholesterol (LDL-C)

Concentration of LDL-C was calculated mathematically from the total cholesterol , the triglycerides , and the HDL-C concentration using Fried Wald's formula equation .[187]

LDL-C = Total cholesterol – (HDL-C + triglycerides / 5)

2.3.2.7 Calculation of very low density lipoprotein cholesterol (VLDL-C)

Concentration of VLDL-C was calculated as one – fifth of the serum TG [188] .

VLDL – C (mg/dL) = TG / 5

2.3.2.8 Determination of serum level 25 (OH) D_3

Concentration of 25 (OH) D_3 was determined by using cobas analyzer .

A. Principle of procedure of test

Total duration of assay 27 minutes

- First incubation is by incubating 15 µl of the sample with pretreatment regent 2 (sodium hydroxide) , bounded 25 (OH) D_3 is released from the vitamin D binding protein .

- Second incubation is by incubation the pretreated sample with ruthenium labeled vitamin D binding protein , a complex between the vitamin D and the ruthenylated vitamin D binding protein is formed .

- Third incubation is after addition of streptavidin coated microparticles and 25 (OH) D_3 labeled with biotin , unbound ruthenium labeled vitamin D binding proteins become occupied . A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated 25(OH $)_3$ D is formed and becomes bound to the solid phase via interaction of biotin and streptavidin .

- The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode . Unbound substances are then removed with procell . Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier [189]. Results are determined via a calibration curve which is instrument specifically generated by 2 – point calibration and a master curve provided via the reagent barcode .

Figure 2.3 : Cobas e 411

2.3.2.9 Determination of serum Para Thyroid Hormone (PTH) level

Concentration of (PTH) was determined by using cobas analyzer .

A- Principle and procedure test

The total duration of assay is 18 minutes

- First incubation is 50 µl of sample , abiotinylated monoclonal PTH – specific antibody and monoclonal PTH-specific antibody labeled with aruthenium complex from a sandwich complex .

- Second incubation is after addition of streptavidin – coated microparticles , the complex becomes bound to the solid phase via interaction of biotin and streptavidin .

- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode . Unbound substances are then removed with pro cell . Application of a voltage to the electrode then induces chemiluminescent emission which is measured by aphotomultiplier .[190]

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

2.3.2.10 Determination of serum Insulin level

A- Principle of test

According to the assay procedure of www. Demeditec .com , the Demeditec insulin ELISA kit was a solid phase Enzyme – Linked Immunosorbent Assay (ELISA) based on the sandwich principle . The microtiter wells were coated with amono clonal antibody directed towards a unique antigenic site on the insulin molecule . An aliquot of a patient sample containing endogenous insulin was incubated in the coated well with enzyme conjugate , which was an anti-insulin antibody conjugated with biotin. After incubation , the unbound conjugate was washed off . During the second incubation step, streptavidin – peroxidase Enzyme complex binds to the biotin – anti insulin antibody . The amount of bound HRP complex was proportional to the concentration of insulin in the sample . Added the substrate solution , the intensity of color developed was proportional to the concentration of insulin in the patient sample [191].

B-Procedures

Insulin was measured by using the (ELISA) kit .

1- Twenty - five µl of each standard , control and sample was added per well .

2- Twenty - five µl of enzyme conjugate was added to each well and incubated for 30 minutes at room temperature .

3- Microwell strip was washed 3 time with 400 µl per well of wash solution and aspirated .

4- Fifty µl of enzyme complex was added to each well and incubated for 30 minutes at room temperature .

5- Microwell strip was washed 3 times with 400 µl per well of wash solution and aspirated .

6- Fifty µl of substrate was added to each well and incubated for 15minutes at room temperature .

7- The enzyme reaction stopped by adding 50 µl of stop solution to each well .

8- The absorbance of each well was determined at 450 nm with a microtiter plate reader .

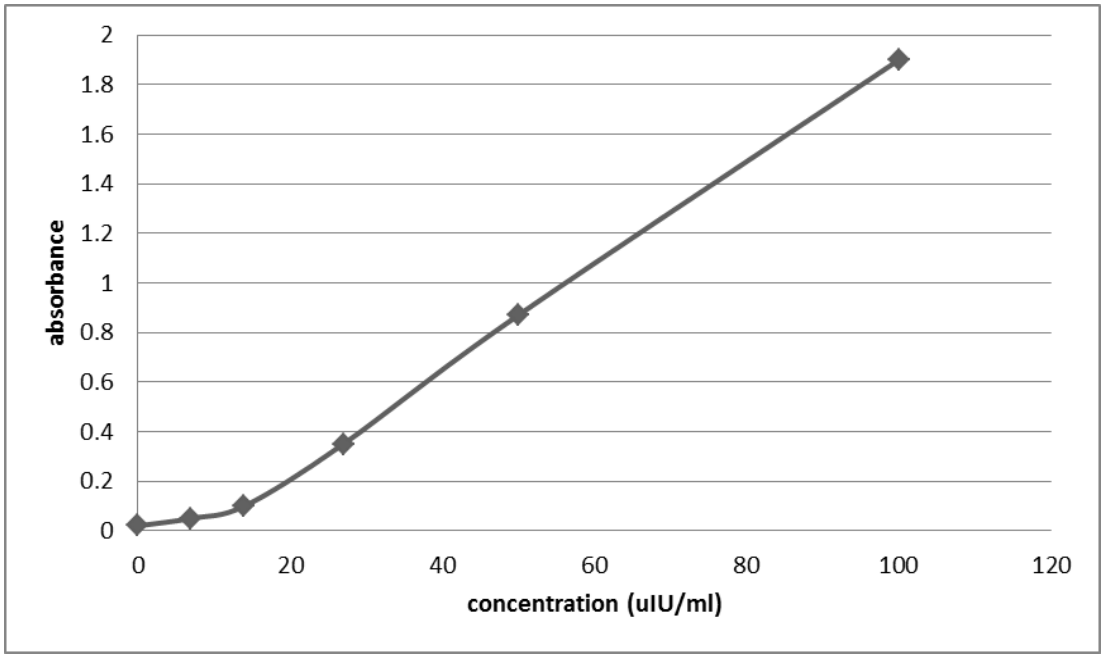

Figure 2.4 : Standard curve of Insulin

2.3.2.11 Calculation of Insulin resistance (IR)

HOMA –IR was calculated using the equation [192]

HOMA – IR = (FPI * FPG) / 405

2.3.2.12 Determination of Insulin – like Growth Factor (IGF1)

Concentration of IGF-1 was determined by using ELISA kit.

A. Principle of test

The kit assay Human IGF-1 level in the sample uses purified Human IGF-1 antibody to coat microtiter plate wells , make solid – phase antibody , then we add IGF-1 to the wells , combined antibody with which HRP labeled , become antibody –antigen –enzyme –antibody complex , after washing completely .We add TMB substrate solution . TMB substrate becomes blue color at HRP enzyme – catalyzed , reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometerically at a wave length of 450 nm. The concentration of IGF-1 in the sample is then determined by comparing the O.D of the sample to the standard curve [193].

B. Procedures

1- Serum – coagulation at room temperature 10 – 20 minutes , centrifugation 20 minutes at the speed of 2000 r.p.m remove supernatant.

2- Sample dilution 40µl was added to 10 µl of testing sample (sample final dilution is 5- fold.

3- This was incubated for 60 min and 37 ℃.

4- Twenty fold wash solution was diluted 20 fold with distilled water and reserved.

5- Washing buffer was added to well , still for 30 sec then drain, repeated 5 times, and dried by pat.

6- Chromogen solution A 50 µl and chromogen solution B were added to well, the light preservation was evaded for 15 min at 37 ℃.

7- Stop solution 50 µl was added to well , stop the reaction (the blue color changed to yellow color).

8- Read absorbance at 450nm after adding stop solution and with 15 min.

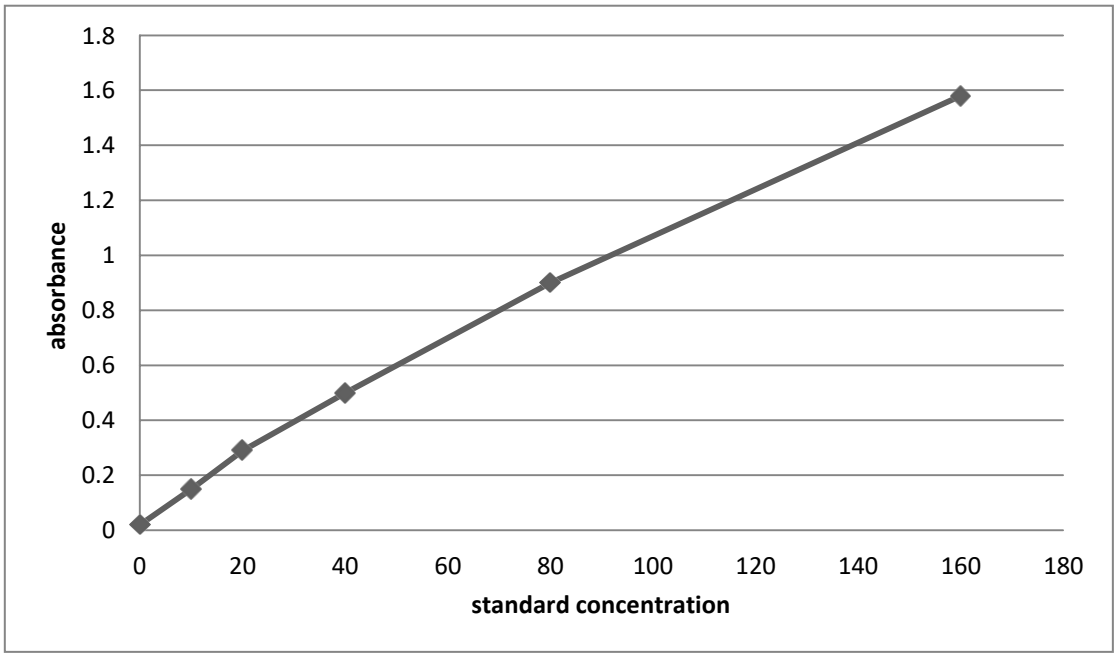

Figure 2.5: Standard curve of Insulin Like Growth Factor 1 (IGF 1)

2.3.2.13 Determination of serum Calcium level

The serum level of calcium was determined by enzymatic colorimetric method .

A- Pinciple of test

The method is based on the specific binding of O - Cresolftalein Complexone (OCC) , a metallochromic indicator and calcium at alkaline pH with the resulting shift in the absorption wave length of the complex . The intensity of the cromophore formed is proportional to the concentration of total calcium in the sample. [194]

$$\text{OCC} + \text{Calcium} \xrightarrow{\text{PH10.7}} \text{OCC- Calcium complex}$$

B- Procedures

1- Bring reagents consisting of OCC indicator (O - Cresolftalein Complexone ,HCL, 8-quinolinol) and OCC buffer PH10.7 and sample at room temperature.

2- Pipette into labeled test tube .

Tubes	**Blank**	**Sample**	**Standard**
Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 µl	-
Standard	-	-	10 µl

3- Mix and let the tubes stand 2 minutes at room temperature .

4- Read the absorbance (A) of the sample and the standard at 570 nm against the blank . the concentration of calcium in sample calculate according to the equation :

$$\textbf{Conc. Of calcium (mg/dl) } = \frac{\textbf{A sample}}{\textbf{A standard}} * \textbf{C}_{\textbf{standard}}$$

2.3.2.14 Determination of serum inorganic Phosphorus level

Serum level of inorganic phosphorus was determined by using enzymatic colorimetric method.

A- Principle of test

Inorganic phosphate reacts with molybdic acid forming phosphomolybdic complex. Its sub sequent reaction in alkaline medium originates a blue molybdenum color which its intensity is proportional to the amount of phosphorus present in the sample [195].

$$PO_4^{3-} + H^+ + (NH_4)_6 MO_7 O_{24} \longrightarrow \text{phosphmolybdic complex}$$

$$\text{phosphmolybdic complex} \xrightarrow{PH > 10} \text{Molybdenum blue}$$

B- Procedures

1- Bring reagents consisting of molybdate reagent , reducting solution (hydroxylamine) and color developer (sodium hydroxide) with sample at room temperature.

2- Pipette into labeled test tubes.

Tubes	**Blank**	**Sample**	**Standard**
Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	50µl	-
Standard	-	-	50µl

3- Mix and let stand for 1 minute and than addition of 0.5ml of color reagent to each tube.

4- Mix and let the tubes stand 10 minutes at room temperature .

5- Read the absorbance (A) of the sample and the standard at 740 nm against the blank.

The concentration of phosphorus in the sample was calculated according to the equation:

$$\textbf{Conc. of inorganic phosphorus (mg /dl)} = \frac{\textbf{Asample}}{\textbf{A standard}} * \mathbf{C_{standard}}$$

2.3.3 Molecular analysis

2.3.3.1 DNA extraction

The following is for the purification of DNA from 100µL whole blood [196].

1- Added 400µl of genomic lysis buffer to 100 µl of blood (4:1). Mixed completely by vortexing 4-6 seconds ,then let stand 5-10 minutes at room temperature .

2- Transferred the mixture to a zymo-spin column in a collection tube and centrifuge at xg rpm , discarded the collection tube with the flow through.

3- Transferred the zymo-spin column to a new collection tube. Added 200 µl of DNA pre wash buffer to the spin column. It centrifuges at xg rpm.

4- Added 500µl of DNA wash buffer to the spin column. It centrifuges at 10000 rpm.

5- Transferred the spin column to a clean micro centrifuge tube , and added ≥50µl DNA elution buffer or water to the spin column . In

cubated 2-5 minutes at room temperature and the centrifuge at top speed for 30 second to elute the DNA.

2.3.3.2 Determination of DNA concentration and purity

Electrophoresis has been done to determine DNA pieces after the process of extraction.

2.3.3.2.1 Prepare of the agarose gel

The agarose gel has been made in 1.5 % concentration by melting 1.5 gm of agarose in 100 ml of previously made tris –borate EDTA (TBE) buffer . Agarose has been heated to boil then left to cool down at (45-50 ℃). The gel has been poured in the pour plate in which the plate of agarose support has been prepared after fixing the comb to make holes that would hold the samples. The gel has been poured gently not to make air bubbles and left 30 minutes to cool down . The comb has been removed gently of the solid agarose . The plate has been fixed to its stand in the electrophoresis horizontal unite represented by the tank used in the electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface [197].

2.3.3.2.2 Preparation of samples

The processor loading buffer (3 µl) has been mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye), after the mixing process, the process of loading is now to the holes of the gel. An electric current of 5 v /c^2 has been exposed for 1: 15 hours till the tincture has reached to the other side of the gel.

The gel has been tested by a source of the UV with 336nm after putting the gel in pool which contains 30µl red safe nucleic acid staining solution and 500 ml from distilled water [180].

2.3.3.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a small number of copies of a piece of DNA [198].

There are three main steps involved in the PCR technique : denaturation, annealing and extension [197].

2.3.3.3.1 preparation of primers

The primers were lyophilized , they dissolved in the free ddH_2O to give a final concentration of 100 p mol \ µl as stock solution and kept at -20˚c to prepare 10 pmol \ µl concentration as work primer suspended , 10 µl of the stock solution in 90µl of the free ddH_2O water to reach a final volume 100µl .

2.3.3.3.1.1 The specific primer rs (10735810) of gene

The sequencing of primer which used to identification the rs (10735810) fragment is illustrated in table 2.4 .[199]

Table 2.4: Sequence of primer rs (10735810) of gene [199]

Primer	Sequence	Tm (℃)	GC (%)	Product size
Forward	5-AGTGGCCCTGGCACTGACTCTGGCTCT-3	68.6	63	270 Base pair
Reverse	5-ATGGAAACACCTTGCTTCTTCTCCCCTC-3	60.5	48.1	

2.3.3.3.1.2 The specific primer rs (1544410) of gene

The sequencing of primers which used to identification the f rs (1544410) ragment is illustrated in table 2.5 [199] .

Table 2.5: Sequence of primer rs (1544410) of gene [199]

Primer	Sequence	Tm (℃)	GC (%)	Product size
Forward	5- CAACCAAGACTACAAGTACCGCGTCAGTGA-3	62.8	50	820 base pair
Reverse	5- AACCAGCGGGAAGAGGTCAAGGG-3	63.3	60.9	

A- Maxime PCR pre mix kit (i-Taq)

Maxime PCR premix kit has not only various kinds of premix kit according to experience purpose , but also a 2X master mix solution . Maxime PCR premix kit (i-Taq) is the product which is mixed every component : i-Taq DNA polymerase , dNTP mixture , reaction buffer and so on – in one tube for 1 rxn PCR .[200]

Table 2.6: The components of Maxime PCR premix kit (I – Taq)

Material	Volume
i-Taq DNA polymeras	5 U/µl
DNTPs	2.5 Mm
Reaction buffer (10x)	1x
Gel loading buffer	1x

B- Diagnosis of Gene

Optimization of Polymerase Chain Reaction was accomplished after several trials .

The following mixtures were adopted table 2-7

Table 2-7: Mixture of the specific interaction for diagnosis gene

Components	**Volume**
Taq PCR premix	5µl
Forward primer	10 picomols/µl
Reverse primer	10 picomols / µl
DNA	1.5 µl
Distill water	16.5 µl
Final volume	25µl

The optimal condition has identified for initial denaturation and annealing after doing several experiments to gain this condition . The temperature has changed through the work for all samples to select the optimal condition , and also changed the concentration for DNA template between (1.5 – 2 µl) since we considered these two factors as important factors in primer annealing with complement .

Table 2-8: The optimum condition of detection rs (10735810) and rs (1544410).

NO	Phase	Tm (℃)	Time	No. of cycle
1	Initial denaturation	95 ℃	3 min	1 cycle
2	Denaturation 2	95 ℃	45 sec	35 cycle
3	Annealing	68 ℃	45 sec	
4	Extension . 1	72 ℃	45 sec	
5	Extension . 2	72 ℃	7 min	1 cycle

The PCR products from amplification of rs (10735810) and rs (1544410) was electrophoresed on 2% agarose gel stained with red safe stain .

2.3.3.4 Restriction fragment length polymorphism (PFLP)

Restriction fragment length polymorphism (PFLP) is a popular technique for genetic analysis. RFLP is used to simultaneously detect the presence of rs (10735810) and rs (1544410) polymorphism .

The PCR product was subjected to RFLP using a rs (10735810) restriction endonuclease and rs (1544410) restriction endonuclease. The digestion reaction is carried out in a 0.5 ml sterile eppendrof tube , where 5µl of product PCR is mixed with 0.5 µl of Restriction Enzyme (R.E), 4.5 µl buffer. Tables 2.9 , 2.10.[(201)]

Table 2.9 : Reaction condition of restriction enzyme rs (10735810) .

Protocol	**Volume**
Product PCR	5 µl
R.E	0.5 µl
Buffer	4.5 µl
Temperature	37 ℃ /30 min

Table 2.10: Reaction condition of the restriction enzyme rs (1544410) .

Protocol	**Volume**
Product PCR	5 µl
R.E	0.5µl
Buffer	4.5µl
Temperature / Time	65 ℃ / 40 min

The digestion products electrophoretically separated on a 3% agaros gel stained with Red Safe .

Chapter Three

Results and Discussion

Results and Discussion

This study included the changes in some clinical parameters in Gestational Diabetes Mellitus patients with controls and genotyping of vitamin D receptor rs (10735810) and rs (1544410) .

3.1 Age and Body Mass Index

The mean value of age for Gestational Diabetes Mellitus (GDM), normal pregnant and controls is shown in figure 3.1.

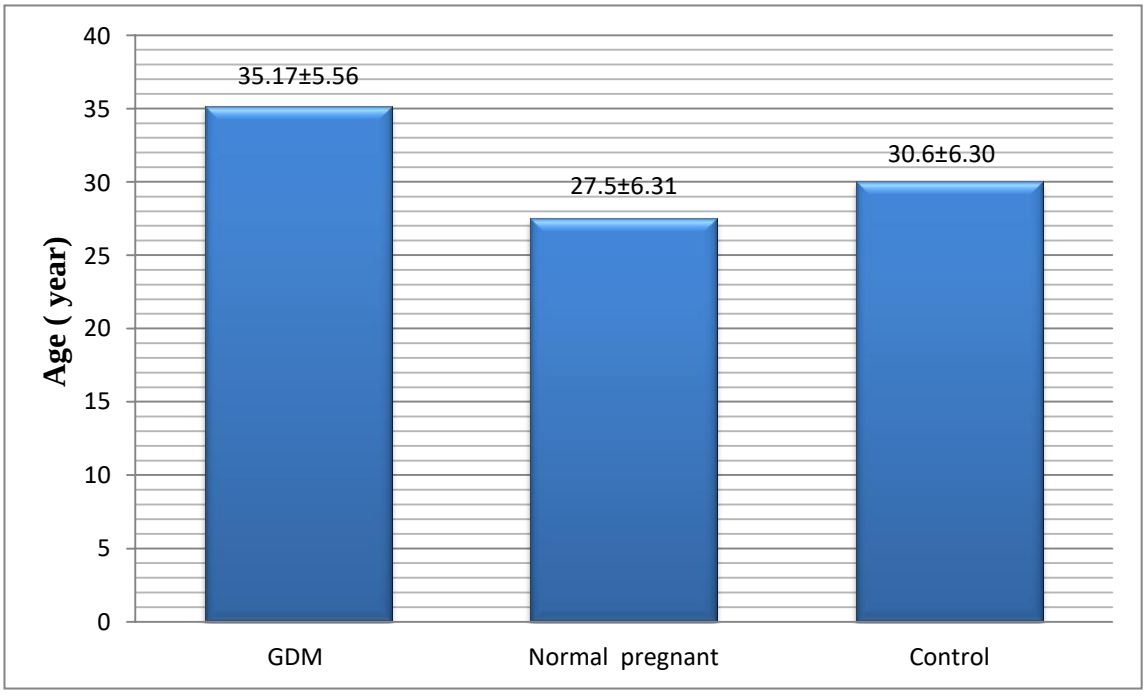

Figure 3.1: Mean age for GDM , normal pregnant and control .

The mean (± SD) value of age of GDM (35.175 ± 5.56) years was significantly higher than that of pregnant without GDM (27.5 ± 6.31) years and control (30.6 ± 6.30) years. The last significant difference LSD $_{p \leq 0.05}$ was 2.95. The diagnosis of GDM usually occurs after the age of 30 years[145].

The mean value of BMI was significantly different between the study groups. It was significantly higher in GDM (30.723 ± 4.108) kg/m^2 compared to pregnant without GDM (26.569 ± 2.438) kg/m^2 and control (24.196 ± 3.298) kg /m^2 LSD $_{P \leq 0.05}$ = 1.686 shown in figure 3.2 below :

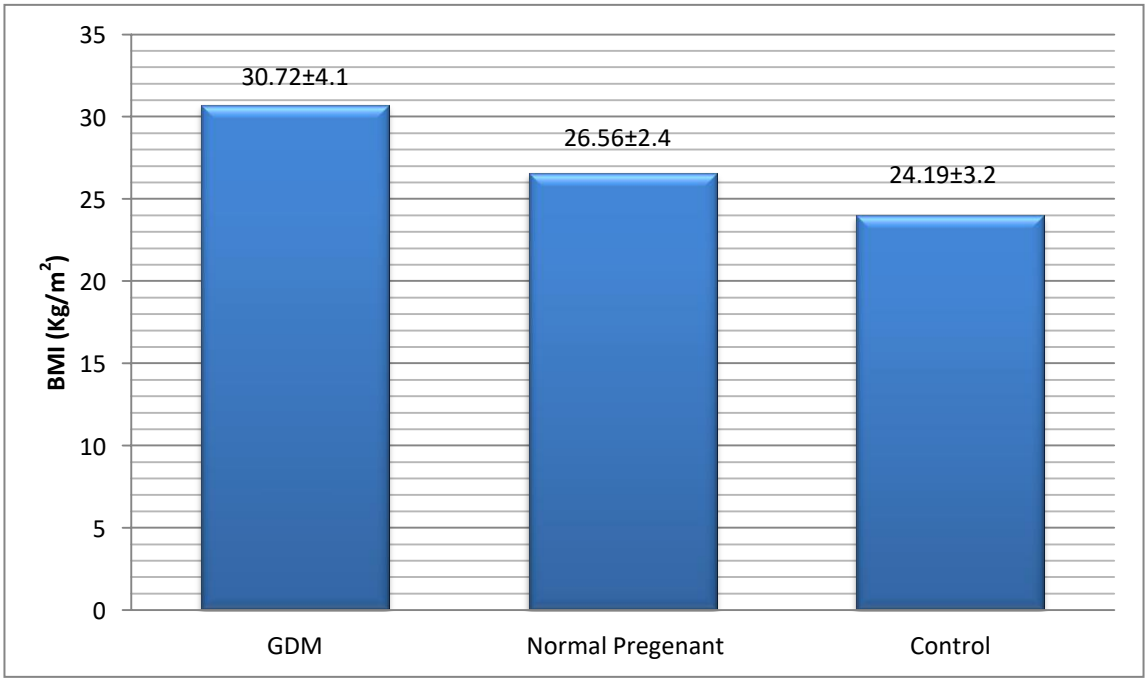

Figure 3.2: The mean value of BMI for GDM , normal pregnant and control .

BMI provides a reliable indicator of body fatness for most individuals and BMI is used as a screen for weight categories that may lead to health problems [(202)]. In the present study, BMI was significantly associated with GDM compared to normal pregnant. Increasing maternal BMI is a significant risk factor for the development of GDM [(203-205)].

Even though the association between BMI and GDM can still be used to counsel women about their risk of developing GDM, BMI as a screening tool does not have high enough sensitivity and specificity to identify a group of women that should not receive GDM diagnostic

testing. This continues to support the notion for continuing universal screening programs for pregnant women rather than stratifying by BMI[(206)].

3.2 Glucose index in study groups.

The average mean of Fasting Blood Glucose (FBG), Glycated hemoglobin (HbA1c), Insulin hormone and Insulin Resistance (HOMA IR) was obviously higher in GDM compared with controls.

3.2.1 Fasting Blood Glucose (FBG)

The mean (± SD) value of FBG was significantly different between the study groups. It was significantly higher in GDM (145.950 ±35.88) mg/dl compared to pregnant without GDM (89.400 ± 9.03) mg/dl and control (86.24 ± 7.69) mg/dl LSD $_{p \leq 0.05}$ = 11.6,as shown in figure 3.3 .

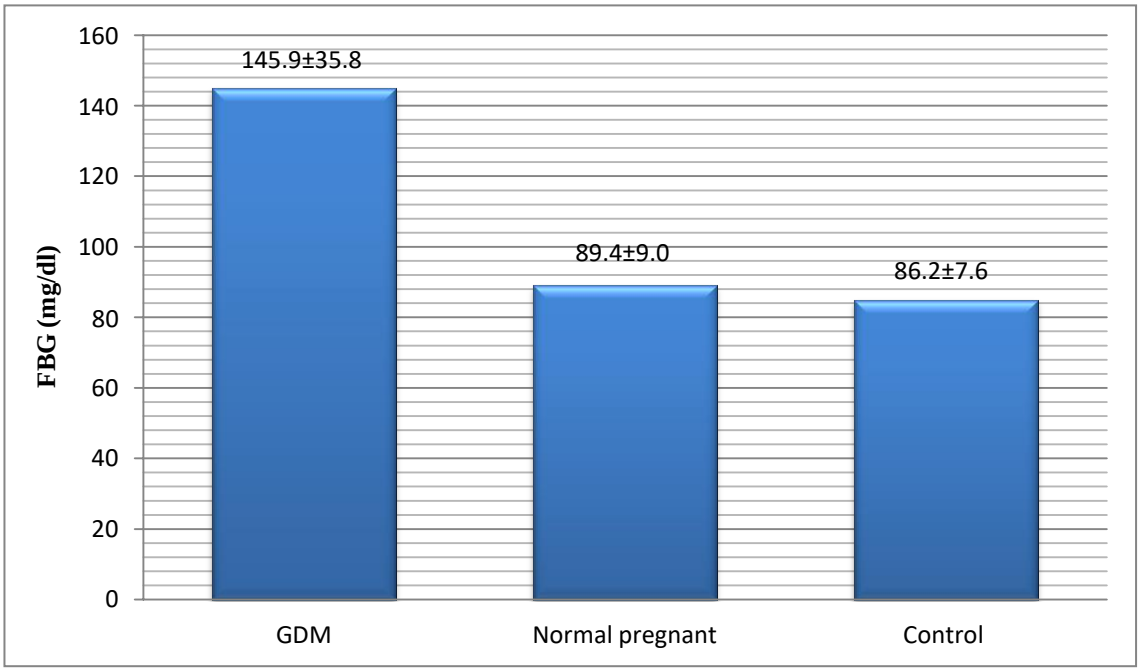

Figure 3.3: Mean of FBG in GDM and normal pregnant compared with control .

The results correspond with the findings of previous studies. FBG values tend to stay constant throughout the entire period of pregnancy . FBG values have less individual variation compared to other glucose values, therefore, abnormal FBG level is a significant indicator in diagnosing GDM. FBG is a good screening test for GDM with advantages such as simple procedure, reasonable cost, reproducibility, easy access, and acceptance[207].

Other studies have reported that abnormal FBG alone is capable of detecting 50% of pregnant women who had already been diagnosed with DM with another screening method [208] .

3.2.2 Glycated hemoglobin (HbA1c)

The mean value of HbA1c was obviously higher in GDM (7.082 ± 1.667 %) respectively as compared with normal pregnant (5.04 ± 0.430%) and control (3.014 ± 0.362 %), LSD $_{p \leq 0.05}$ = 0.539 as shown in figure 3.4

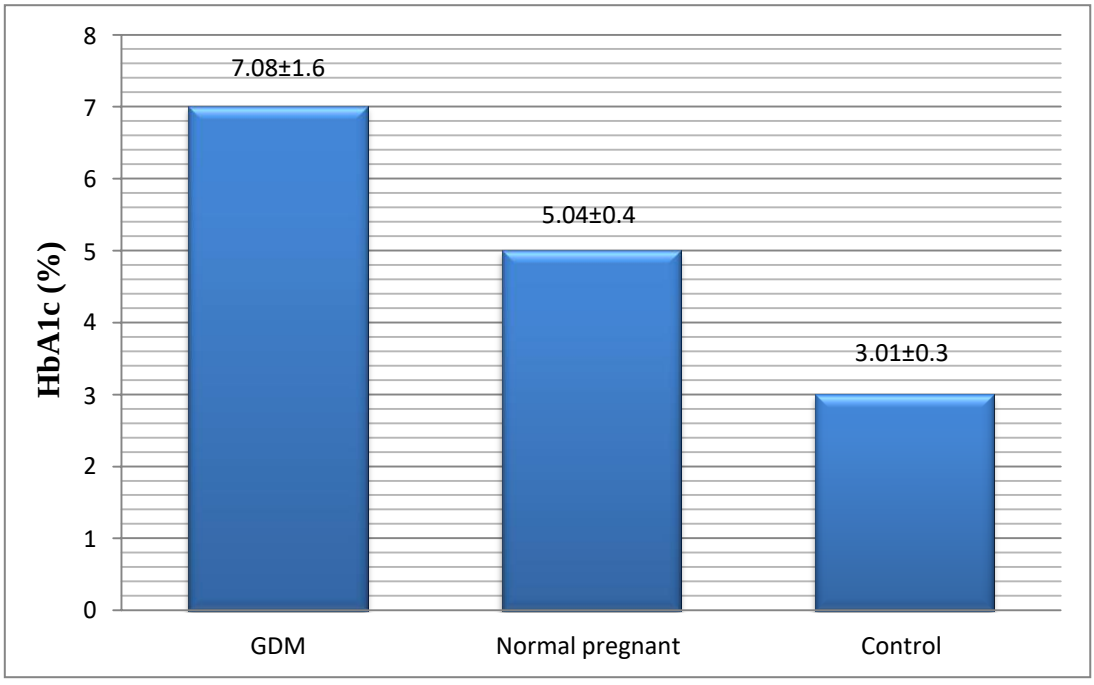

Figure 3.4: Mean of HbA1c in GDM , normal pregnant and control .

This result is in agreement with the previous studies which proved that concentration of HbA1c was higher in GDM [145,209].

HbA1c test is currently considered to be the best measure and the gold standard for assessing glycemic control, it measures the amount of glucose that is bound to hemoglobin molecule, reflects average plasma glucose over the previous 2-3 months in a single measure which can be performed at any time of the day and does not require any special preparation such as fasting [210].

3.2.3 Insulin hormone

Serum Insulin lever was higher in GDM (14.783 ± 9.296) µIU/ml compared to normal pregnant (9.293 ± 4.636) µIU/ml and control (10.707 ± 4.245) µIU/ml, LSD $_{p\leq0.05}$ = 3.346 as shown in figure 3.5 below :

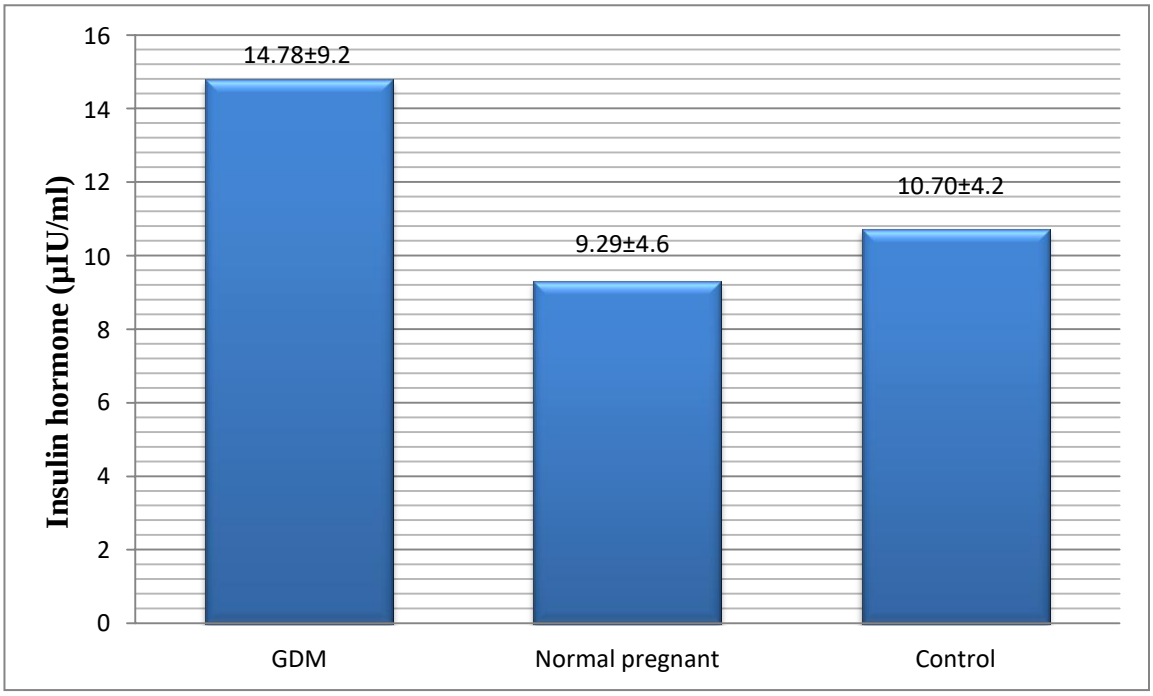

Figure 3.5: Mean of Insulin level in GDM, normal pregnant and control .

This result is agreement with the previous studies which proved that concentration of Insulin hormone was higher in GTM than in a normal pregnancy [211], women with GDM have an increase in Insulin production in the ß-cell of the pancreas [212].

β-cell adaption refers to the change that pancreatic islet cells undergo during pregnancy in response to maternal hormones in order to compensate for the increased physiological needs for mother and baby. These changes in the ß-cells cause increased Insulin secretion as a result of increased ß-cell proliferation [213].

Pregnancy causes increased Insulin resistance and so higher Insulin demand. The ß-cell must compensate this by either increasing insulin production or proliferating [214].

3.2.4. Insulin Resistance (HOMA -IR)

Homeostatic model assessment insulin resistance (HOMA- IR) was found of higher significance in gestational diabetes mellitus compared with controls group, and with mean 4.9123 ± 2.835% for GDM, 2.048 ± 1.0686 % for normal pregnant and 2.2457 ± 0.875 % for control , LSD $_{\leq 0.05}$ = 0.956 as shown in figure 3.6 .

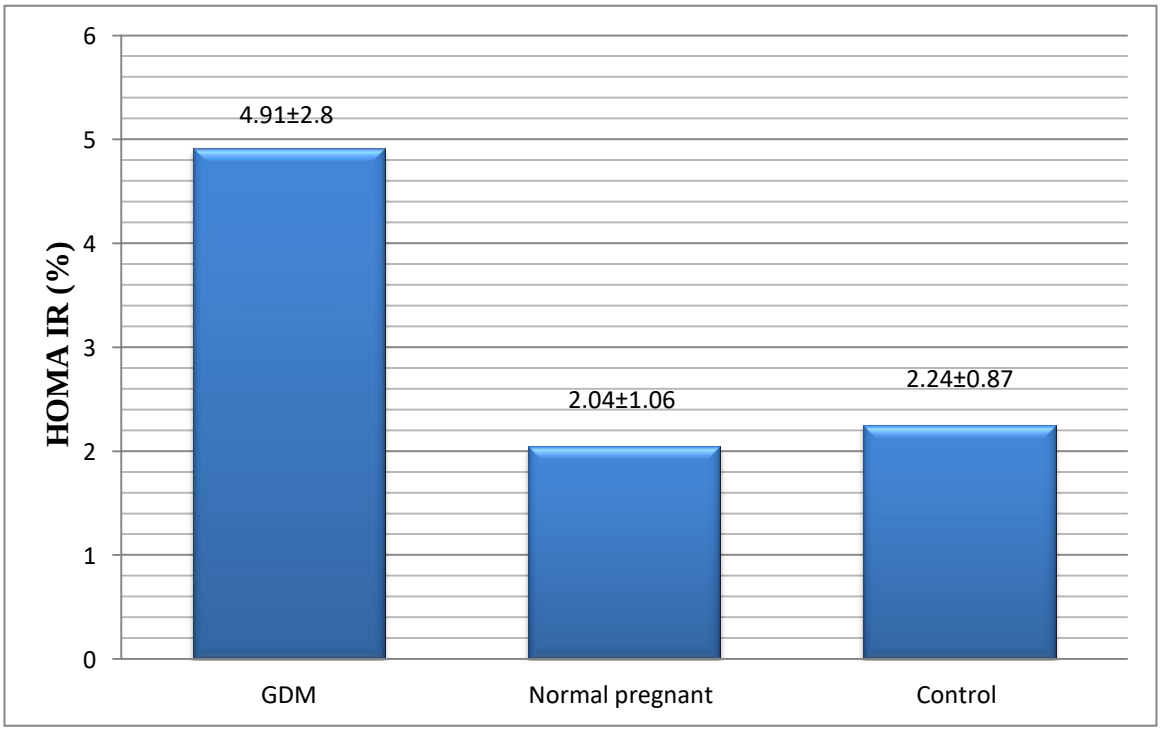

Figure 3.6: Mean of HOMA- IR in GDM , normal pregnant and control.

GDM can be regarded as the early pathogenesis of T2DM and it shares some physiological characterize [(215)].

Insulin resistance in pregnancy is consequent to the physiological adaption necessary to provide glucose to the growing fetus [(216)].

HOMA IR in women with GDM increased significantly during pregnancy [(217)], mainly in 2nd and 3rd trimesters of pregnancy [(218)].These findings are in agreement with a study performed in south Asians and Middle Eastern that showed gestational women were more insulin resistant as compared with western Europeans [(219)].

3.3 Lipid profile

3.3.1 Total serum cholesterol

In our study the results showed that the total serum cholesterol was significantly higher in GDM as compared with both normal pregnant without diabetes mellitus and control .

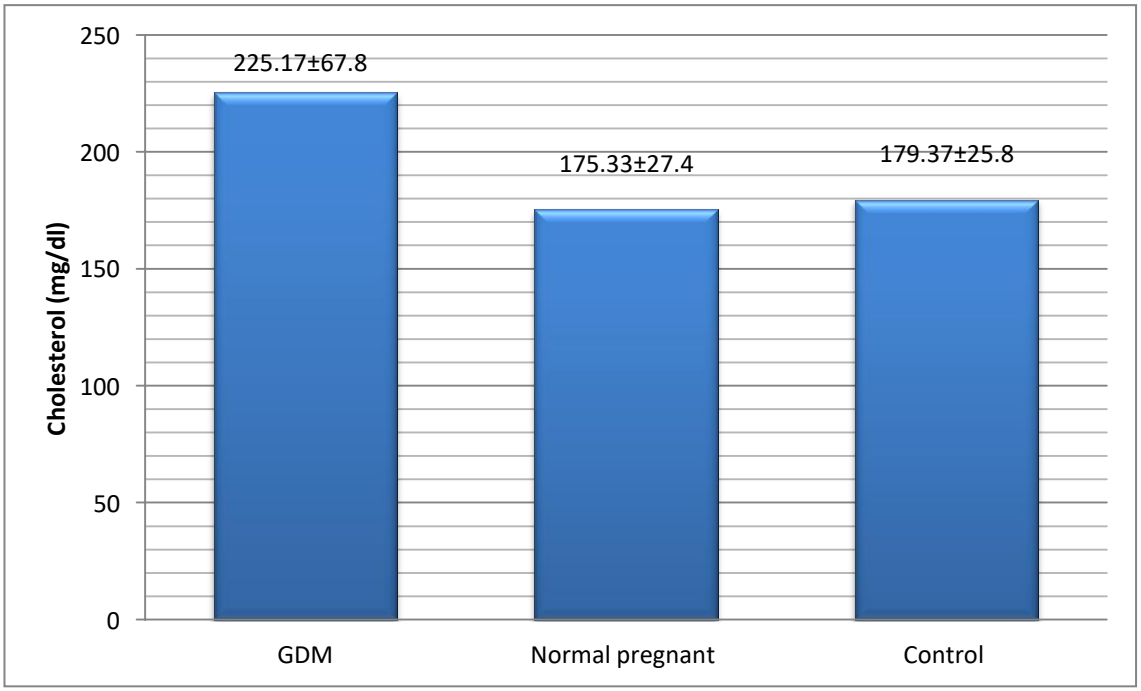

Figure 3.7: The mean of serum total cholesterol in GDM , normal pregnant and control .

The mean serum T-cholesterol was significantly higher in GDM (225.17 ± 67.82) mg/dl , than in pregnant without GDM (175.33 ± 27.40) mg/dl, and controls (179.37 ± 25.84) mg/dl LSD $_{p\leq 0.05}$ = 23.38.

The significant increase in total cholesterol concentration in GDM compared with controls in the study is a result of the fact that GDM significantly alters cholesterol metabolism leading to dyslipidaemia. These findings are consistent with reports by Amraei and Azemati [220], who

reported significant difference in total cholesterol levels between pregnancy complicated by GDM and normal pregnancy. In a study done in Pakistan on the lipid profile and serum insulin levels in gestational diabetes mellitus [221] , reported significantly higher total cholesterol levels in women with GDM than the controls. The results of this study are in agreement with reports by Asare- Anane ,et al [222]. who reported that there was increasing in levels of total cholesterol in GDM compared with controls.

3.3.2 Serum Triglycerides

The results of our study clearly indicate a high significance in the studied group, the mean of the serum concentration of triglyceride (TG) for GDM was 194.55 ± 45.27 mg/dl , while the mean serum of TG for pregnant without GDM was 164.56 ± 20.25 mg/dl , for control was 133.5 ± 23.03 mg/dl LSD $_{p\leq 0.05}$ = 16.3 as shown in figure 3.8

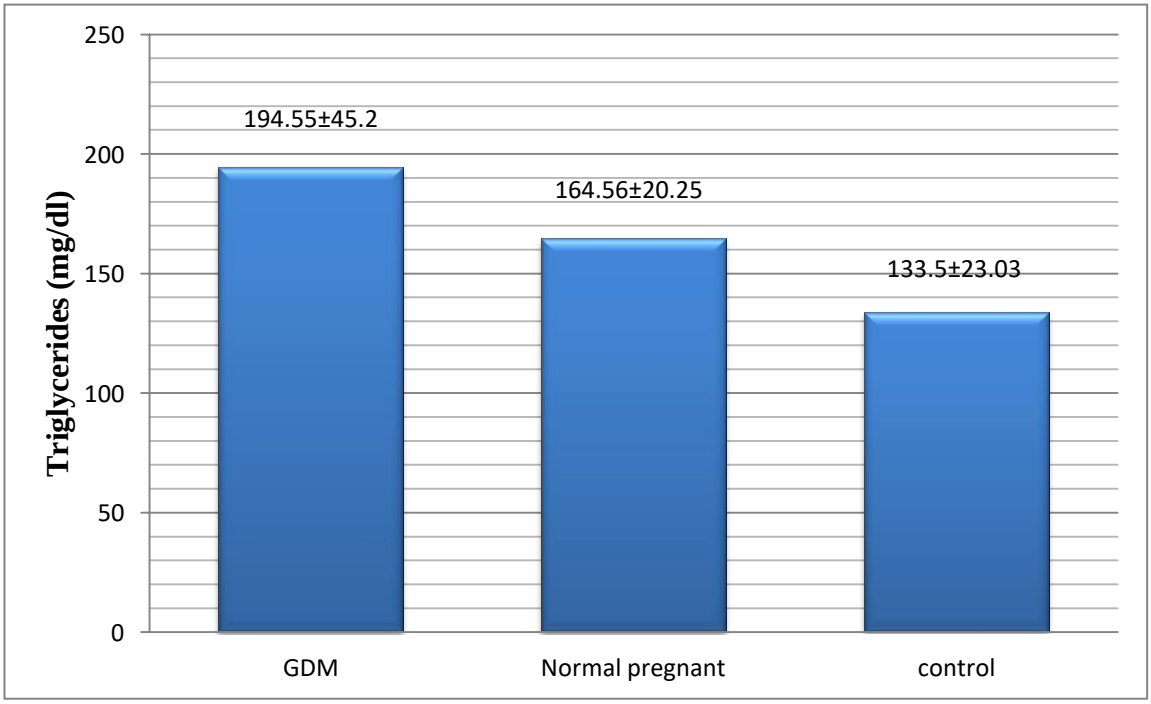

Figure 3.8: Mean of serum triglycerides in GDM ,normal pregnant and control.

Lipid profile changes in normal pregnancy are characterized by marked elevations of total serum triglyceride and total cholesterol levels as a result of increased liver synthesis of TG in response to elevated estrogen levels[223].

The results of this study agreed with a study by Amraei and Azemati[220], who reported a significant increase in the triglycerides levels in pregnancy complicated by glucose intolerance as compared to normal pregnancy and agreed with a study by Asare –Anane , et al [222].

3.3.3 Serum High Density Lipoprotein cholesterol (HDL - C)

Mean HDL-C for the GDM was significantly lower in GDM (38 ± 8.85) mg/dl compared to control (41.83 ± 8.01) mg/dl and did not find significance with normal pregnant women (35.567 ± 5.78) mg/dl $LSD_{p\leq 0.05}$=3.82,figure3.9

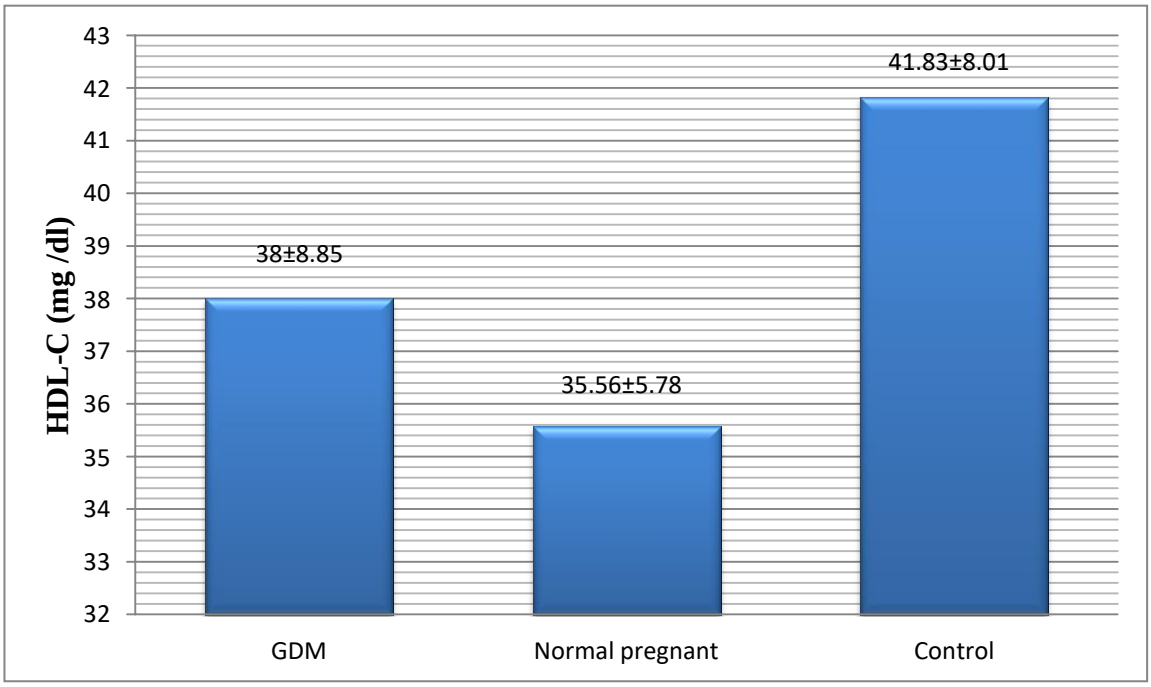

Figure 3.9: Mean of serum HDL-C in GDM, normal pregnant and control.

This result was consistent to the report by koukkou et al and Wiznitize et al [224,225] who did not find significant difference in HDL-C levels between GDM and normal pregnant women.

3.3.4 Serum low density lipoprotein cholesterol (LDL)

The results showed that the mean ± SD value of LDL -C level for the GDM (146.78 ± 65.49) mg/dl , was statistically significantly higher than normal pregnant (107.53 ± 29.28) mg/dl and control (111.06 ± 24.06) mg/d LSD $_{p\leq 0.05}$ =22.76 , as shown in figure 3.10 .

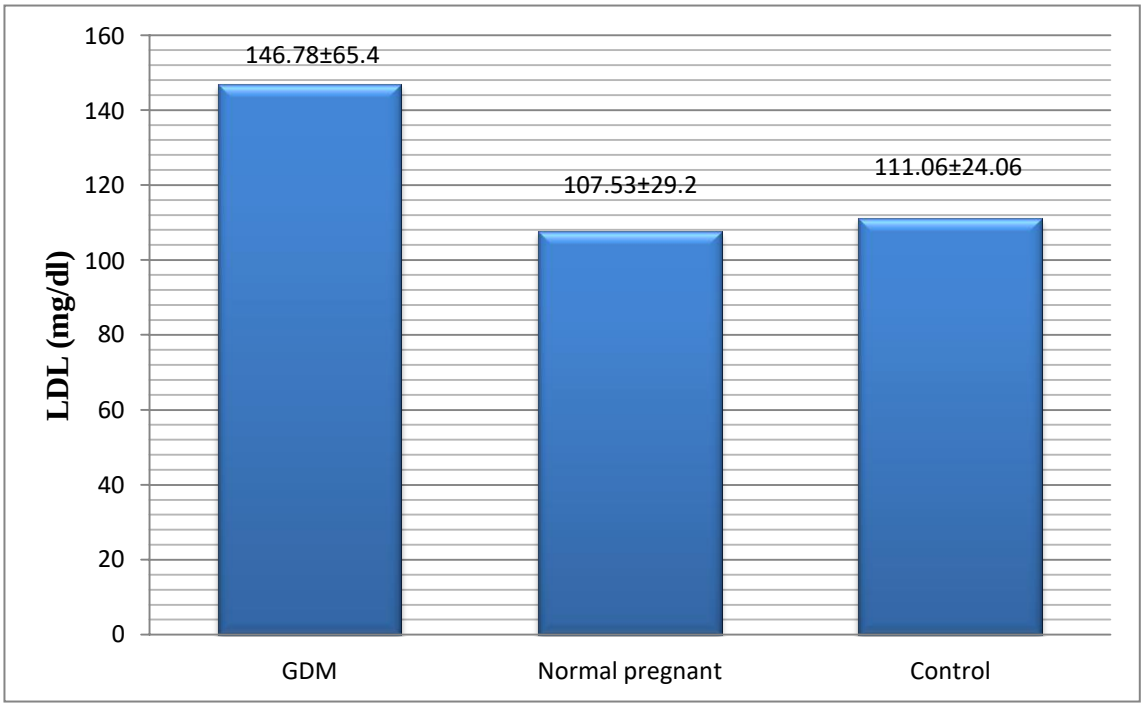

Figure 3.10: The mean of serum LDL-C in GDM as compared with normal pregnant and control .

LDL-C is formed from VLDL-C which is the principal transport form of triglyceride in the blood and so in GDM, when triglyceride concentration increases, LDL-C increases accordingly. In this study,

LDL-C was significantly higher in GDM than the normal gestational. These results are in agreement with previous reports an increases in LDL-C levels significantly during pregnancy complicated by GDM [220-222,226].

3.3.5 Serum Very Low Density Lipoprotein cholesterol (VLDL)

The results showed a mean ±SD value of VLDL-C level was 38.910 ± 9.055 mg/dl for the GDM, 32.9 ± 4.075 mg/dl for normal pregnant women and 26.37 ± 4.206 mg/dl for control, respectively LSD $_{p\leq0.05}$ = 3.224, as shown in figure 3.11 .

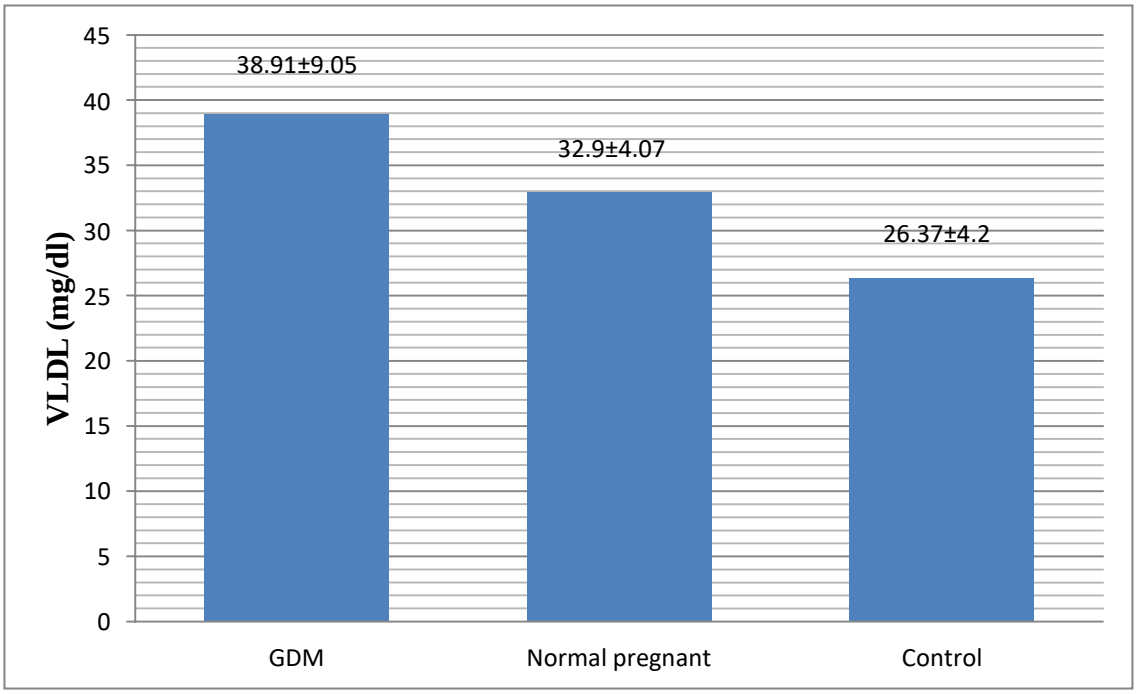

Figure 3.11: Mean of serum VLDL-C in GDM , normal pregnant and control.

VLDL-C levels for the GDM were significantly higher than in gestational without diabetes mellitus and controls. This could be as a result of the high triglycerides levels observed in this study. VLDL-C is formed

from triglycerides synthesized in the liver denova or by re-esterification of free fatty acids. Therefore, VLDL-C level increases when triglyceride levels increases. The results of this study are in agreement with reports by Amraei and Azamati, Aziz and Mahbob, Asare –Anane .[220-,222]

The increased levels of triglycerides, total cholesterol, LDL-C and VLDL-C observed in GDM are a result of increase fat storage [227] and progesterone[226] in the second trimester of pregnancy.

Elevation of triglyceride and decrease of HDL-C levels in diabetic patients were documented by several authers [228-231].

The general increase of levels of serum lipid in diabetic may be mainly attributed to increase in the mobilization of free fatty acid from fat tissue, then excess fatty acids in serum are converted into triglycerides, phospholipids and cholesterol in liver [232,233] .

3.4 Serum level of Parathyroid Hormone (PTH)

Serum PTH level in GDM group was 67.43 ± 12.10 pg /ml and 57.16 ±10.89 pg /ml in no diabetic gestational group, whereas in control group was 45.22 ± 8.79 pg /ml respectively , LSD $_{p\leq 0.05}$= 4.02 as shown in figure 3.12.

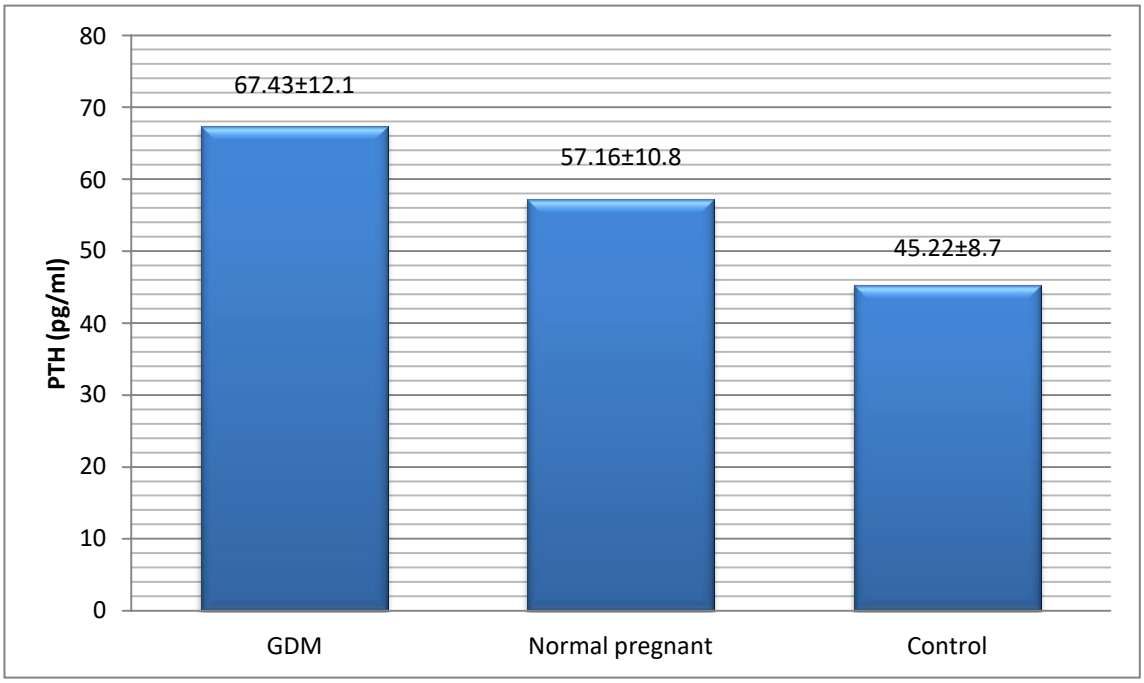

Figure 3.12: Mean of serum PTH in GDM , normal pregnant and control.

In our study, PTH levels was elevated in GDM group more than that in normal pregnant as compared with control . A previous study showed that PTH concentration was significantly higher in women with GDM as compared with pregnant without GDM [234,235]. Some studies had reported that increased PTH levels were associated with impaired glucose tolerance[236-238]. The net effect of PTH excess on regulation of lipolysis would most likely promote an increase in fat mass. Furthermore, the up-regulation of insulin secretion associated with PTH induced that insulin resistance would be expected to potentiate this effect[239,240].

3.5 Serum level of Vitamin D_3

In our study we have found high prevalence of deficiency in overall study sample. Given the high overall prevalence of vitamin D_3 deficiency, we did not find any statically significant difference in 25 (OH) D levels between women with GDM (6.83 ± 3.547) ng/ml and pregnant without GDM (8.73 ± 4.158) ng/ml LSD $_{P\leq 0.05}$ =2.161 as shown in figure 3.13.

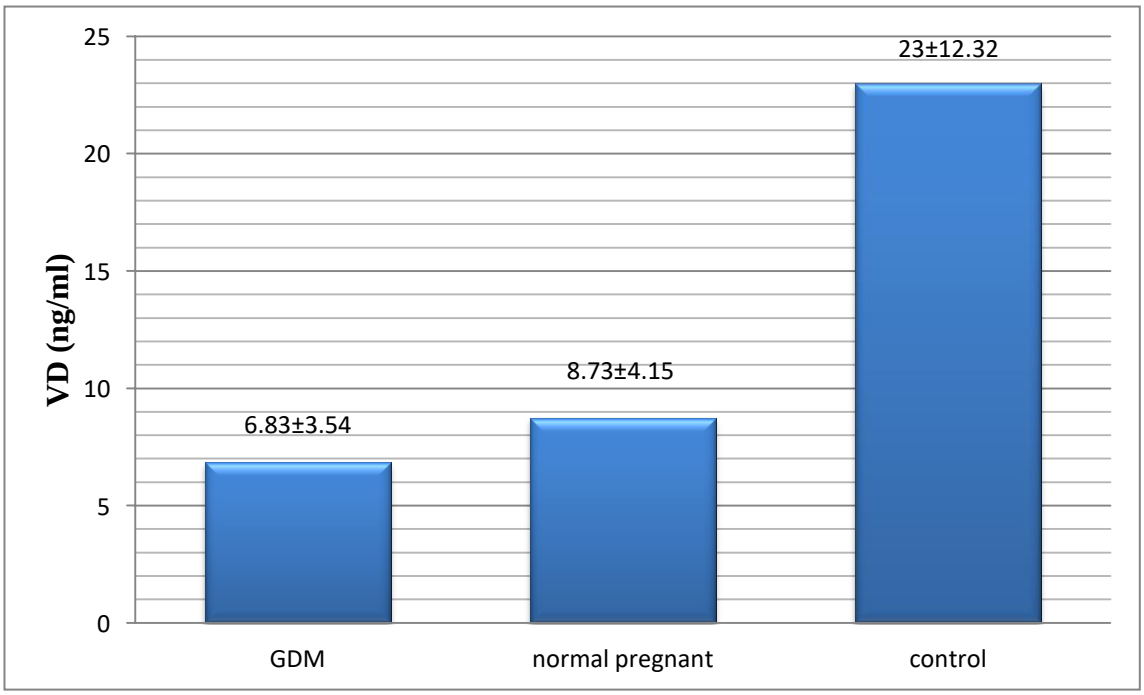

Figure 3.13: Mean of serum vitamin D_3 in GDM as compared with normal pregnant and control.

In accordance with other studies, our results imply that in a vitamin D deficiency population, the association between 25 (OH) D and GDM may not exist [241-243]. Similarity, Helena H ,et al ,[244] stated that GDM status was not associated with pregnancy 25 (OH) D, GDM is a multi-factorial disease involving various risk factors for example lifestyle factors, obesity, rapid weight gain and predisposing genetic factors. Furthermore, some of these factors are related with poor vitamin D

status[245,246], which further increases the challenge when dissecting independent effect. It is possible that in previous studies no adjustment for confounding factors was performed , the association between 25 (OH) D and GDM reflects shared factors such as unhealthy lifestyle or adiposity[247].

Yet, contrary to many studies, association between high 25 (OH) D and GDM have been reported [248,249]. Although a biological mechanism between low vitamin D status and diabetes is plausible [250], only a few interventions have been conducted, and these have not proved an effect of vitamin D supplementation on risk of GDM [251].

3.6 Serum level of Insulin-like Growth Factor 1 (IGF-1)

The mean (± SD) value of IGF-1 was not significantly different between GDM (99.350 ± 45.05) ng/ml and pregnant without GDM (90.517 ± 17.41) ng/ml. However, it was significantly higher than control (63.100 ± 21.62 ng/ml), $LSD_{p\leq 0.05}$ =15.86 as shown in figure 3.14 .

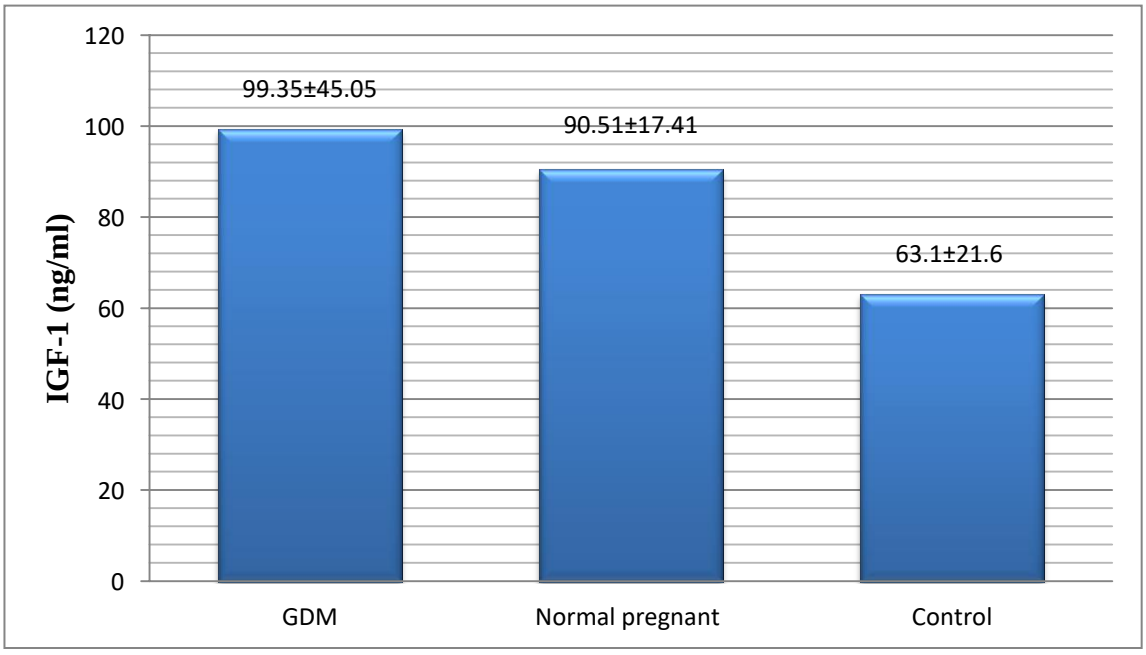

Figure 3.14: Mean of IGF-1 in GDM ,normal pregnant and control.

During pregnancy, IGF-1 is important for growth of fetal and maternal tissues [252]. In pregnancy, placental growth hormone stimulates maternal production of IGF-1 [253,354], that could promote placental development and transfer of nutrients to the fetus [255]. Thus, some studies have reported that low maternal IGF-1 is associated with low birth weight [256], but the association could not conformed by others [253,257,258]. The biologic activity of IGF-1 is influenced by IGF binding proteins that leave only a fraction of IGF-1 in its biologically active form. Maternal insulin-like growth factor binding protein (IGFBp-1) increases early in pregnancy[258-260].

3.7 Serum level of Calcium ion (Ca^{+2})

The data of this study shows that there is significant differences in Ca^{+2} level between various groups. The mean ± SD for GDM was 7.855 ± 0.834 mg/dl , for normal pregnant was 8.503 ± 0.735 ng/d, while for control was 9.136 ± 0.630 ng/dl, $LSD_{p \leq 0.05}$ = 0.367. as shown in figure 3.15.

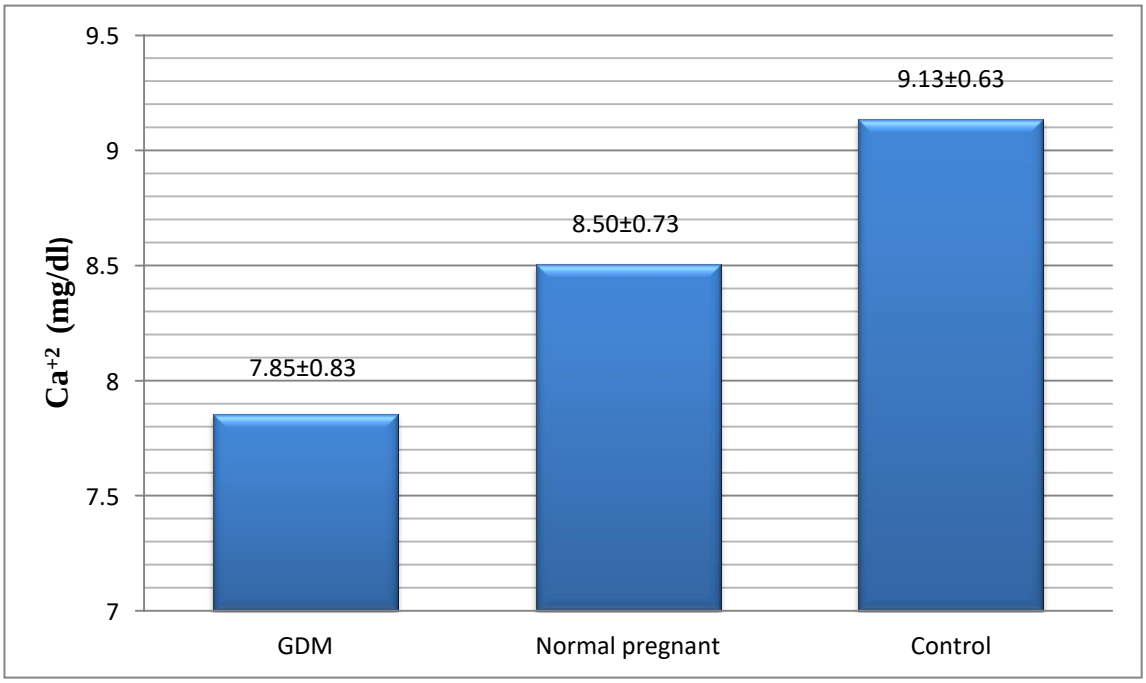

Figure 3.15: Mean of serum Calcium ion level in GDM, normal pregnant and control.

As indicated in the current study, we found an association between Ca^{+2} and gestation [261-263]. Calcium is actively transported across the placenta, with the transfer from mother to fetus beginning by week 12 of gestation and peaking at weak 36 [264] .

Placental calcium transport is dependent upon transport protein located in the syncyiotropholblast, which forms a barrier between the mother and fetus [248].

Serum Ca^{+2} in GDM is lower than normal pregnant, this result agrees with another study performed by Yanez CO et al [266], that proves higher levels of maternal periconceptional dietary calcium intake which are associated with lower GDM risk. Previous studies focused on dairy foods instead of calcium intake in GDM risk [267,268]. Our findings are largely consistent with existing literature reporting associations of calcium

intake with lower risk of incident type2 diabetes in non-pregnant women[269,270]. The biological mechanisms underlying associations of diabetes risk and dietary calcium might involve regulation of intracellular calcium affecting both insulin sensitivity and insulin release [271], as well as appetite regulation and related fat intake [272].

3.8 Serum level of inorganic phosphorus ion $(PO_4)^{-2}$

Serum phosphorus ion value was lower in each of GDM (3.672 ± 0.626) mg/dl and normal pregnant (3.51 ± 0.342) mg/dl compared to control (4.183 ± 0.743) mg/dl, There was statically significant difference between pregnant and control at LSD $_{p\leq 0.05}$ = 0.2931 that is shown in figure 3.16.

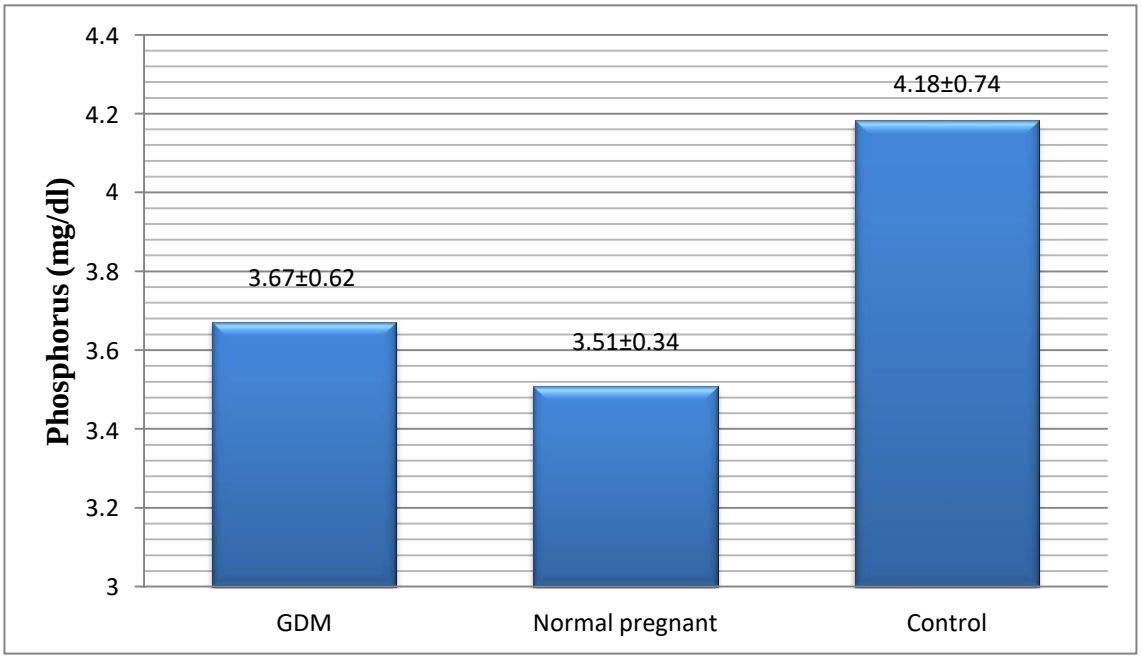

Figure 3.16: Mean of serum inorganic phosphorus ion in GDM, normal pregnant and control

This result agreed with the findings of Khastiga G [273] ,who also noted a significant decrease in the later state of pregnancy between seven and eight months. These findings could also be explained by the fact that most of the mineral transfer from the maternal circulation into the developing fetus occurs during the second and third trimesters of pregnancy. The increase in fetal requirement as it progressively matures is met from the mother's plasma pool and this brings about a decrease in the mean material level of phosphate [274].

3.9 :Correlation between 25 (OH) D and variance biochemical parameters studied.

The correlation of 25(OH)D level with demographic and clinical parameters for diabetic and non-diabetic pregnant women is shown in table 3.1 .

Table3.1: Correlation of 25-hydroxy vitamin D level with anthropomorphic and biochemical parameters of study

Demographic and clinical parameters	25(OH)D (ng/ ml)			
	GDM		Normal pregnant	
	R	P	R	P
Age (years)	0.153	0.173	-0.389	0.017
BMI (Kg /$m^{2)}$	0.088	0.295	-0.420	0.010
FBG(mg/dl)	-0.148	0.181	-0.144	0.223
HbA1c(%)	-0.049	0.385	0.024	0.450
Cholesterol (mg/dl)	-0.079	0.313	-0.479	0.004
TG(mg/dl)	-0.193	0.166	-0.263	0.080
LDL-C (mg/dl)	-0.014	0.467	-0.450	0.006
VLDL-C (mg/dl)	-0.193	0.116	-0.273	0.072
Ca^{+2} (mg/dl)	-0.010	0.476	0.220	0.122
$(PO_4)^{-2}$ (mg/dl)	0.111	0.247	0.394	0.016
Insulin hormone(µIU/ml)	-0.232	0.075	0.130	0.247
PTH (pg/ml)	-0.198	0.111	0.391	0.016
IGF(ng/ml)	-0.177	0.138	-0.091	0.316
HOMA IR	-0.384	0.014	0.108	0.285

In the GDM group , serum 25(OH) D had a significant negative correlation with HOMA IR (P=0.014) , and tendency towards significant negative correlation with insulin hormone (P=0.075) . There was no association between GDM and low 25(OH)D .

Previous reports showed the association between vitamin D deficiency and GDM [275,276]. and in other studies performed in several countries like Australia [277], Iran [278] and the United state [279]. On the contrary and similar to our results, no significant association was reported by flood – Nichols et al [280]. and Rodriguez et al [281]. and in different countries too, like India [249] and the United kingdom [282]. On the other hand, an association of vitamin D deficiency with impaired insulin secretion has been observed in different studies [283,284]. In addition, a significant correlation was described between 25(OH) D level and insulin sensitivity in pregnant women with GDM [285] .Our study and many other studies reported no significant association between vitamin D and BMI[285,286] .

In agreement with Farrant et al and Torloni M [249,287], the present study showed no association between vitamin D and HbA1c . Despite this, other studies showed a potential interaction between 25(OH)D and blood glucose in pregnancy [288-290].

3.10 Molecular analyses

3.10.1 DNA detect

The purity and concentration of the nucleic acid were detected by nanodrop UV spectrophotometer . (figure 3.17)

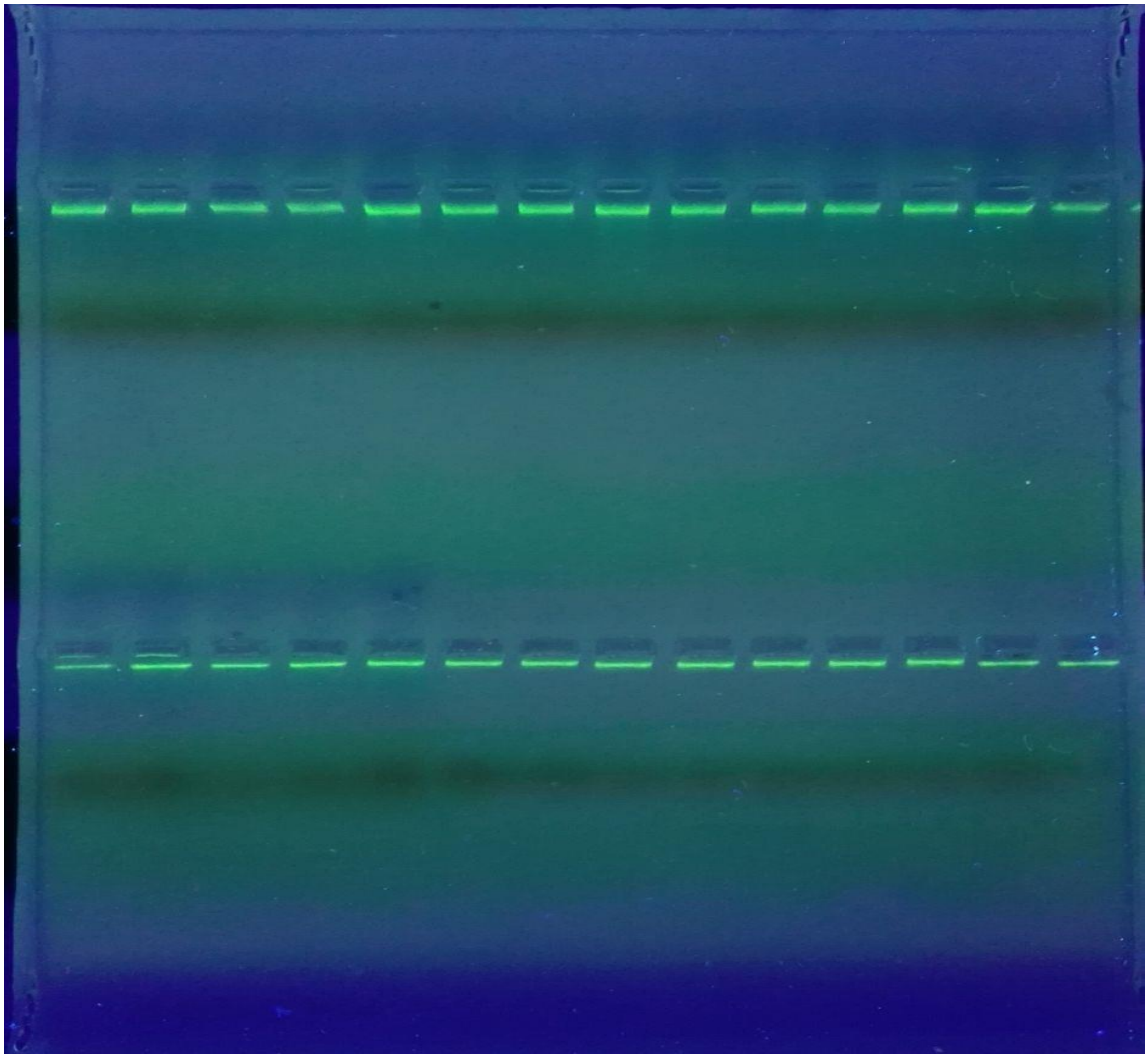

Figure 3.17: Gel electrophoresis of genomic DNA extraction from blood ,1.5% agarose gel at 5 vol / cm for 1:15 hour.

3.10.2 polymerase chain reaction (PCR)

3.10.2.1 rs (10735810) polymorphism .

To analyse the rs (10735810) polymorphism (exon 2 of VDR) , it was amplified using the PCR technique . After PCR , a 270 bp fragment of genomic DNA was obtained to view the fragment , the PCR product was loaded on to a 1.5% agarose gel and electrophoresis at 5 volt/cm^2 was performed (Figure 3.18) .

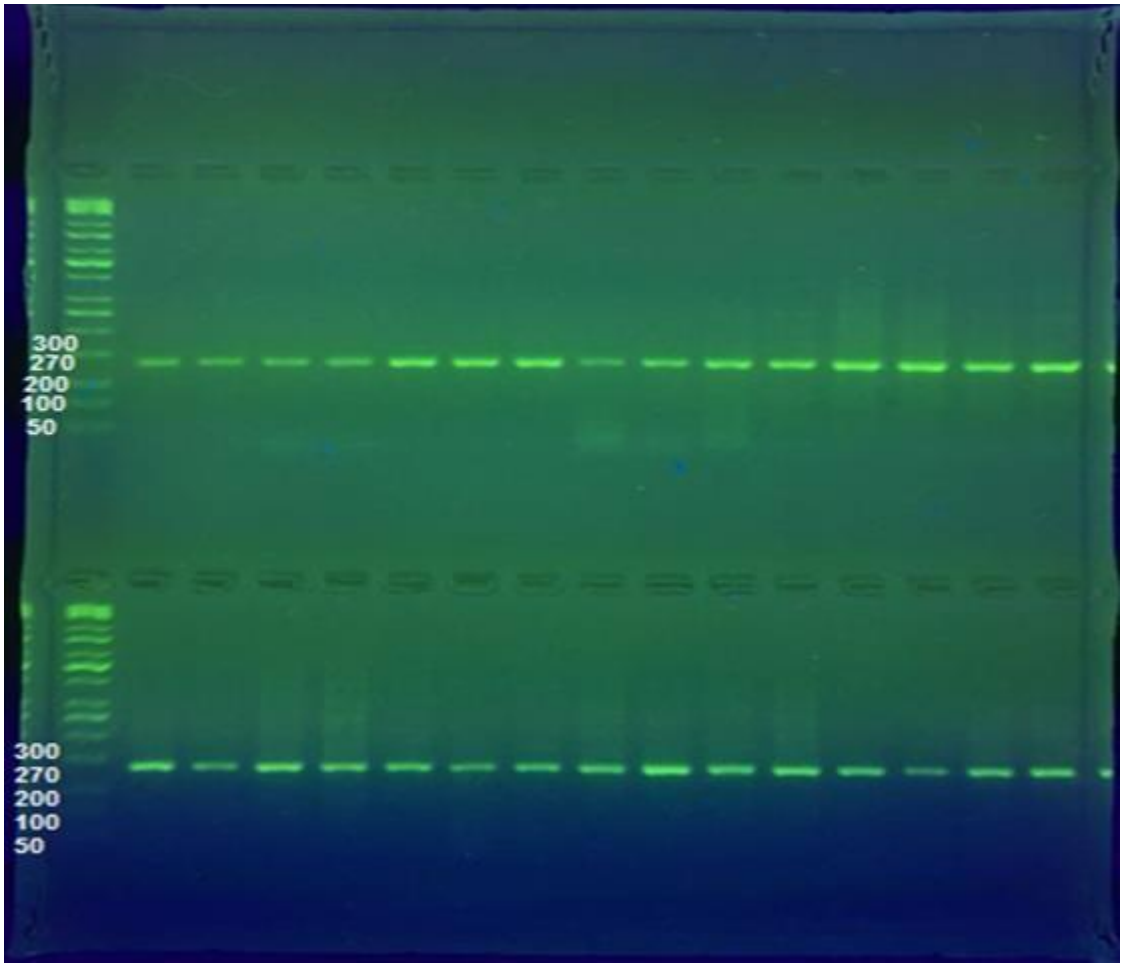

Figure 3.18: PCR product of the VDR gene rs (10735810) at the band size 270 bp, at 1.5 % agarose gel at 5 volt/cm^2

3.10.2.2 rs (1544410) polymorphism

The rs (1544410) polymorphis located in intron 8 of VDR , was also amplified through the PCR technique .

The amplified fragment , with a size 820 bp , could be viewed in the 1.5 % agarose gel after electrophoresis at 5 volt/cm2 . (Figure 3.19)

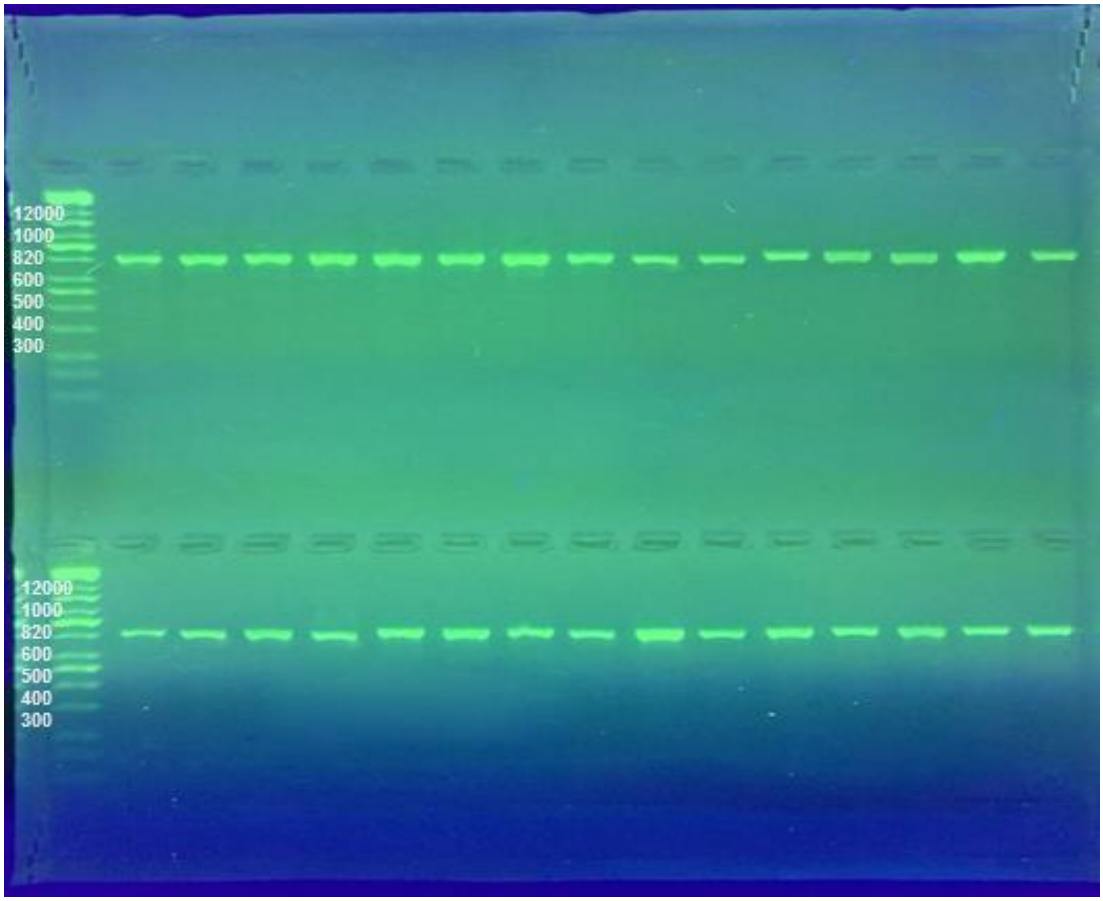

Figure 3.19: PCR product of the VDR gene rs (1544410) at the band size 820 bp, at 1.5 % agarose gel at 5 volt/cm^2

3.10.3 Restriction fragment length polymorphism (RFLP) .

3.10.3.1 rs (10735810) polymorphism

Amplified PCR product was digested with rs (10735810) restriction enzyme. Genotype which was determined according to the fragments length homozygote CC remained undigested in 270 pb . Homozygote TT genotype was digested to 210 and 60 bp , and heterozygote CT digested to 270, 210 and 60bp as shown in figure 3.20

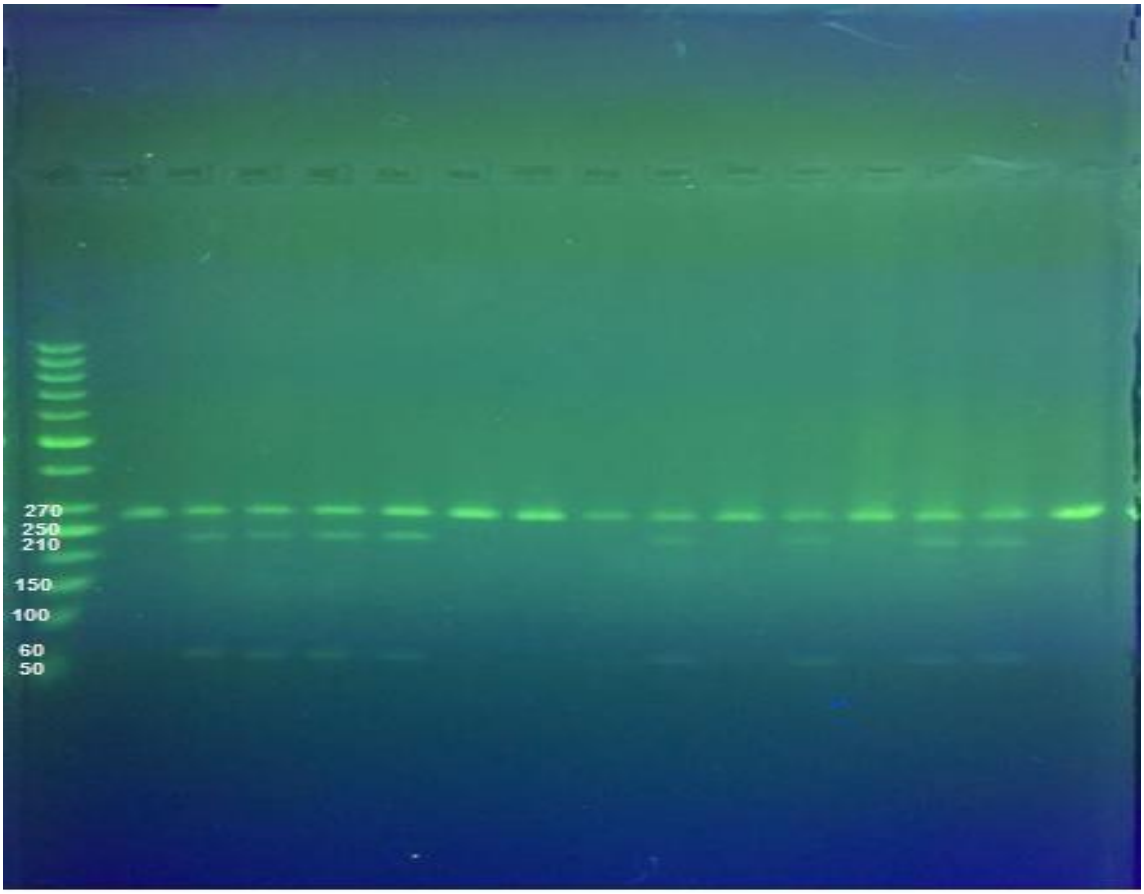

Figure 3.20: Electrophoresis pattern of PCR product digested with rs (10735810) restriction enzyme

Genotype and allele frequency of VDR rs (10735810) gene polymorphism in GDM , normal pregnants and controls showed no significant differences ($p>0.05$) . These findings are in agreement with results of the study that showed VDR rs (10735810) and rs (1544410) polymorphism which are not associated with Saudi GDM [199].

An indian study suggested that VDR gene polymorphism is associated with type 2 diabetes mellitus [291] .

Genotyping analyzing results by using RFLP-PCR technique and hardy Weinberg equilibrium offer three genotyping patterns in studying groups (CC , TT , CT) .

Results elucidated variation in genotyping frequency, where ratio of CC genotype in controls was higher than GDM and normal pregenants (60%), no significant difference was observed between controls, GDM and normal pregenants OR < 1 which represents the pattern as preventive factor from GDM . TT genotype ratio in patients with GDM was higher than controls, non significant differences are observed between studying groups ($p > 0.05$) , OR > 1, which represent genotype pattern which increases GDM risk factor. CT genotype showed ratio top of a little in GDM in comparison with control , OR = 1.07 , also CT pattern appears as genotype related with risk of GDM as shown in table 3.2.

Table 3.2 : Genotype of rs (10735810) polymorphism .

Gene	Genotype	Gestational No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs (10735810)	CC	14 (45.45%)	18 (60.00%)	0.56 (0.18 - 1.73)	0.241
	CT	16 (54.55%)	11 (36.00%)	2.13 (0.68 - 6.71)	0.163
	TT	0 (0.00%)	1 (4.00%)	0.36 (0.02 - 8.75)	0.532

Gene	Genotype	GDM No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs(10735810)	CC	22 (54.17%)	18 (60.00%)	0.79 (0.26 - 2.39)	0.451
	CT	15 (37.50%)	11 (36.00%)	1.07 (0.34 - 3.33)	0.574
	TT	3 (8.33%)	1 (4.00%)	2.18 (0.19 - 24.50)	0.484

Gene	Genotype	Gestational No. (%)	GDM No.(%)	OR (95% CI)	*P* value
VDR rs(10735810)	CC	14 (45.45%)	22 (54.17%)	0.71 (0.23 - 2.20)	0.812
	CT	16 (54.55%)	15 (37.50%)	2.00 (0.63 - 6.33)	0.928
	TT	0(0.00%)	3 (8.33%)	0.20 (0.01 - 4.12)	1.000

The above results showed that CT and TT genotypes are associated with risk and development of GDM . However , the correlation between TT genotype and the risk of GDM was higher than that of CT genotype , so it was found that the TT genotype can be used as an indicator with the risk of GDM . The results also showed that homozygous CC is important as preventive genotype of GDM . These results were confirmed by many studies and agreed with them [199,292] .

Results of allele frequency for found T showed that the results are closed between GDM and normal pregnant .

Table 3.3 also shows that frequency distribution dose not have significant differences between patients with GDM and control samples ($p > 0.05$) .

Table 3.3 : Allele frequency of rs (10735810) polymorphism .

Gene	Allele	Gestational No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs(10735810)	C	22(72.73%)	23(78.0%)	0.75 (0.30-1.91)	0.361
	T	8 (27.27%)	7(22.0%)	1.33 (0.52-3.38)	0.361

Gene	Allele	GDM No. (%)	Control sample No.(%)	OR (95% CI)	*P* value
VDR rs (10735810)	C	29(72.92%)	23(78.0%)	0.76 (0.30-1.89)	0.363
	T	11(27.08%)	7(22.0%)	1.32 (0.53-3.29)	0.363

Gene	Allele	Gestational No. (%)	GDM No.(%)	OR (95% CI)	*P* value
VDR rs(10735810)	C	22(72.73%)	29 (72.9%)	0.99 (0.40-2.46)	0.601
	T	8(27.27%)	11 (27.1%)	1.01 (0.41-2.51)	0.601

Where odds ratio (OR) to allele C < 1 , this ratio represents the allele C as a preventive allele (preventive factor) .

The results of our study are consistent with those arrived at by Aslani S and Alzaim M [293,294], who suggested that C allele may have a role in

decreased incidence of GDM . T allele was higher in patients with GDM compared to the control, but it is not significant stastically . The value of OR >1 and high allele frequency T in patients shows the extent of the role of this allele with the risk of GDM . These finding were confirmed by several studies [295]. Another study showed significant frequency similar to our study for C and T allele which showed that C allele appears as preventive factor (OR <1) while T allele acts as etiological fact (OR >1) [296] .

3.10.3.2 rs (1544410) polymorphism

Amplified PCR product was digested with rs (1544410) restriction enzyme. Genotype was determined according to fragment length . Homozygote GG genotype remained undigested in 820bp . Homozygote AA genotype was digested to three band 820 , 650 and 170 bp . There was not band for genotype GA in 650 and 170 bp in our studying as shown in figure 3.21

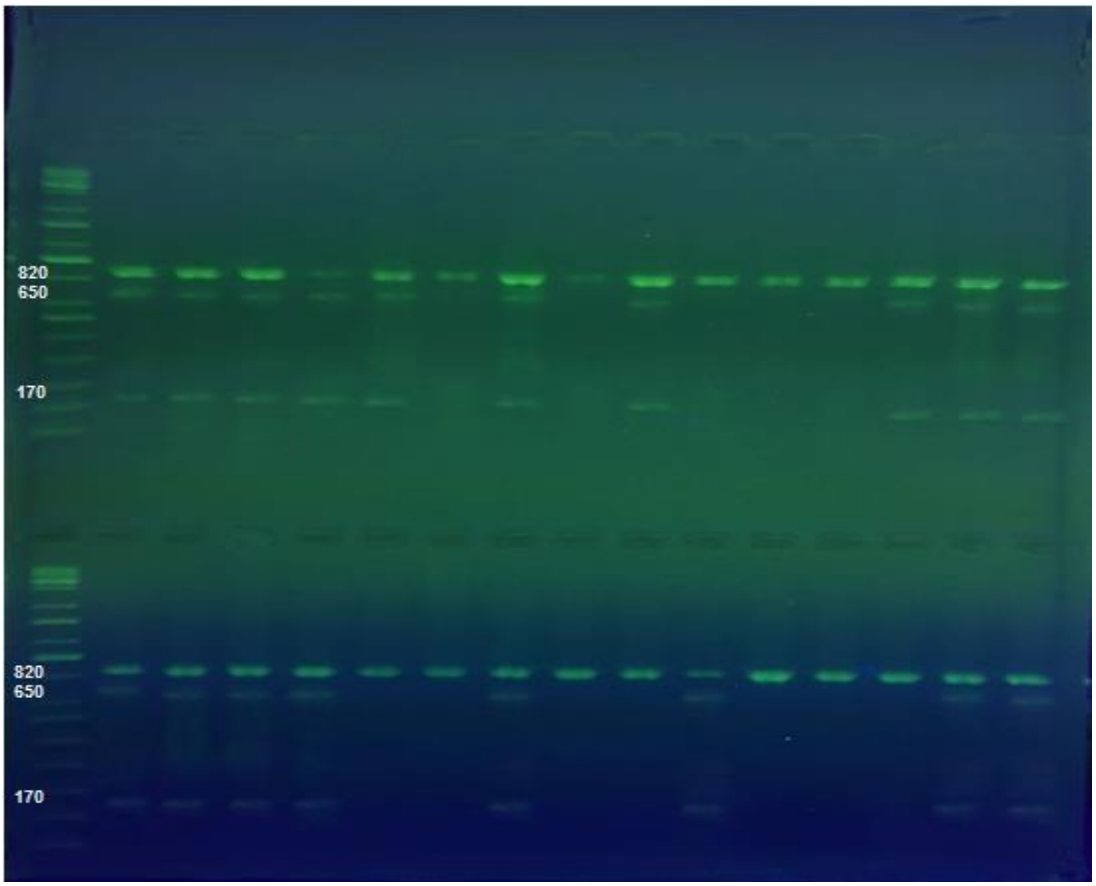

Figure 3.21: Electrophoresis pattern of PCR product digested with rs (1544410) restriction enzyme

Genotype frequency of VDR rs (1544410) gene polymorphism in GDM , normal pregnant and controls are non stastically significant ($p > 0.05$).

Results obtained by different investigators who studied rs (1544410) polymorphism varied among diabetics , rs (1544410) polymorphism has been linked to susceptibility to diabetes in several countries [111,297] , however , studies in other countries could not establish association between rs (1544410) and existence of diabetes [110,199,298].

Results elucidated variation in genotyping frequency between studying groups . GG genotype in healthy control was higher than patients with GDM , but no significant difference was observed , where odds ratio was equal to 0.76 with confidence intervals 95 % CI (0.25-2.34) , which represents preventive factor from GDM . While ratio of GA pattern appears as genotype related with risk of GDM (OR=1.31) with confidence intervals 95% (0.43 - 4.01) as shown in table 3.4 .

Table 3.4 : Genotype of rs (1544410) polymorphism .

Gene	Genotype	Gestational No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	GG	11(36.36%)	13 (44.00%)	0.73 (0.23 - 2.29)	0.408
	GA	19(63.64%)	17 (56.00%)	1.37 (0.44 - 4.34)	0.408
	AA	0 (0.00%)	0 (0.00%)	1.00 (0.07 - 18.27)	1.000

Gene	Genotype	GDM No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	GG	15 (37.50%)	13(44.00%)	0.76 (0.25 - 2.34)	0.432
	GA	25(62.50%)	17 (56.00%)	1.31 (0.43 - 4.01)	0.432
	AA	0 (0.00%)	0(0.00%)	1.00 (0.07 - 18.27)	1.000

Gene	Genotype	Gestational No. (%)	GDM No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	GG	11(36.36%)	15 (37.50%)	0.95 (0.29 - 3.08)	0.649
	GA	19(63.64%)	25 (62.50%)	1.05 (0.33 - 3.39)	0.649
	AA	0 (0.00%)	0 (0.00%)	1.00 (0.07 - 18.27)	1.000

Results of allele frequency for G and A showed variable results between studying groups . The allele G represents as preventive factor from GDM (odds ratio less than one) . The value of odds ratio >1.0 and high allele frequency A in patients shows the extent of the role played by this allele with the risk of GDM . Table 3.5 .

Table 3.5 : Allele frequency of rs (1544410) polymorphism .

Gene	Allele	Gestational No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	G	20(68.18%)	22 (78.0%)	0.83 (0.35 - 2.00)	0.429
	A	10(31.82%)	8 (22.0%)	1.33 (0.52 - 3.38)	0.361

Gene	Allele	GDM No. (%)	Control samples No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	G	28(68.75%)	22(78.0%)	0.76 (0.30 - 1.89)	0.363
	A	12(31.25%)	8(22.0%)	1.06 (0.44 - 2.52)	0.538

Gene	Allele	Gestational No. (%)	GDM No.(%)	OR (95% CI)	*P* value
VDR rs (1544410)	G	20(68.18%)	28(68.75%)	0.97 (0.41 - 2.33)	0.612
	A	10(31.82%)	12(31.25%)	1.03 0.43 - 2.45	0.612

Hong YJ, et al showed that G allele in VDR may be a protective factor in the development of diabetic retinopathy [299]. El-Beshbishy, et al reported no evidence of allelic association of the rs (1544410) of VDR gene with GDM in Saudi women [199] .

Conclusions and Recommendations

Conclusions

1. Vitamin D deficiency was more prevalent among Iraqi women .

2. There was a relationship between age and gestational diabetes mellitus and also between body mass index and gestational diabetes mellitus .

3 . The results showed s showed that vitamin D receptor FokI and BsmI polymorphisms not associated with Iraqi gestational dibetes mellitus .

4. Homozygous FF and homozygous BB turned out to be as a prenetive genotype of gestational diabetes mellitus ,while Ff ,ff ,Bb and bb turned out to be related with risk of GDM.

5. The allele F and allele B represent a preventive allele (preventive factor) , while the allele f and allele b act as etiological factor .

Recommendation

1. In take vitamin D as supplement for people and for all ages .

2. Risk of gestational diabetes mellitus(GDM) may be reduced by knowing the allele that acts as preventive factor and the allele represented as etiological factor .

3. Using more than single nucleotide polymorphism (SNP) for another receptor to see the effect of all SNP on GDM .

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الخالصة

سكري الحمل هو احد انواع السكري ويعرف بعدم القدرة على تحمل الكلوكوز اثناء الحمل, ويوصف بأن افراز خاليا β في البنكرياس غير كافية لتلبية حاجة الجسم من االنسولين ربما بسبب امراض المناعة الذاتية او مقاومة االنسولين او بسبب تشوهات وراثية .

قد يتراوح انتشار مرض سكري الحمل بنسبة 1 % الى 14 % بين النساء الحوامل, اعتمادا على المعايير السكانية ومعايير التشخيص المدروسة . من اهم العوامل المسببة لسكري الحمل هي البدانة والحمل في عمر متأخر .قد ينتج عن حالة االيض غير الطبيعية اثناء الحمل واالصابة بسكري الحمل الى التأثير على نمو الجنين وصحة االم في المستقبل . يتم تشخيص حالة االصابة بسكري الحمل بأجراء اختبار تحمل الكلوكوز عن طريق اخذ 75 غرام من الكلوكوز ولمدة ساعتين .

تهدف هذه الدراسة الى قياس تركيز نيتامين D للنساء العراقيات غير الحوامل وممن هم في عمر االنجاب وكذلك للنساء الحوامل االصحاء وغير المصابات بسكري الحمل ونساء حوامل مصابات بسكري الحمل ودراسة العالقة بين تركيز نيتامين D وعدد من المقاييس الكيموحيوية مثل تركيز الكلوكوز والسكر التراكمي وتركيز هرمون االنسولين وهرمون الغدة الجار الدرقية وهرمون النمو شبيه االنسولين التي تم قياسها في المصل وكذلك تم قياس نسبة تركيز ايونات الكالسيوم والفوسفور والكولسترول والدهون في المصل . وكذلك هدفت هذه الدراسة الى التعرف على العالقة بين نوعين من مستقبالت نيتامين D)1544410(rs و)10735810 (rs واألصابة بسكري الحمل .

جمعت نماذج الدم التي استخدمت في الدراسة من مستشفى الرمادي التعليمي للنسائية واالطفال ومن مختبر المولى للتحليالت المرضية, واجريت التحاليل البايولوجية في مختبر المولى واجريت الدراسة الجينية في مكتب وهج الدنا العلمي للفترة من االول من شهر ايلول 2017 الى نهاية شهر نيسان 2018.اجريت الدراسة على 40 حالة مصابة بسكري الحمل و30 حالة لحوامل اصحاء وغير مصابات بسكري الحمل و30 حالة لنساء غير حوامل وفي عمر االنجاب ولديهن تراكيز طبيعية من نيتامين D ,وجمعت نماذج الدم)5 مل(عند الصباح بعد 10- 12 ساعة من الصيام ,وتم حفظ 2 مل من الدم في انبوب يحتوي على ايثين ثنائي امين رباعي حامض الخليك (EDTA) وتم استخدامه لقياس السكر التراكمي (HbA1c(والستخالص ال DNA الذي اجريت عليه الدراسة الجينية , و3 مل من الدم استخلص منها المصل واجريت عليها التحليل البايولوجية .

أظهرت النتائج ان معدلات تراكيز فيتامين D كانت منخفضة لدى الحوامل االصحاء (8.73±4.158ng/ml) والحوامل المصابات بسكري الحمل (6.83±3.547ng/ml) وال يوجد فرق معنوي بين المجموعتين , بينما كشف اختبار االنحدار اللوجستي عن وجود فروق معنوية في تراكيز الكلوكوز والسكر التراكمي وهرمون االنسولين وهرمون الغدة الجار الدرقية وتركيز الكالسيوم ونسبة مقاومة االنسولين والكولسترول بين المجاميع المدروسة, واظهرت الدراسة عدم وجود فروق معنوية في تراكيز هرمون النمو شبيه االنسولين وتراكيز البروتينات الدهنية عالية الكثافة وكذلك تركيز ايون الفوسفور بين المجاميع المدروسة .كانت هناك عالقة عكسية بين تركيز فيتامين D ومقاومة االنسولين (P= 0.014) عند مجموعة الحوامل المصابات بسكري الحمل ,نتائج الدراسة بينت ان هناك عالقة بين السمنة واالصابة بسكري الحمل وكذلك هنالك عالقة بين الحمل عند سن متاخر واالصابة بسكري الحمل .

بينت الدراسة الجينية ان مستقبالت فيتامين D (rs1544410) و(rs10735810) ليس لها عالقة باألصابة بداء سكري الحمل عند النساء العراقيات وبينت ان CC وGG يمثالن عوامل وقاية من االصابة بسكري الحمل (OR<1) بينما CT , TT, GA يمثلون عوامل خطورة ومسببات لسكري الحمل (OR ≥1) لكن االليل C واالليل G يمثالن عاما وقاية من االصابة بداء سكري الحمل

(OR<1) بينما االليل T واالليل A يمثالن عوامل مسببة لسكري الحمل (OR≥1) .

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

((وَلَوْلَا فَضْلُ ٱللَّهِ عَلَيْكَ وَرَحْمَتُهُۥ لَهَمَّت طَّآئِفَةٌ

مِّنْهُمْ أَن يُضِلُّوكَ وَمَا يُضِلُّونَ إِلَّآ أَنفُسَهُمْ ۖ وَمَا

يَضُرُّونَكَ مِن شَىْءٍ ۚ وَأَنزَلَ ٱللَّهُ عَلَيْكَ ٱلْكِتَٰبَ

وَٱلْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ ۚ وَكَانَ فَضْلُ ٱللَّهِ

عَظِيم

الع

عل ٰيك عظيما

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سورة النساء

جمهورية العراق وزارة
التعليم العالي والبحث العلمي
جامعة الانبار-كلية العلوم
قسم الكيمياء

العلاقة بين مستقبلات جين فيتامين D_3 وبعض المتغيرات الكيموحيوية للعراقيات المصابات بسكر الحمل

أطروحة

مقدمة إلى مجلس كلية العلوم– جامعة الانبار

وهي جزء من متطلبات الحصول على درجة الدكتوراه فلسفة في علوم الكيمياء

من قبل

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