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Study the effect of Asprosin and Vitamin D_3 in Patients with Nephropathy Type2 Diabetic Mellitus

A Thesis

Submitted to the Council of the College of science for Woman, University of Babylon in partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

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بسم الله الرحمن الرحيم

(يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ
وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ)

صدق الله العلي العظيم

المجادلة: الآية (١١)

Supervisor Certification

I certify that this thesie entitled (Study the effect of Asprosin and Vitamin D_3 in Patients with Nephropathy Type 2 Diabetic Mellitus) was prepared by Ola Hussien under my supervision at the Department of Chemistry, College of Science for Woman, Babylon University, in partial requirements for the degree of Master of science in chemistry.

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Date / /2023

DEDICATION

I dedicate my work to my loving parents for their encouragement and their patience.

I dedicate my wok to my companion and Supporter in my life, my husband.

also I dedicate my work to my lovely brothers and sisters.

also I dedicate my work to my beloved

Daughters;

(Fatima, Maryam, Ruqayah and Asinat)

OLA....

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OLA HUSSIEN AL-MOSAWY

Chapter One

Introduction
And
Literature Review

1- Introduction and Literature review

1-1 Diabetes Mellitus (DM) is a series of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels[1].

Several pathogenic processes are involved in the development of diabetes mellitus. These range from autoimmune destruction of the pancreatic beta cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action[2]. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action [3].

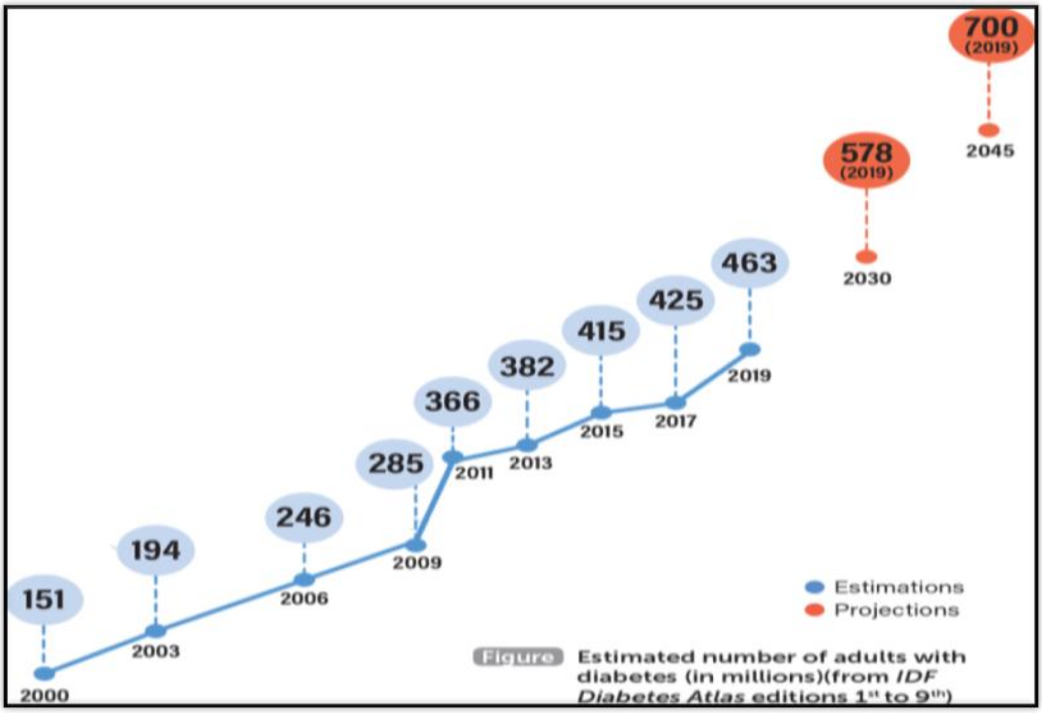
The vast majority of cases of diabetes mellitus. fall into two broad categories: In one category, type 1 diabetes, accounts for 5–10% of total DM cases worldwide and occurs due to slow and progressive autoimmune destruction of insulin-producing pancreatic β-cells leading to an absolute deficiency of insulin production. In the other, much more prevalent category, type 2 diabetes mellitus., the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. As a result, a degree of hyperglycemia sufficient to cause pathological and functional changes in various target tissues, but without clinical symptoms, may be present for a long period of time before diabetes is detected[3].

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia and blurred vision. Impairment of growth and susceptibility to certain infection may also accompany chronic hyperglycemia. Acute life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis[4].

1-1-2 Prevalence of diabetes Mellitus

The number and prevalence of people with diabetes mellitus are rapidly rising. Diabetes mellitus. is chronic progressive condition that results in significant morbidity, premature death, and economic burden to any healthcare system [5]. According to a World Health Organization global report on diabetes released on April 2016, the prevalence of diabetes in adult population globally was 8.5% (422 million) in 2014, which is nearly twofold of (4.7%) in 1980 [6].

The report of the International Diabetes Federation in 2019, a total of 463 million people are estimated to be living with diabetes, representing 9.3% of the global population (20-79 years). This number is expected to increase to 578 million (10.2%)in 2030 and 700 million (10.9%) in 2045 (Figure 1-1) [7]. The prevalence of diabetes in women in 2019 is estimated to be 9.0% and 9.6% in men. The increase of diabetes prevalence with age leads to prevalence of 19.9% (111.2 million) in people aged 65-79 years. The prevalence is higher in urban (10.8%) than rural (7.2%) areas, and in high-income (10.4%) than low-income countries (4.0%). One in two (50.1%) people living with diabetes do not know that they have diabetes mellitus[8].The global prevalence of impaired glucose tolerance is estimated to be 7.5 %(374 million) in 2019 and is projected to reach 8.0% (454 million) by 2030 and 8.6%(548 million) by 2045 [7].



Figure(1-1):The estimated number of adults with Diabetes Mellitus [7].

1-1-3 Classification of Diabetes Mellitus

Diabetes can be classified into the following general categories:

1-1-3-1 Type 1 Diabetes Mellitus

Type 1 Diabetes mellitus (T1DM), also known previously as (juvenile DM) or (insulin-dependent DM) (IDDM) is a chronic autoimmune disease characterized by elevated blood glucose levels (hyperglycemia), which are result from the insulin deficiency that occurs as the consequence of the loss of the pancreatic islet beta-cell [9,10]. However,the vast majority of patients (70-90%), the loss of β-cells is the consequence T1DM-related autoimmunity due to production of TIDM-associated autoantibodies, these patients have autoimmune TIDM (also known as type 1 a diabetes mellitus) [11]. In a smaller subset of patients, no immune responses or autoantibodies are preserved, and the cause of β-cell destruction is known (idiopathic type 1diabetes mellitus) [12].The T1DM represents only around 10% of the diabetes cases worldwide but occurs with increasing incidence much earlier in life. Although it may occur at any age, T1DM is most commonly seen in children and adolescents [13]. Affected individuals have impaired glucose metabolism and are prone to chronic complication from hyperglycemia, and acute complication from hypoglycemia and ketoacidosis [14].

1-1-3-2 Type 2 Diabetes Mellitus

Type 2 Diabetes mellitus that referred to as (noninsulin-dependent diabetes mellitus) or (adult-onset diabetes) is the most prevalent from of diabetes, accounting for (90%) of all diabetes cases [15]. It is characterized by hyperglycemia due to insulin resistance peripheral tissues and impaired insulin secretion from pancreatic beta cells.

Also ,T2DM develops when beta cells fail to compensate for the increasing demand for insulin caused by insulin resistance [16]. As a result, deregulation of carbohydrate, lipid, and protein metabolism will occur.Environmental factors that have been associated with an increased risk of T2DM canteens age, obesity, and lack of physical activity [17].

Most patients with this type of diabetes are obese, which is major cause of insulin resistance [18]. and patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region [19].

Because T2DM is regarded as a "disease of lifestyle", it is usually identified in people who have a high body mass index (BMI), blood pressure, and other cardiovascular risk factors. Therefore, management of T2DM includes changes in the diet and exercise routine, combinations of anti-hyperglycemia medicines (oral and often insulin), antihypertensive, lipid –lowering , and types of medication [20].

1-1-3-3 Gestational Diabetes

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy [21].This definition encompasses glucose tolerance, which normalizes following delivery, and diabetes mellitus, which was undiagnosed before or began concomitantly during pregnancy. The latter include T2DM, and rarely T1DM or monogenic diabetes [21].

Recently, the American Diabetes Association (ADS) defined it is as " Diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes" [22]. GDM is one of the most frequent metabolic diseases during pregnancy and approximately affects 7% (range:2-18%) of all pregnancies [23].

It has been estimated that the worldwide prevalence of GDM is approximately 17%, varying by regions, race or ethnicity, and socioeconomic status of individuals [24]. As a consequence of increasing obesity prevalence and advancing maternal age, the incidence of GDM is increasing worldwide, constituting a major economic burden for the public health care system [25].

1-1-4 Pathophysiology of Diabetes Mellitus Type 2

The major causative of T2DM is due to the combination of defective insulin secretion by pancreatic beta cells and the inability of insulin-sensitive tissues to respond properly to insulin due to peripheral insulin resistance (IR) [26]. According to the pathophysiology of the disease, a failure of the feedback loops between insulin action and insulin production result in unusually increasing blood glucose levels.

In the case of beta cell dysfunction insulin secretion is diminished, reducing the body's capacity to maintain physiological glucose levels. On the other hand, the IR causes increased glucose production in the liver as well as decreased glucose uptake in muscle, liver, and adipose tissue. Beta cell dysfunction is usually more severe than IR, even if both processes occur early in the pathogenesis and contribute to the development of the disease. However, when both Beta-cell dysfunction and IR are found hyperglycemia is amplified leading to the progression of T2DM [27].

The increasing levels of glucose in the blood after a meal is detected by the beta cells that respond by releasing insulin. Insulin lowers the blood glucose concentrations by enhancing glucose uptake and use by target tissues like fat cells and skeletal muscle. This increase in blood glucose suppresses the release of glucagon, which in turn reduces the generation of glucose from other sources such glycogen breakdown [28].

Obesity and physical inactivity lead to insulin resistance together with a genetic predisposition, place stress on β-cells, leading to a failure of β-cell function and a progressive decline in insulin secretion. IR is not only present in muscle and the liver, the two tissues responsible for the majority of glucose disposal following carbohydrate ingestion, but also adipose, kidney, gastrointestinal tract, vasculature and brain tissues, and pancreatic β-cells. In muscle, multiple abnormalities contribute to insulin resistance, including defects in insulin signaling, glucose transport, glucose phosphorylation, glycogen synthesis, pyruvate dehydrogenase complex activity, and mitochondrial oxidative activity[29].

In the liver, insulin resistance, in combination with insulin deficiency, hyperglucagonemia, increased glucagon sensitivity, and increased substrate delivery (fatty acids, lactate, glycerol, and amino acids), causes increased gluconeogenesis, which is responsible for increased basal glucose production and fasting hyperglycemia [30].

Impaired suppression of hepatic glucose production, decreased hepatic glucose uptake, muscle insulin resistance, and excessive renal glucose reabsorption leads to postprandial hyperglycemia in T2DM [31]. In T2DM, as a result of insulin secretion impairment and resistance, glucose use and release by the target tissues is disturbed, resulting in hyperglycemia [32].

It is commonly assumed that the condition starts with insulin resistance and is followed by increased secretion of insulin by the pancreatic beta in order to maintain glucose homeostasis. At a later stage of T2DM and because of the long-term compensation mechanism offered by the beta cells to keep a high insulin demand, β-cells subsequently undergo further damage and apoptosis. As a result of the inability to respond to the last demand of insulin release, plasma glucose levels rise [33].

1-1-5 Etiology of Type 2 Diabetes Mellitus

The cause of T2DM like other common human complex diseases infects the human (such as obesity, hypertension, etc.) included both genetic and environmental factors. Additionally, the risk of T2DM determine by the interaction of the genetic factors with the various environmental exposures encounter during life span of individual [34].T2DM characterized by the following causes:

1-1-5-1 Lifestyle Factors:

Several lifestyle factors affect the incidence of T2DM. Obesity, weight gain, and physical inactivity elevate the risk of the disease [35].Cigarette smoking is a risk factor for IR a precursor for diabetes mellitus by directly alter intracellular glucose transport, altering the distribution of body fat, and smoking tobacco may be toxic to the pancreatic tissue [36]. Furthermore, unhealthy lifestyle leads to metabolic syndrome, which could the leading cause of T2DM [37].

1-1-5-2 Genetic Factors

Polygenic DM is one of the causes that make T2DM a complex disease. The genetic effect leads to variation in an unlimited number of the site in the genome, some play a protective effect and other predisposing cause [38]. Most DM cases include several genes, About more than 36 genes were found to contribute in the increment the risk of T2DM, these genes contribute together approximately 10% of the total heritable component of the disease. There are some rare cases of DM that increased due to an abnormality in a single gene known as" monogenic forms of diabetes", such as (Maturity onsent dibetes of the youny) [39].

1-1-6 Complications of Diabetes Mellitus

There are two types of complications for diabetes mellitus, acute (short-term) complication and chronic (long-term) complication.

1-1-6-1 Acute complications:

1-Diabetic Ketoacidosis (DKA) is formed a hallmark of type 1 diabetes, it results from absolute insulin deficiency or abstinence from using insulin, but it also occurs in patients with T2DM [40].

2-Hyperosmolar non ketosis coma is life threatening endocrine emergency with type 2 and characterized by sever hyperglycemia (600 mg/dl), sever hyperosmolarity and dehydration in the absence of ketone bodies [41].

3-Hypoglycemia is mostly caused by accidental over administration of insulin or meglitinides or sulphonylureas. Precipitating causes include a too high dose of insulin or hypoglycemic drug; Conversely, the patient may have missed a meal or taken vigorous exercise following the administration usual dose of insulin or oral hypoglycemic drugs [42].

1-1-6-2 Chronic vascular complications

The chronic complications of diabetes mellitus affect many organ systems and are responsible for the majority of morbidity and mortality. It can be divided into micro and macro-vascular complications [43]

1-1-6-2-1 Macrovascular Complications of Diabetes

The macrovascular complications consist of cardiovascular disease (CVD), Cerebro vascular disease, and Peripheral artery disease (PAD). Diabetic foot syndrome has been defined as the presence of foot ulcer associated with neuropathy, PAD, and infection, and it is a major cause of lower limb amputation [44].

The central pathological mechanism in macro vascular disease is the process of atherosclerosis, which leads to the narrowing of arterial walls throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. In response to endothelial injury and inflammation, oxidized lipids from low density lipoprotein (LDL) particles accumulate in the endothelial wall of arteries. Monocytes then infiltrate the artery wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells induce macrophage proliferation and attraction of T-lymphocytes.The T-lymphocytes, in turn, cause smooth muscle proliferation in the arterial walls and collagen accumulation. The net result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of this lesion leads to acute vascular infarction [45].

1-1-6-2-2 Microvascular Complications

The microvascular complications divided into:

1-Diabetic nephropathy

2-Diabetic retinopathy

3-Diabetic neuropathy

1-1-7 Pathogenesis of Diabetic Complications (mechanisms of hyperglycemia -induced damage)

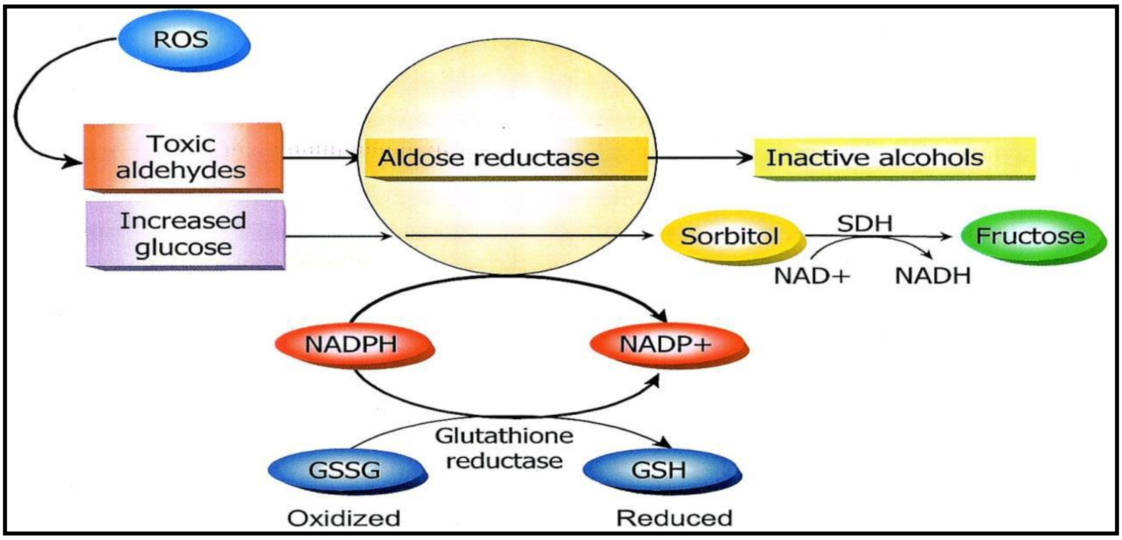
The pathogenesis of micro-and macrovascular complications of diabetes mellitus is complex and multifactorial. The main driver for the development of these complications is hyperglycemia, which activates several pathways leading to increased inflammations, endothelial dysfunction, and microvascular disease [46].

The possible biochemical mechanisms include: increased polyol pathway flux; increased formation of advanced glycation end-products (AGEs); activation of protein kinase C isoforms; and increased hexosamine pathway flux [47].

1- Increased polyol pathway flux: The polyol pathway is the first pathway to be defined linking hyperglycemia to microvascular complications. Aldose Reductase (AR), the first and rate-limiting enzyme in this pathway, has a low affinity to glucose at normal concentrations; however, in a hyperglycemic environment, when the glucose concentration in the cell becomes too high, AR reduces that glucose to sorbitol, using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor[48].

Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH), which uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor. The buildup of sorbitol has damaging effects in cells, including osmotic damage [49].

The oxidation of sorbitol by NAD+ increases the cytosolic NADH:NAD^+ ratio, which inhibits glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and leads to increased triose phosphate levels, which results in increased AGE and diacylglycerol (DAG). Moreover, activation of the polyol pathway reduces the amount of reduced glutathione due to NADPH consumed by AR and reduces the ability of cells to respond to oxidative stress [50]. Figure (1-2)[47] demonstrates the role of polyol pathway in complication of diabetes mellitus.

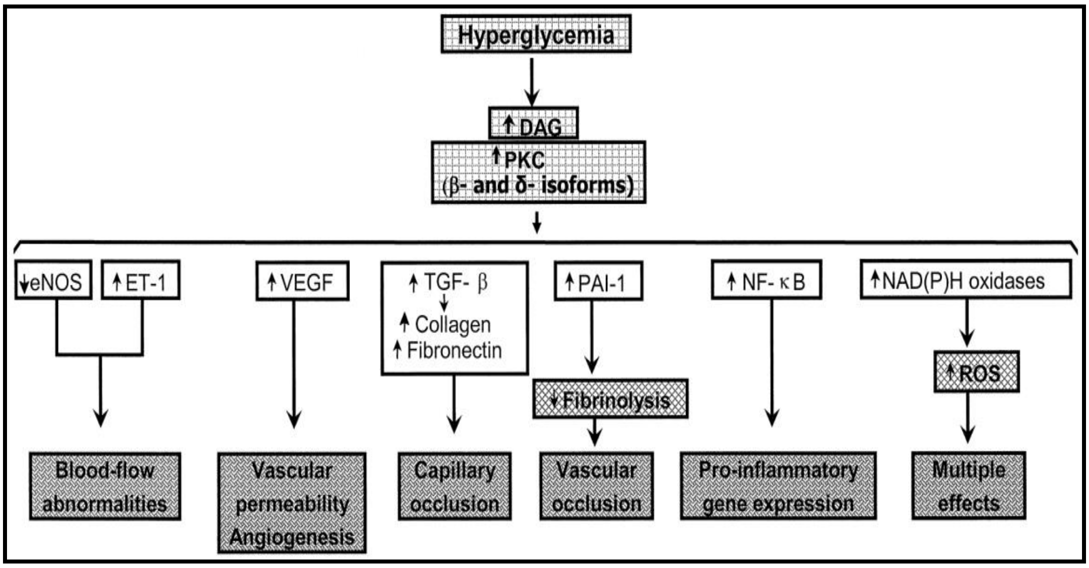


Figure(1-2): Aldose reductase and the polyol pathway. when the glucose concentration in the cell becomes too high, AR reduces that glucose to sorbitol, using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH),which uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor. GSH= Reduced glutathione ,GSSG = Oxidized glutathione,SDH=Sorbitol dehydrogenase,ROS= Reactive oxygen species. **[47]**

2- Advanced glycation end-products: During the maillard process, glucose interacts with amino groups on proteins to make Amadori products, which produce irreversible glycation products called AGEs throughout time. Essential proteins are cross-linked by AGEs, which alter their function and cause cellular destruction. AGEs bind to cell surface receptors, principally the receptor for AGE (RAGE), which trigger harmful downstream signaling pathways controlled in part by nuclear factor-kappa B (NF-B) activation[51].

3- Protein kinase C (PKC): is a family of serine-threonine kinases. It appears to be activated in a range of diabetic tissues including the retina, kidney, heart, and aorta as a result of hyperglycemia by increasing the synthesis of diacylglycerol (DAG), which is a critical activating cofactor for the classic isoforms of protein kinase-C [52].

PKC activation leads to decreased production of endothelial nitric oxide synthase (eNOS); increased endothelin-1, tissue growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and plasminogen activator inhibitor-1 (PAI-1); and activation of nuclear factor-KB (NF-KB). The net effect is inflammation, endothelial and vascular dysfunction, and cellular/tissue damage [47]. Figure (1-3) [47] show the activation of PKC as Consequences of hyperglycemia**.**



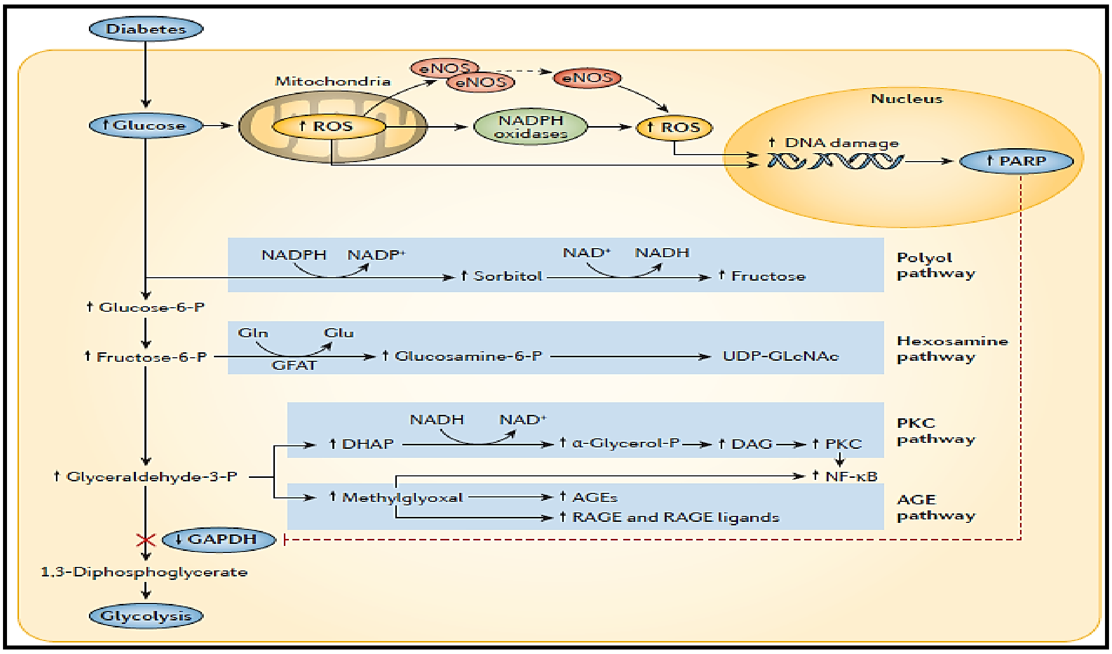
Figure(1-3): Consequences of hyperglycemia-induced damage activation of protein kinase C (PKC) [47]. eNOS**(**endothelial Nitric Oxide Synthase**)**, VEGF(Vascular Endothelial Growth Factor**)**, TGF-β **(**tissue growth factor-β, PAI-1**(**plasminogen activator inhibitor-1),ET-1(endothelin-1),NF-KB(nuclear factor-KB)

4- The hexosamine pathway: some of the fructose-6-phosphate resulting from the metabolism of glucose is transformed into glucosamine-6 phosphate by glutamine: fructose-6 phosphate amidotransferase in the hexosamine pathway and finally to uridine diphosphate N-acetyl glucosamine. N-acetyl glucosamine contributes modifications that enhance microvascular occlusion/dysfunction, such as increased PAI-1 expression [47].

5- Reactive oxygen intermediate theory(oxidative stress): A common feature of cells that are damaged by hyperglycemia is the presence of free radical species, such as Reactive Oxygen Species (ROS) and reactive nitrogen species [52].

ROS activates these pathways via the key glycolytic enzyme GAPDH. When GAPDH activity is inhibited, the levels of all the upstream glycolytic intermediates increase, including methylglyoxal (increasing AGE formation), DAG (activates PKC), fruc-tose-6 phosphate (activating the hexosamine pathway), and intracellular glucose levels (activating the polyol pathway) [48].

Oxidative stress inhibits GAPDH via activation of polymers of ADP-ribose polymerase (PARP) (Figure 1-4)[53]. In addition to the excess in superoxide production, hyperglycemia results in a reduction in the antioxidant defense system, further exacerbating oxidative stress diabetes



Figure(1-4): The central role of reactive oxygen species (ROS) in diabetic complications [53]**.** AGE, advanced glycation end-product; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; GFAT, glutamine fructose-6-phosphate amidotransferase; NF-κB, nuclear factor-κB; PKC, protein kinase C; RAGE, receptor for AGE; UDP-GLcNAc, uridine diphosphate N-acetylglucosamine.

1-2 Diabetic Nephropathy

Diabetic nephropathy (DN) is defined is a microvascular complication of diabetes characterized by injury to the capillaries, mesangial cell expansion, extracellular matrix accumulation, thickening of the glomerular basement membrane (GBM), and podocyte and glomerular injury, leading to glomerular sclerosis and tubulointerstitial fibrosis[55].

(DN) is the leading cause of Chronic Kidney Disease (CKD) and end-stage renal disease (ESRD) worldwide, accounting for nearly 50% of all ESRD cases that required treatment with dialysis or renal transplantation and approximately 40% of patients with T2DM and 30% of those with T1DM will eventually develop CKD (54).Clinically, Not everyone with diabetes mellitus will develop DN. In patients with

T1DM, DN is more probable to progress 5 to 10 years or maybe more after the onset of diabetes. patients with T2DM may discover that they previously have a small amount of protein in the urine at the time which diabetes is diagnosed [56].

Three major histologic alterations happen in the glomeruli of during diabetic nephropathy. Firstly; mesangial expansion is directly influenced by hyperglycemia, may be via increased matrix production or glycation of matrix proteins. Secondly; increase thickness of the glomerular basement membrane (GBM) takes place. Thirdly; glomerular sclerosis is caused by intraglomerular hypertension, which is caused by dilatation of the afferent renal artery or from ischemic injury caused by narrowing of the vessels providing the glomeruli by blood [57].These changes damage the kidney's glomeruli, which leads to the characteristic feature of albumin in the urine which (called albuminuria) [58].

The pathogenesis and progression of diabetic nephropathy are likely to be as a result of interplay between metabolic and hemodynamic pathways, which are often disturbed in the setting of diabetes .In hemodynamic pathways, there is an imbalance in afferent and efferent arteriolar resistance, resulting in increased glomerular hydrostatic pressure and hyperfilteration. Activation of renin-angiotensin system (RAS) increase angiotensin II levels, leading to efferent arteriolar vasoconstriction. In addition, increased levels of endothelin-1 also contribute to vasoconstriction[59].

In metabolic pathways, Activation of glucose-dependent pathways inside the kidney leads to: enhanced oxidative stress, renal polyol formation, and accumulation of advanced glycation end products[60].

Activation of all these pathways results in increased renal albumin permeability and accumulation of extracellular matrix leading to increased proteinuria, glomerulosclerosis, and finally tubulointerstitial fibrosis as shown in Figure (1-5)[61] .

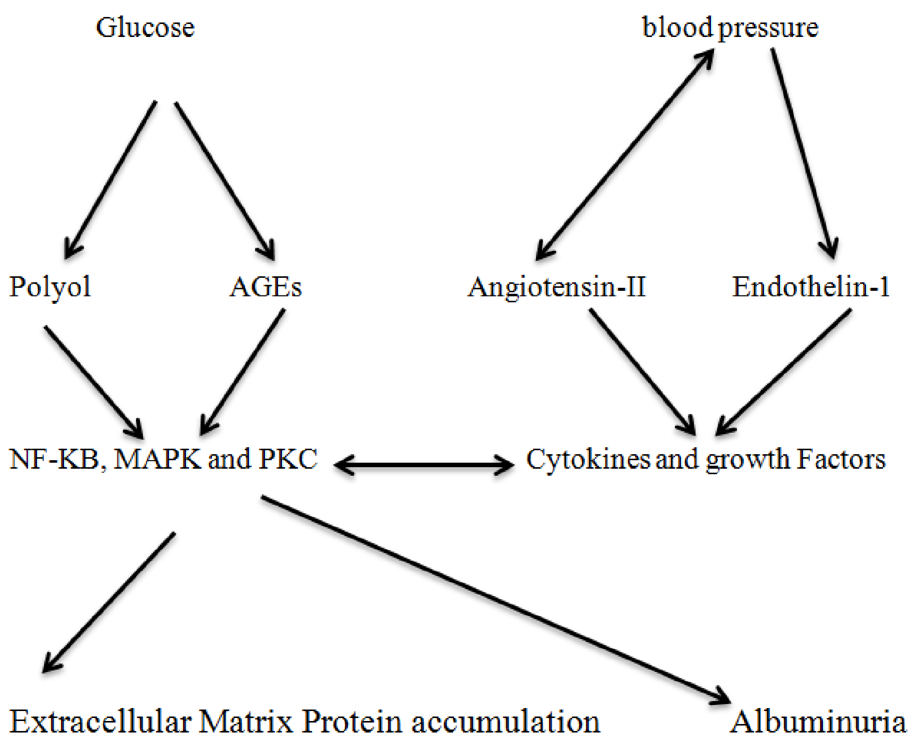


Figure (1-5):Interaction of hemodynamic and metabolic pathways in diabetic Nephropathy[61].

1-2-1 Risk Factors for Development of Diabetic Nephropathy

Current evidence suggests that both genetic and environmental factors determine the risk for and susceptibility to develop DN. The two main potentially modifiable initiation and progression risk factors for DN are hyperglycemia and arterial hypertension. However, the ethnicity, family history, dyslipidemia, obesity, and insulin resistance are the major risk factors of diabetic nephropathy and other putative risk factors include elevated glycosylated hemoglobin level (HbA1c), elevated blood pressure, and smoking [62].

1-3 Asprosin

Asprosin is primarily produced by white adipocytes, which have the highest FBN1 mRNA expression across all tissues[63]. Since FBN1is expressed in a variety of human tissues, white adipose is unlikely to be the only source of plasma Asprosin. Asprosin secretion from dermal fibroblasts has been documented by Romere *et al*. [63], implying that it is secreted from the skin. Lee *et al*.[66] discovered that pancreatic cells secrete Asprosin. According to these findings, Asprosin can be produced by the skin and pancreatic cells. More recently, Asprosin has been detected in human saliva samples [67]. Asprosin is a fasting-induced gluconeogenic protein hormone, discovered and first identified by Romere *et al*. in a study of Neonatal Progeroid Syndrome (NPS) patients in 2016 [63].

1-3-1 Synthesis of Asprosin

Fibrillin1gene,is located in chromosome 15q21.1 [64]. It encode a proprotein called (profibrillin) with 2871 amino acids in length. Subsequently, the translated profibrillin is cleaved at its C- terminus by activated protease furin, which generates mature fibrillin-1, and 140-amino-acid long Asprosin. One of the main functions of fibrillin-containing microfibrils is their essential contribution in providing a scaffold for elastic fiber formation in elastic tissues. The C-terminal Asprosin is released into the circulation [65].

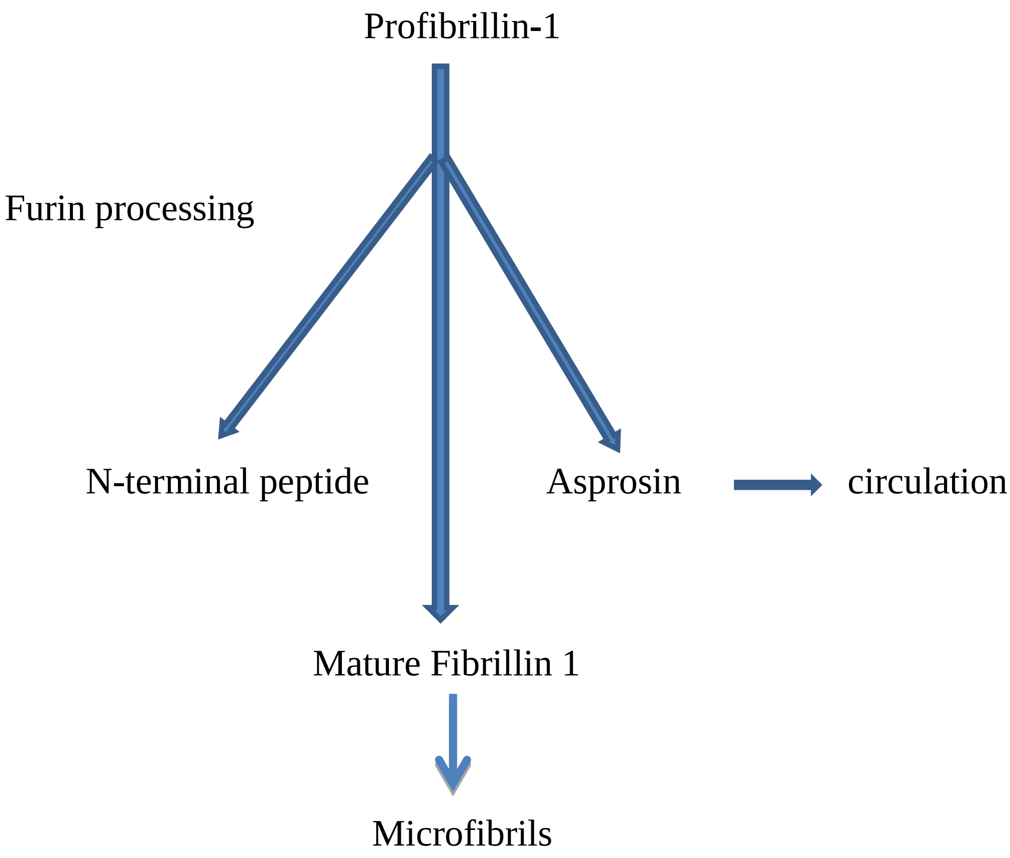


Figure (1-6): Schematic represents the synthesis of Asprosin [65].

1-3-2 Metabolic effects of Asprosin

Asprosin is a recently discovered hormone released during fasting states mainly by adipocytes from white adipose tissue. As a glucogenic protein, it induces the release of glucose from hepatocyte by binding to the OLFR734 receptor and causing the activation of the G protein-cAMP-PKA pathway [63].

As it crosses the blood–brain barrier, it also acts as an orexigenic protein that stimulates food intake via activation of AgRP+ neurons in the hypothalamus; thus, Asprosin take part in maintaining the body's energy homeostasis [68].

1-3-2-1 Glucogenic action of Asprosin

The main target organ of asprosin action is the liver, where it stimulates glucose production and release[63]. As reported by Li *et al* [69]. Asprosin works via the Olfactory 734 (Olfr734) receptor to induce glucose output in the liver in both fasting and obesity states as shown in Figure1-7. Asprosin, a fasting-induced gluconeogenic hormone, activates protein kinase A (PKA) in the liver through the G protein and cyclic AMP (cAMP) messenger system, increasing glucose release from hepatocytes [70]. Furthermore, as reported by Romere *et al*., the action of Asprosin is independent of glucagon and catecholamine axis activation, which is also involved in glucose release [63]. Figure (1-8)[69] shows the action of asprosiin the liver and brain.

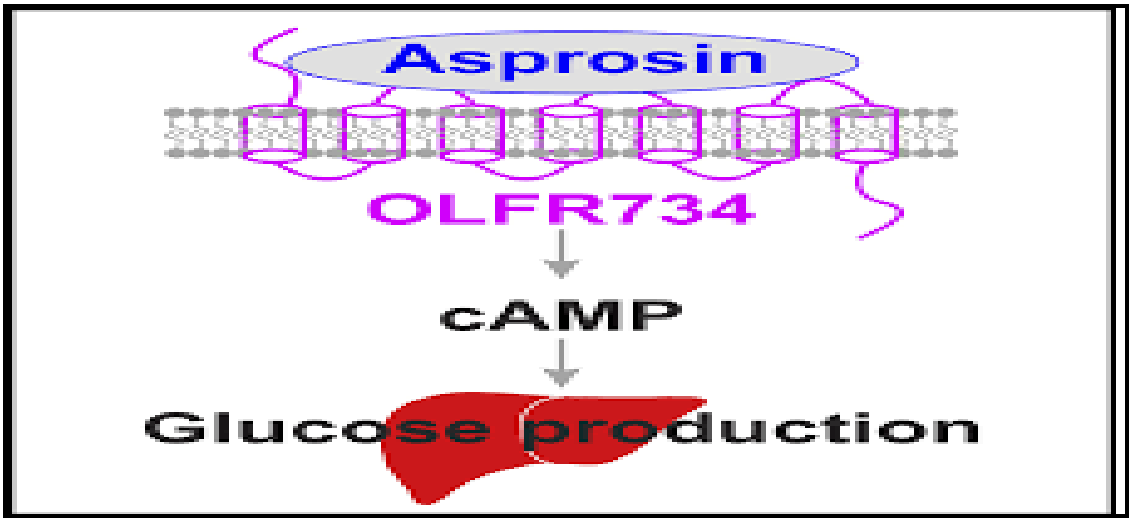


Figure (1-7): OLFR734 receptor of Asprosin [69]. Asprosin works via the Olfactory 734 (Olfr734) receptor, activates protein kinase A (PKA) in the liver through the G protein and cyclic AMP (cAMP) messenger system, increasing glucose release from hepatocytes

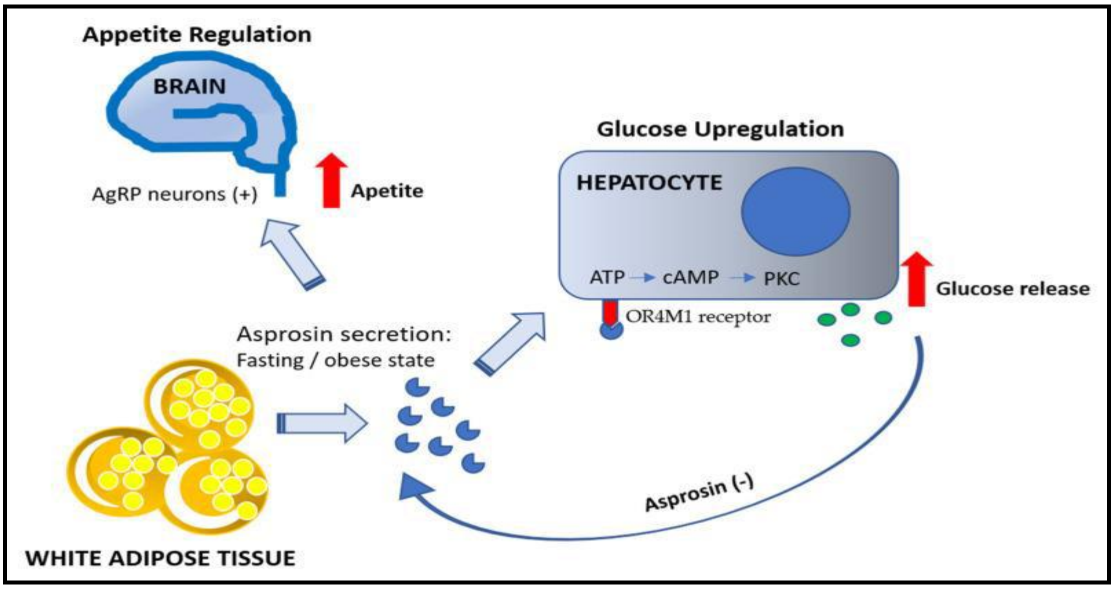


Figure (1-8): Mechanisms of asprosin action in fasting and obese state [71]. asprosin crosses the blood–brain barrier and affects appetite stimulation directly by activating orexigenic agouti-related peptide (AgRP) neurons in the hypothalamus.It has been shown that the effect of asprosin is linked with the activation of the Gαs–cAMP–PKA axis**,** Asprosin works via the Olfactory 734 (Olfr734) receptor, activates protein kinase A (PKA) in the liver through the G protein and cyclic AMP (cAMP) messenger system, increasing glucose release from hepatocytes.

The level of asprosin varies according to the circadian rhythm; after an overnight fast, it rises dramatically, then drops after a meal [71].

1-3-2-2 Orexigenic action of Asprosin

Asprosin is an important hormone in appetite regulation. According to Duerrschmid *et al*. [68], asprosin crosses the blood–brain barrier and affects appetite stimulation directly by activating orexigenic agouti-related peptide (AgRP) neurons in the hypothalamus.It has been shown that the effect of asprosin is linked with the activation of the Gαs–cAMP–PKA axis. Food intake and energy homeostasis are controlled by AgRP+ neurons [72].

1-4 Vitamin D_3 (1,25,dihydroxy-cholecalciferol)

There are two common different types of vitamin D, the first type is D_2 (ergocalciferol) which presents in plants and fish and cannot synthesize in human body, another form is D_3 (cholecalciferol) that presents in human body, full human vitamin D_3 requirement can tak from either exposure to sunlight for enough time or by supplementation [73].Vitamin D_3 considered as a chief natural form of vitamin D because the human bodies can synthesize vitamin D_3 after exposing the epidermal cell to UV-B from sunlight, main vitamin D_3 source is many types of oil-rich fish such as salmon, mackerel and herring. Ingested vitamin D (represents for D_2 or D_3 or both) is assembled withchylomicrons then absorbed to the lymphatic system and enter venous blood [74].

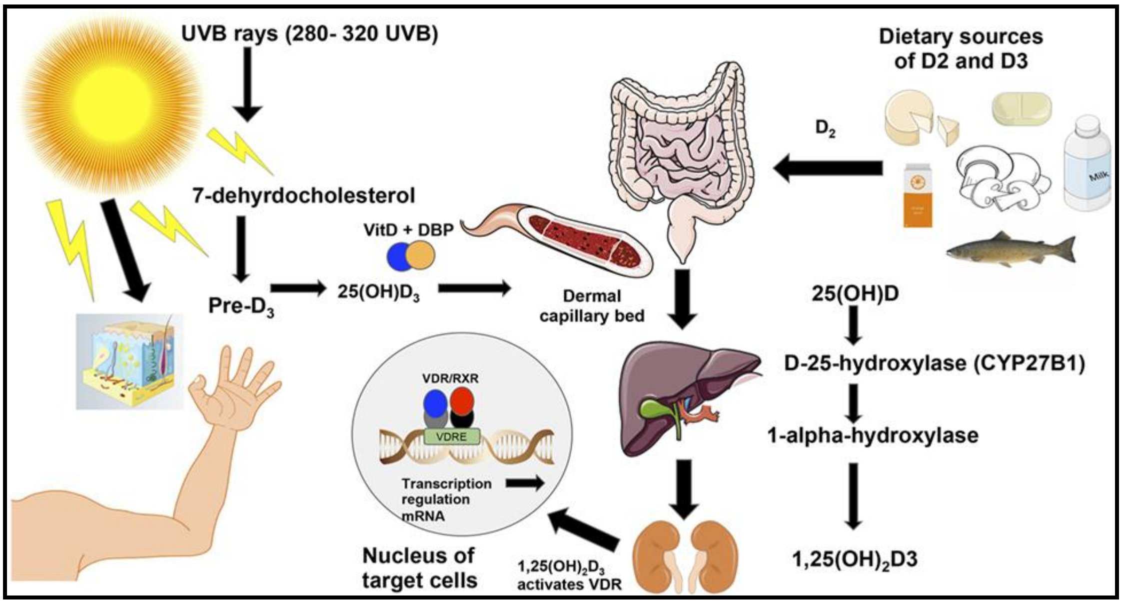
Calcium and phosphate homeostasis and immune modulator role is the major vitamin D_3 function in human body. The different study observes vitamin D_3 deficiency can result in multiple diseases probably to anti-inflammatory, an immune-modulatory role that influences on cytokine production [73].

1-4-1 Synthesis of Vitamin D_3

Vitamin D3 synthesizes in the skin by exposure to the ultraviolet (UV) Brays. The time that needs to produce adequate amount of vitamin D_3 from the skin is different from one person to another and it depends upon the strength of the UV-B rays, In another word, it depends on the resident place of people, length of consumed time in exposure to the sunlight (more than 15 minutes) and skin melanin pigment concentration [75].

In the keratinocyte plasma membrane, 7-dehydrocholecaliceferol are photolyzed into pre-vitamin D_3 after exposing the human skin to UV-B light, in the presence of skin temperature this pre-vitamin D_3 transformed into vitamin D_3 in two steps [76]. Skin vitamin D_3 and dietary vitamin D undergo tow respectively hydroxylation, the first one by hepatic 25α- hydroxylase and the second by renal 1α –hydroxylase enzyme to produce 25(OH)D and $1,25[OH]_2D_3$ respectively, as in the figure 1-8. High solar UV-B exposure cannot result in vitamin D_3 toxicity due to augmented vitamin D_3 and previtamin D_3 are catabolized to inactive photoproduct called calcitoric acid [76].

Keratinocytes are also capable for hydroxylation of 25(OH)D to produce$1,25(OH)_2D_3$ and this is due to the presence of 1 α- hydroxylase enzyme in the cell [76]. Human epidermal keratinocytes contain the complete machinery needed to produce the hormone $1\alpha,25(OH)_2D_3$ (calcitriol) from its initial precursor 7-dehydrocholesterol (7-DHC) [77].Calcitriol can bind with high affinity to the vitamin D receptor (VDR) caused to increase of intestinal absorption calcium and phosphorus, bone formation, resorption and mineralization [72].



Figure(1-9) Synthesis of Vitamin D_3 [89]. Skin vitamin D_3 and dietary vitamin D undergo tow respectively hydroxylation, the first one by hepatic 25α- hydroxylase and the second by renal 1α –hydroxylase enzyme to produce 25(OH)D and $1,25[OH]_2D_3$

1-4-2 Physiological actions of Vitamin D3

Calcitriol has a much shorter half-life in the human body than calcidiol, calcitriol half-life is 15 hr, while for calicidiol15 days [78]. The active D3 form (1,25 [OH]2D3) concentration is much lower than 25(OH)D and it has a higher affinity for binding with vitamin D receptor that distributed in the entire body [79]. Different body cells including a cell of vascular wall systems have 1α-hydroxylase and subsequent transformation of calcidiol to calcitriol to exhibit its effect on an individual cell prior to its degradation into inactive calcitoric acid [80].

Aims of the study

1. To evaluate the diagnostic utility of Vitamin D3 and Asprosin as a biomarkers for early detection of nephropathy in type 2 diabetes mellitus patients

2. To study the correlation between Vit D3 and Asprosin with traditional renal function parameters(Urea ,creatinine, and albumin).

3. To determine specificity, sensitivity and cutoff value for both serum Vitamin D3 and Asprosin through ROC analysis in order to see whether one or both of them acts as early dignositic marker of nephropathy in T2DM.

Chapter two

Materials and Methods

2- Materials and Methods

2-1 Chemicals

The chemicals were used in the current study listed in Table 2-1.

Table 2-1 Chemicals used in the current study

Chemicals	Company	Origin
Total Cholstrole Kit	BIO LAB	France
HDL Cholstrole Kit	BIO LAB	France
Triglyceride kit	BIO LAB	France
Albumin Kit	SANYMED	Italy
A1Care™ HbA1c cartridge	I-SENS	Korea
Creatinine kit	LINEAR	Spain
Urea Kit	LINEAR	Spain
Fasting serum glucose kit	LINEAR	Spain
Human Asprosin,ASPROSIN ELISA Kit	BT LAB	Zhejiang, china
Human Vitamin D,VD ELISA Kit	BT LAB	Zhejiang, china

2-2 Instruments and Tools

Table (2-2) list for commonly used instruments and tools are used in the study

Table 2-2: Instrumental and tools used in this study

Instruments and Tools	Company	Origin
Eppendorf tube (1.5 µL)	BC	China
pipette tips 0.2 mL	BC	China
pipette tips 1 mL	BC	China
Disposable test tube (10 mL)	Meheco	Chine
Spectrophotometer	C-cell	England
Centrifuge	Hettich	Germany
Deep freeze	GFL	Germany
Incubator	Memmert	Germany
Micropipettes (5-50 µL), (2-20 µL), (20-200 µL), (100-1000 µL)	Slamed	Germany
Water bath	Memmert	Germany
Test tube with separation gel	AFCO	Jordan
EDTA tube (5ml)	AFCO	Jordan
Elisa reader and washer	Biotek	USA

2-3 Subjects

This study was conducted by fasting blood samples collection in outpatients clinic at Diabetes mellitus and Endocrine Center in Marjan

Medical City in Hilla city, Babylon province from 25 October 2022 till 31 January 2023.

2-3-1 Study groups

The current study was designed as case control study, involved 120 individuals, 40 patients with type 2 diabetes mellitus (T2DM) group and 40 patients with Diabetes mellitus (type2) Nephropathy and 40 participants who are apparently healthy was used as control group. As shown in Figure (2-1).

All patients were diagnosed by physicians and according to ADA criteria(26). They were enrolled in Diabetes and Endocrine Center in Marjan Medical City in Babylon / Hilla city.

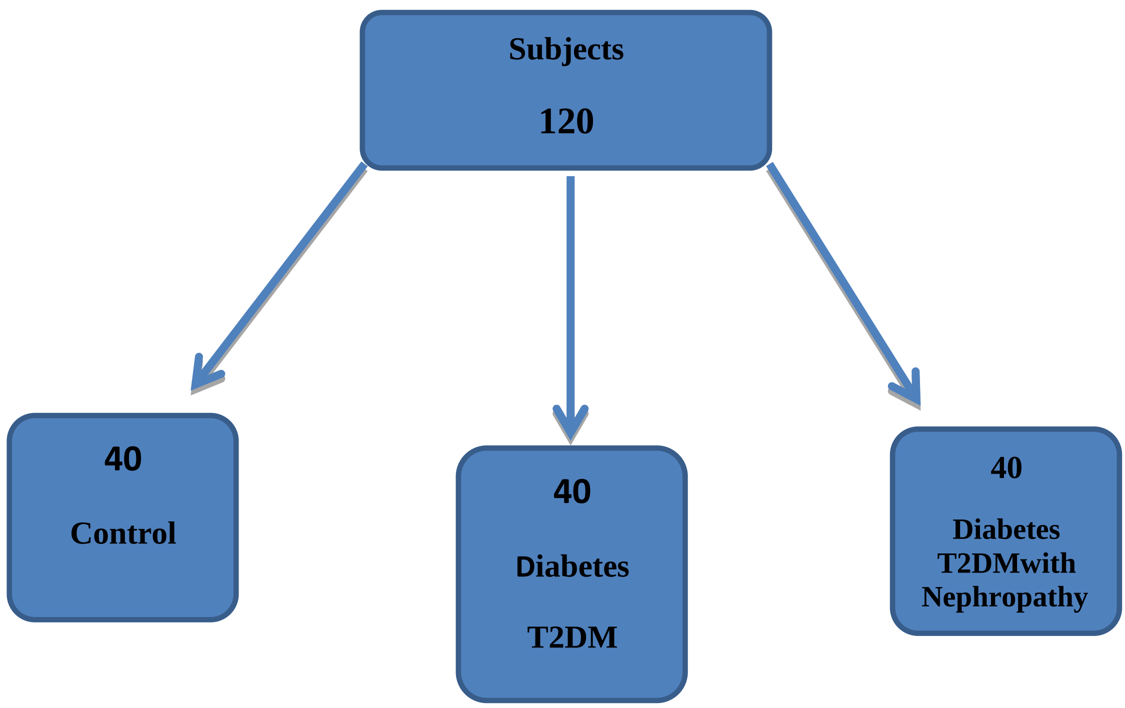


Figure (2-1) Simplified Scheme represents the study groups.

2-3-2 Inclusion criteria

1-Type 2 Diabetic mellitus.

2-Age between 40-60 years old.

3-Diabetes mellitus duration is more than 3 year.

2-3-3 Exclusion criteria

1- Type 1 diabetic mellitus

2- Urinary tract infection

3- Chronic Hypertension

4- History of cardiovascular diseases (coronary artery disease, cerebrovascular or peripheral artery diseases).

5- Acute and chronic inflammatory diseases.

6- Pregnant women.

7- Women taking an oral contraceptives.

8- Surgery or trauma within 6 months.

9-Malignancies.

10- Proteinuria due to causes other than DM.

2-3-4 Samples collection

●Serum
●whole Blood

2-3-4-1 Blood Samples

Five milliliters of fasting blood samples were obtained from each participant in this study. Each blood samples divided into two parts:-

Part 1: Two milliliters were collected into an EDTA tube for measuring glycated hemoglobin (HbA1c).

Part 2: Three milliliters of the blood sample was left for 20 minutes into a plain tube at room temperature. After coagulation, serum was separated by centrifugation at 1000 xg for 10 minutes.

Serum was subdivided into two aliquots in plain tubes for:-

Aliquot 1: Immediate measurement of glucose (FSG), creatinine, urea,TC,TG,HDL and AlbuminThis analysis were measured by the colorimetric method.

Aliquot 2: Stored at -40C^{o} for the subsequent assay of serum neutrophil gelatinase-associated lipocalin and Asprosin which were measured by using an ELISA technique.

2-3 Methods

2-3-1 Determination of Body Mass Index

Body Mass Index (BMI) was calculated in all subjects according to ratio depend on weight and height obtained by applying a mathematical equation, in which the weight in kilogram was divided by the square height in meter, and the results were considered as follows:

●BMI less than 18.5, falls within the underweight range.

●BMI of 18.5 to <25, falls within the normal.

● BMI of 25.o to <30, falls within the overweight range.

● BMI of 30.0 or higher, falls within the obesity range.[81]

2-3-2 Determination Blood Glucose Concentration

Principle

By using an enzymatic colorimetric method with a commercially available kit, the Fasting Serum Glucose (FSG) was determined. It is based on the principle the glucose is oxidized to D-gluconate by the glucose oxidase (GOD) with formation of hydrogen peroxide, in the presence of Peroxidase (POD) a mixture of phenol and 4-aminoantipyrine (4-AA) is oxidized by hydrogen peroxide, to form a red quinoneimine dye[82]. as the following reactions

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

$$\text{4-AA} + \text{Phenol} + H_2O_2 \xrightarrow{\text{POD}} \text{Quinonimine} + H_2O$$

Reagents composition :

Table 2-3: Reagents for measuring blood glucose level.

R1// Monoreagent. Phosphate buffer 100 mmol/L pH 7.5, glucose oxidase > 10 KU/L, peroxidase > 2 KU/L, 4- aminoantipyrine 0.5 mmol/L, phenol 5 mmol/L.
CAL// Glucose standard. Glucose 100 mg/dl (5.55 mmo/l).

Procedure

1- Reagents and samples were brought to room temperature.

2- Into labeled tubes was Pipetted

Tubes	Blank	Sample	Standard
R1.Monoreagent	1.0 mL	1.0 mL	1.0 Ml
Sample	---	10 µL	---
CAL. Standard	---	---	10 µL

3. The tubes were mixed and let stand 10 minutes at room temperature or 5 minutes at 37ºC.

4. The absorbance (A) of the samples and the standard were read at 500 nm against the reagent blank.

Calculation:

Glucose (mmol/L) = $\frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$

●Standard concentration = 5.56 mmol/L.

2-3-3 Determination of Glycated hemoglobin (HbA1c):

principle:

The level of HbA1c among total hemoglobin was determined optically through a series of biochemical and enzymatic reactions[83]. As the following color change occurs during a chain reaction, the HbA1c concentration was quantified using a color change. The total hemoglobin level was assayed using a methemoglobin method on the same test cartridge. All measurements and calculations were performed automatically by the A1Care™ Analyzer. As the following steps:

Step 1: Red blood cell + lysing buffer $\rightarrow$ HbA0 + HbA2 + HbA1c

Step 2 : HbA1c + protease $\rightarrow$ glycated Val – His + other residues

Step 3 : Glycated Val – His + FPOX $\rightarrow$ H_2O_2

Step 4 : H_2O_2+ POD + dye $\rightarrow$ color change

Reagent composition:

Table 2-4: Reagents for measuring HbA1c level

MES (2-(N-morpholino)ethansulfonic acid) : 0.058 mg.
Fructosyl-amino acid oxidase: 0.18 mg.
Peroxidase: 0.01 mg.
10-(carboxymethylaminocarbonyl)-3,7-bis(dimethylamino)-phenothiaazine.
Sodium salt: 1.8 µg

Procedure:

Un coagulated blood(2.5µL) in EDTA tube was entered to the equipment and analyzed automatically to measure HbA1c.

Calculation:

The instrument automatically performs the calculation of HbA1c ratio in % or mmol/mol from total hemoglobin.

2-3-4 Determination of Blood Urea:-

Principle:

By using an enzymatic colorimetric method with a commercially available kit, the serum urea was determined. It is based on the principle that urea is hydrolyzed by urease into ammonia and carbon dioxide. The ammonia generated reacts with alkaline hypochlorite and sodium salycilate in presence of sodium nitroprusside as coupling agent to yield a green cromophore[84]. As the following reactions:

$$\text{Urea} + H_2O \xrightarrow{\text{UREASE}} 2NH_3 + CO_2$$

$$NH_4 + \text{Salycilate} + NaCIO \xrightarrow[OH^-]{\text{NITROPRUSSIDE}} \text{Indophenol} + NaCI$$

Reagents composition :

Table 2-5: Reagents for measuring blood urea level.

■ **R1 = Enzyme reagent**. Urease > 500 U/mL.
■ **R2 = Buffered chromogen**. Phosphate buffer 20 mmol/L pH 6.9, EDTA 2 mmol/L, sodium salycilate 60 mmol/L, sodium nitroprusside 3.4 mmol/L.

■R3 **Alkaline hypochlorite.** Sodium hypochlorite 10 mmol/L, NaOH 150 mmol/L.
■ **CAL Urea standard.** Urea 50 mg/dL (8.3 mmol/L).

Reagent preparation:

1 volume of **R1** was mixed with 24 volume of **R2** to yield working reagent which stable for 4 weeks at 2-8ºC, and for 7 days at 15-25ºC.

Procedure:

1. Reagents and samples were brought to room temperature.

2.Into labeled tubes was pipetted:

Tubes	Blank	Sample	Standard
Working reagent	1.0 ml	1.0 ml	1.0 ml
Sample	---	10 Ml	---
CAL. Standard	---	---	10 µL

3. The tubes were mixed and let stand 10 minutes at room temperature or 5 minutes at 37ºC.

4. Pipetted:

R3	10.0 ml	10.0 ml	10.0 ml

5. The tubes were mixed thoroughly and let stand 5 minutes at 37ºC in water bath.

6. The absorbance (A) of the samples and the standard were read at 600 nm against the reagent blank.

Calculation:

urea (mmol/L) = $\frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$

●Standard concentration= 8.33mmol/L

Samples with concentrations higher than 300 mg/dL (50 mmol/L) should be diluted 1:5 with saline and assayed again then Multiply the results by 5.

Normal reference range: 15-40 mg/dl **(**2.5 - 6.6 mmol/L).

2-3-5 Determination of serum creatinine:

Principle:

By using kinetic colorimetric method with a commercially available kit, the serum creatinine was determined. The principle that based upon a modification of original picrate reaction (Jaffe reaction) creatinine under alkaline condition reacts with picrate ions forming reddish complex[85].

$$\text{Creatinine} + \text{Picric acid} \xrightarrow[37^{o}C]{pH\sim 12} \text{Red complex}$$

Reagents composition:

Table 2-6: Reagents for measuring serum creatinine level

■ R1 Picric acid (25 mmol/L)
■R2 Alkaline buffer. Phosphate buffer 300mmol/L, pH 12.7.
■CAL **Creatinine standard.** Creatinine 2mg/dl(177 micro mol/L).

Reagent preparation:

1 volume of **R1** was mixed with 1 volume of **R2** to yield working reagent which stable for 1 week at room temperature.

Procedure:

1. Working reagent, samples, and standard were brought to reaction temperature(37ºC).

2. The photometer was let to zero with distilled water.

3. Into cuvette was Pipetted:

Working reagent	1.0 ml
Sample or standard	100 Ml

4. The cuvette was mixed and inserted into the instrument and a stopwatch was started.

5. Two absorbances at 510 nanometers were recorded, the first one after 30 seconds (A1), and the second one after 90 seconds (A2).

Calculation:

Concentration of creatinine in serum or plasma:

$$\frac{(A2-A1)Sample}{(A2-A1)Sample} \times \text{Conc. of standard } \left(\frac{\text{mg}}{\text{dl}} creatinine\right)$$

• **Normal reference range:**

Serum or plasma:

Men = 0.70 – 1.20 mg/dl (62 – 106 micromol/l).

Women= 0.50 – 0.90 mg/dl (44 – 80 micromol/l).

2-3-6 Determination of Lipid Profile

2-3-6-1 Determination of Serum Total Cholesterol

The total Serum cholesterol is determined by the colorimetric method for the quantitative in *vitro* diagnostic measurement using a kit.

Principle :

This procedure is used in the measurement of total serum cholesterol using three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO), and peroxidase (POD). The mixture of phenol and 4.aminoantipyrin (4AA) is condensed into a quinonimine dye proportionately to the cholesterol concentration of in the sample the Serum cholesterol is measured using the enzymatic method based on the following reactions:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\textit{Cholesterol esterase}} \text{Cholesterol} + \text{Free Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{Cholesterol oxidase}} \text{Cholesterol .4.en.one3} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4.amino.antipyrine} \xrightarrow{\textit{Peroxidase}} \text{Quinonimine (pink)} + 4H_2O$$

Reagent composition:

Table 2-7: Reagents for measuring Serum Total Cholesterol level.

Reagent	Content	Concentration
Reagent 1Buffer	Phosphate buffer Chloro.4.phenol Sodium Chelate Triton ×100 Preservative	100mmol/L 5mmol/L 2.3mmol/L 1.5mmol/
Reagent 2Enzyme	4.amio.antipyrine Cholesterol Esterase Cholesterol Oxidase Peroxidase	0.25mmol/L ≥1.70 IU/l ≥ 100 IU/l ≥ 1200 IU/l
Reagent 3 Standard	Cholesterol	200 mg/DI

Procedure:

1.Three sets of tubes (Sample, standard and blank) were prepared as the following

Tubes	Blank	Sample	Standard
Working reagent	1.0 mL	1.0 mL	1.0 mL
DW	10 μL	---	---
Sample	---	10 μL	---
CAL. Standard	---	---	10 μL

2. The solutions were mixed and incubated for at least 5 min at 37 ºC.
3. The absorbance of samples and standards were measured at wave length 500 nm against blank.

Calculation :

The total cholesterol (mg/dl) $= \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$

●$\text{Conc. of standard} =$ 200 (mg/dl)

Reference Values :

The total cholesterol concentration in serum is
< 200 mg/dL (< 5.2 mmol/l).

2-3-6-2 Determination of Serum Triglycerides

Serum triglycerides were determined by the colorimetric method for the quantitative in *vitro* diagnostic measurement using a kit**.**

Principle :

The process is based on enzymatic serum or plasma triglyceride hydrolysis by lipoprotein lipase (LPL) to glycerol and free fatty acids (FFA). Glycerol is phosphorylated to form glycerol.3.phosphate (G.3.P) and adenosine diphosphate (ADP) in the presence of glycerol kinase (GK). G.3.P is oxidized to form dihydroxy.Acetone phosphate (DHAP) and hydrogen peroxide by glycerol.Phosphate oxidase (GPO).
A red chromogen is produced by peroxidase (POD) catalyzed coupling of 4. ami.noantipyrine (4.AA) and phenol with hydrogen peroxide (H2O2) proportional to the concentration of triacylglycerol in the sample. The Serum triacylglycerol is measured using an enzymatic method based on the following reactions

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol.3.phosphate} + \text{ADP}$$

$$\text{Glycerol.3.phosphate} + O2 \xrightarrow{GPO} \text{Dihydroxacetonephosphate} + H_2O_2$$

$$H_2O_2 + \text{4.Chlorophenol} + \text{4.AA} \xrightarrow{POD} \text{Quinoneimine} + H_2\text{O}$$

Reagents composition :

Table 2-8: Reagents for measuring Serum Triglycerides

Reagent	**Content**	**Concentration**
Reagent 1Buffer	PIPES Magnesium chloride Chloro .4.phenol Preservative	100mmol/L 9.8mmol/L 3.5mmol/L
Reagent 2Enzyme	4.aminoantipyrin(4.AA) Adenosine triphosphateNa(ATP) Lipase Peroxidase(POD) Glycerol.3.phosphate oxidase(GPO) Glycerol Kinase (GK)	0.5mmol/L 1.3 mmol/L ≥1000IU/l ≥1700IU/l ≥3000IU/l ≥660IU/l
Reagent 3 Standard	Glycerol equivalent to triglyceride 200mg/dI	2.28mmol/L

Procedure :

1.Three sets of tubes (Sample, standard and blank) were prepared as the following

Tubes	**Blank**	**Sample**	**Standard**
Working reagent	1.0 mL	1.0 mL	1.0 Ml
Demineralized water	10 μL	---	---
Sample	---	10 μL	---
CAL. Standard	---	---	10 μL

2. The solutions were mixed up and incubated at least for five minutes at 37 ºC.

3. The absorbance of samples and standards were measured at wavelength 500 nm against blank.

Calculation :

$$\text{Triglyceride (mg/dl)} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$$

• Conc. of standard = 200 (mg/dl)

Reference Values :

TG concentration in serum is between 35-160 mg/dl (< 2.66 mmol/l)

2-3-6-3 Determination of Serum High. Density Lipoprotein Cholesterol

Serum HDL.C was determined by the colorimetric method for the quantitative in vitro diagnostic measurement using a kit.

Principle :

This technology used a separation method based on the VLDL, LDL, and chylomicron selective precipitation from the phosphotungstic acid and $MgCl_2$ specimen. After centrifugation, HDL.Cholesterol is then measured by total cholesterol. Serum HDL.C is measured by the Burstein method.

Reagents composition :

Table 2-9: Reagents for measuring Serum High. Density Lipoprotein Cholesterol

Reagent	Content	Concentration

HDL Cholesterol R1	Phosphotungstic acid Magnesium Chloride	13.9 mmol/L 570 mmol/L

Procedure :

A. Precipitation:

1. To achieve HDL supernatant, 0.5 mL of the serum was mixed with 50 μL of HDL reagent.

2. The solution was mixed well, incubated for 10 min at room temperature, then mixed again and centrifuged for 10 min, at 4000 rpm.

3. After centrifugation, the clear supernatant from precipitate was separated to determine the HDL cholesterol concentration

B. HDL.C Determination

1. Three sets of tubes (sample, standard and blank) were prepared as in the following

Tubes	Blank	Sample	Standard
Cholestesterol reagent	1.0 mL	1.0 mL	1.0 Ml
HDL supernatant	---	25 μL	---
Standard (HDL)	---	---	25 μL
DW	25 μL	---	---

2. The solutions were mixed and incubated at least for 5 min at 37 ºC.

3. The absorbance of the sample and standard were measured at wavelength 500 nm against blank.

Calculation :

$$\text{HDL concentration (mg/dl)} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$$

• Conc. of standard = 100 (mg/dl)

Reference Values :

HDL.C concentration in serum is between (35 – 55) mg/dl (> 1.17 mmol/l)

2-3-6-4 Determination of Serum Low Density Lipoprotein Cholesterol

The estimated LDL-C and LDL-C serum level was determined by the Friedwald method. The levels for LDL-C and LDL-C were calculated by the Friedwald formula. In 1972 Friedwald had proposed a formula for LDL-C value calculation based upon the assumption that VLDL-C is present in serum at a concentration equal to one-fifth of the concentration of triglyceride

Cho=HDL+LDL+VLDL

VLDL=TG/5

LDL=Ch-(HDL+VLDL)

Reference Range :

Normal value of LDL cholesterol less than 100 mg/dL (< 3.4 mmol/l)

2-3-7 Determination of serum Albumin levels

Principle:

The method is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acid pH produce a color change of the indicator from yellow –green to green –blue with the resulting shift in the absorption wavelength of the complex.

$$BCG + Albumin \xrightarrow{\text{Acid pH}} \text{BCG-albumin complex}$$

Reagents composition :

Table 2-10: Reagents for measuring serum Albumin level

■ R1 Succinate buffer 61.0 mmol/l, bromocresol green (BCG) 0.18 (mmol/l)
■ STD Albumin standard 5 g/dl (0.746 mmol/l).

Procedure :

1.Three sets of tubes (Sample, standard and blank) were prepared as the following :

TUBES	**Blank**	**Sample**	**Standard**
Reagent	1.5 ml	1.5 ml	1.5 ml
Sample	---	10 µL	---
Standard	---	---	10 Ml
DW	10 µL	---	---

2. The solutions were mixed and incubated at least for 5 min at 37 ºC.

3. The absorbance of the sample and standard were measured at wavelength 630 nm against blank.

Calculation :

$$\text{Albumin concentration (g/dl)} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Conc. of standard}$$

$$\text{Conc. of standard} = 5 \text{ (g/dl)}$$

Reference Values :

Albumin concentration in serum is between 36– 52 g/dl

2-3-8 Determination of serum Asprosin levels

Principle:

Enzyme-Linked Immunosorbent Assay (ELISA) kit was used. The kit used a double-antibody sandwich enzyme-linked immune sorbent one-step process to assay (Asprosin and Vitamin D_3) in Human serum. The plate has been pre-coated with human Asprosin antibody. Asprosin present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human Asprosin antibody was added to wells and binds to Asprosin in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated Asprosin antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human Asprosin bound. The color development was stopped and the intensity of the color was measured at 450 nm.

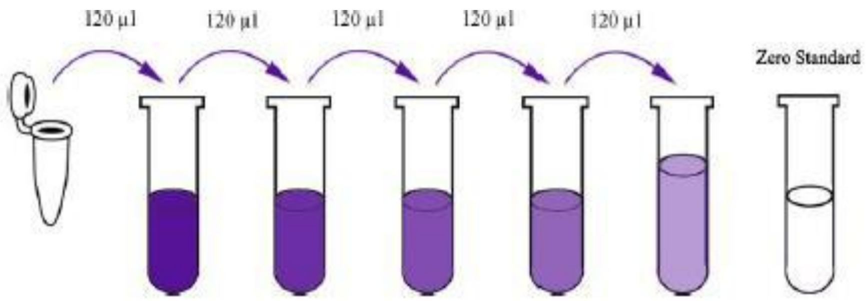
Reagent preparation

1-Before used, all reagents were need to be elevated to room temperature.

2-The original density of standard sample was diluted as the following table:

64 ng/ml	Standard No. 5	120µl Original Standard + 120µl Standard Diluent
32 ng/ml	Standard No. 4	120µl Standard No.5 + 120µl

		Standard Diluent
16 ng/ml	Standard No. 3	120µl Standard No.4 + 120µl Standard Diluent
8 ng/ml	Standard No. 2	120µl Standard No.3 + 120µl Standard Diluent
4 ng/ml	Standard No. 1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentratin	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
128 ng/ml	64 ng/ml	32 ng/ml	16 ng/ml	8 ng/ml	4 ng/ml

Figure (2-2): Concentration of standards of Asprosin.

3- A volume of 500 ml of concentrated (1x) Wash Buffer were diluted into 20ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

Procedure:

1. Before used, all reagents were put to room temperature. The assay was performed at room temperature.

2. The number of stripes that were necessary for the test were identified. The strips that used place inside the frames. The strips that were not used must be kept at 2 to 8°C.

3. A volume of 50µl of standard was added to well standard.

4. A volume of 40µl from sample were added and then added 10µl of anti- Asprosin antibody to sample well, then 50µl of Streptavidin-HRP to both sample and standard wells. Mixed well. The over plate covered with sealer and incubated for 60 min at 37 °C.

5. The coating eliminated and cleaned the plate 5 times over with a washed buffer. For each wash, soak well for 30 sec. to 1 min., with at least 0.35 ml wash buffer. Aspired for automatic washing of all wells, by washing with a wash buffer 5 times, filling wells with a wash buffer. The plate was blotted into paper towels or other absorbing material.

6. A volume of 50 µl substrate solution A was added and then 50µl substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

7. To each well 50 µl of stop solution was added, and the color changed from the blue yellow immediately.

8. The Absorbance was determined of each well immediately at 450 nm within 10 minutes after adding the stop solution.

Calculation:

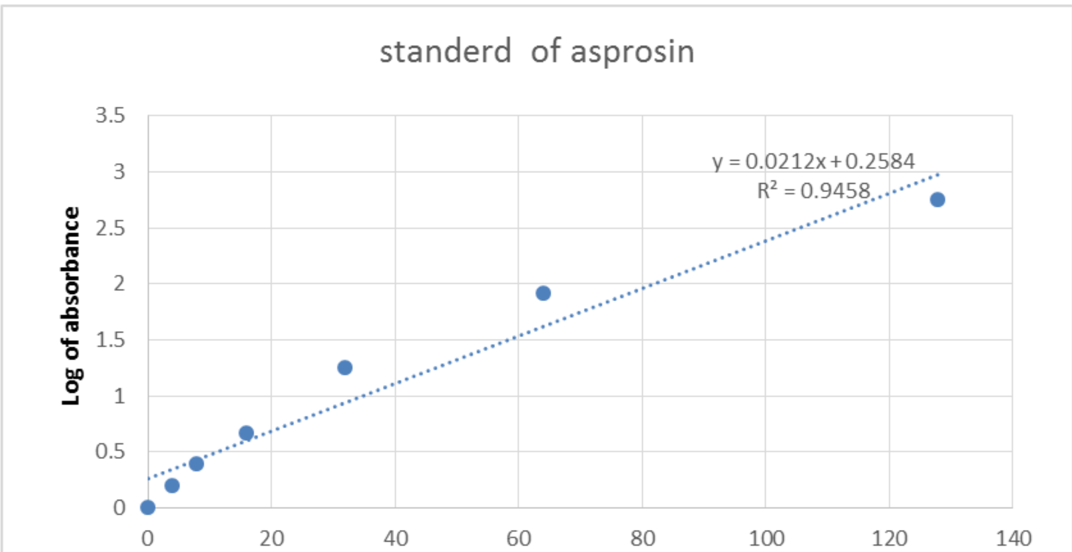


Figure (2-3) Standerd curve for Human Asprosin

2-3-9 Determination of serum Vit D3 (1,25 dihydroxy-cholecalciferol) **levels**

Principle:

This kit is anEnzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with HumanVDantibody. VDpresentin the sample is added and binds to antibodies coated on the wells. And then biotinylated HumanVDAntibody is added and binds to VDin the sample. Then Streptavidin-HRP is added and binds to the Biotinylated VD antibody. After incubation, unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of HumanVD. The reaction is terminated by addition of acidic stop solution and absorbance is measured at450 nm.

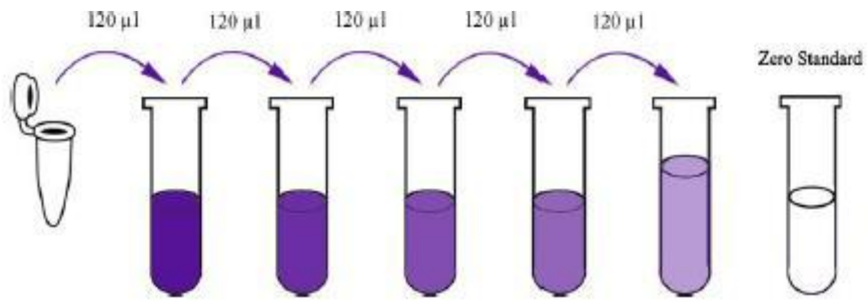
Reagent preparation :

1-Before used, all reagents were need to be elevated to room temperature.

2-The original density of standard sample was diluted as the following table:

64 ng/ml	Standard No. 5	120µl Original Standard + 120µl Standard Diluent
32 ng/ml	Standard No. 4	120µl Standard No.5 + 120µl Standard Diluent
16 ng/ml	Standard No. 3	120µl Standard No.4 + 120µl Standard Diluent
8 ng/ml	Standard No. 2	120µl Standard No.3 + 120µl

		Standard Diluent
4 ng/ml	Standard No. 1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentratin	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
128 ng/ml	64 ng/ml	32 ng/ml	16 ng/ml	8 ng/ml	4 ng/ml

Figure (2-4): Concentration of standards of Vit d3

3- A volume of 500 ml of concentrated (1x) Wash Buffer were diluted into 20ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

Procedure:

1. Before used, all reagents were put to room temperature. The assay was performed at room temperature.

2. The number of stripes that were necessary for the test were identified. The strips that used place inside the frames. The strips that were not used must be kept at 2 to 8°C.

3. A volume of 50 µl standard was added to well standard .

4. A volume of 40 µl from sample were added and then added 10µl anti-VD antibody to sample wells, then 50µl of streptavidin-HRP to both sample wells and standard wells. Mixed well. The over plate was covered with a sealer and incubated 60 minutes at 37°C.

5. The coating eliminated and cleaned the plate 5 times over with a washed buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 ml wash buffer. Aspired for automatic washing of all wells, by washing with a wash buffer 5 times, filling well with a wash buffer. The plate was blotted into paper towels or other absorbing material.

6. A volume of 50µl substrate solution A was added and then 50µl substrate solution B for each well. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

7. To each well 50µl of stop solution was added, and the color changed from the blue to yellow immediately.

8. The Absorbance was determined of each well immediately at 450 nm within 10 minutes after adding the stop solution.

Calculation :

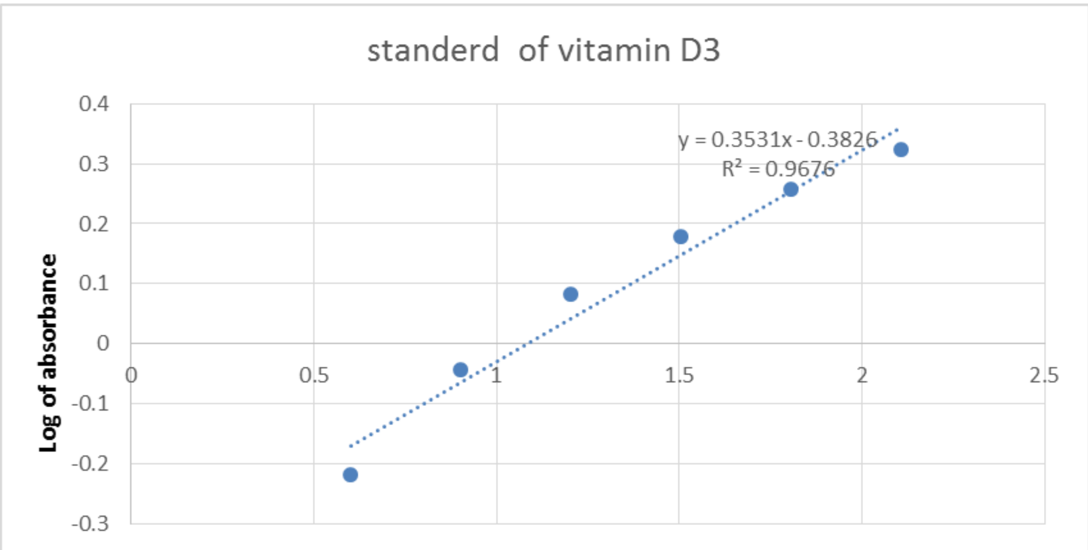


Figure (2-5) Standerd curve for Human Vitamin D3

2.4 Data Analysis

Statistical analysis was carried out using SPSS version 23. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. Pearson correlation coefficient was used to assess the relationship between two continuous variables. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of Vitamin D3 and Asprosin the context of early detection of nephropathy in patients with type 2 diabetes mellitus. The area under the curve (AUC) provides a useful tool to compare different biomarkers. Whereas an AUC value close to 1 indicates an excellent diagnostic and predictive marker[86]. A p-value of ≤ 0.05 was considered as significant**.**

Chapter Three

Results

And

Discussion

3- Results and Discussion

3-1 General Characteristics of Studied Group

3-1-1 Age Distribution in Studied group

The distribution of Studied group according to age is shown in Table (3.1).The results presented age showed that there was no significant difference (P value > 0.05) between the study groups (T2DM, T2DM with Nephropathy, and the control group). This matching is important to eliminate any effects on the results that may arise from the difference in these characteristics.

Table 3-1: The mean age according to studied groups

Characteristics	Study groups	No.	Mean $\pm$ SD	P-value
Age(years)	Control	40	50.62 $\pm$ 5.63	0.264
	Diabetes Mellitus	40	48.56 $\pm$ 6.34	
	Diabetic Nephropathy	40	48.07 $\pm$ 4.24	

P value $\leq$ 0.05 significant
P value > 0.05 non-significant

3-1-2 BMI Distribution in Studied group

The distribution of studied group according to BMI is shown in Table (3-2).The results presented BMI showed that there was no significant difference (P value >0.05) between the study groups (T2DM, T2DM with Nephropathy, and the control group).This matching is important to eliminate any effects on the results that may arise from the difference in these characteristics.

Table 3-2 :The mean BMI according to studied groups

Characteristics	Study groups	No.	Mean ±SD	P-value
BMI(Kg/m^2)	Control	40	26.3 ± 3.08	0.221
	Diabetes Mellitus	40	27.83 ± 5.16	
	Diabetic Nephropathy	40	27.9 ± 3.43	

P value ≤ 0.05 significant
P value > 0.05 non-significant

3-1-3 Gender Distribution in Studied group

The association between study groups and gender shown in Table (3-3) with no statistical difference (p-value >0.05).

Table 3-3: Association between studied groups and gender:

Gender	Study group			Total	+P v alue
	Control	**Diabetes mellitus**	**Diabetic Nephropathy**		
male	22	22	22	66	0.835
Female	18	18	18	48	
Total	40	40	40	120	

P value ≤ 0.05 was significant
P value > 0.05 was non-significant

3-1-4 Duration of Diabetes mellitus

the distribution of patients according to the duration of diabetes including (5-10 years, 10-15 years, and ≥ 15 years) as a shows in the figure (3-1). A majority (No.=27, 45%) of patients presented with a duration of DM between 5-10 years.The duration of DM had important effects on development of complication of diabetes. ADA criteria for microvascular complications considered that long-standing duration of diabetes is one of the typical presentations of diabetic kidney disease [86].

The present study agreed with (Vitasari Indriani *et al.*) who show the frequency of microalbuminuria was strongly associated with diabetes duration ($P < 0.001$) [87]. Another study (El-Ashmawy NE *et al.*) showed that the duration of diabetes was as a predictor for nephropathy in T2DM patients and the development of nephropathy in diabetic patients was linked to the duration of diabetes mellitus [88]. This is because a long period of hyperglycemia induced tissue damage and leads to the progression of the disease. The duration of DM from history not always the true duration of type 2 DM as many had delay in diagnosis [89].

3-2 Fasting Serum Glucose

The mean concentrations of FBG level was significantly increased in T2DM patients with nephropathy and those without nephropathy compared with the control group, as shown in Table (3- 4).

Table 3-4: The level of serum FBG in patients (T2DM ,T2DMNP) with control groups.

Variables	Study groups	No.	Mean ± SD	P-value
FBG (mmol/L)	Control	40	5.47 ± 0.766	<0.001
	Diabetes mellitus	40	12.65 ± 4.21	
	Diabetic Nephropathy	40	14.56 ± 4.63	
HbA1C %	Control	40	4.34 ± 0.5	0.025
	Diabetes mellitus	40	8.36 ± 1.68	
	Diabetic Nephropathy	40	9.43 ± 1.92	

According to the results of the present study that showed the levels of FSG and HbA1c were significantly elevated in T2DM patients with nephropathy and without nephropathy compared with the apparently healthy control. These results support previous studies that have been suggested that hyperglycemia is the driving force behind the development of DN [90].

The cellular mechanisms that contribute to hyperglycemia-mediated renal damage include accelerated generation of advanced glycation end products and activation of their receptor (RAGE), which induces increases in protein kinase C, nuclear factor-kappa B, transforming growth factor-β, and connective tissue growth factors. The resultant generation of reactive oxygen species (ROS) and a chronic subacute inflammatory process play a pivotal role in the development of DN [91].

The high concentration of HbA1c results from the presence of high levels of glucose in the blood, which leads to the addition of glucose moiety to hemoglobin in a spontaneous and non-enzymatic reaction, which is proportional to the lifespan of the red blood cell, so it is considered as a good marker of hyperglycemia for a long period, and this is in agreement with the previous study [92].

In the current study, poor glycemic control (elevated HbA1c) increased in T2DM with nephropathy and this in agreement with the study done by Al-Eisa AA etal who have reported that a poor glycemic control is a risk factor of diabetic nephropathy, the finding of a higher level HbA1c in patients with nephropathy in this study supports the fact that nephropathy is most likely due to poor glycemic control [93].

3-3 Renal Function Parameters

In this study, parameters included in renal function tests (urea and creatinine) recorded higher levels in T2DM patients with nephropathy as compared without nephropathy and the control group (P-value <0.001). As shown in Table (3-5).

Table(3-5): Renal Function Parameters in studied groups

Paremeters	Study group	No.	Mean $\pm$ SD	P-value
Serum urea (mmol/L)	Control	40	5.05 $\pm$ 1.04	$<$ 0.05
	Diabetes Mellitus	40	5.7 $\pm$ 0.95	
	Diabetic Nephropathy	40	12.51$\pm$ 3.27	
Serum creatinine (mg/dl)	Control	40	0.35 $\pm$ 0.12	$<$ 0.05
	Diabetes mellitus	40	0.41 $\pm$ 0.21	
	Diabetic Nephropathy	40	1.21 $\pm$ 0.56	

(P-value <0.05* is considered to be statically significant)

Blood tests for urea and creatinine are the simplest way to monitor renal function.These substances are normal metabolic waste products that are excreted by the kidneys. Urea is byproduct of protein breakdown. In kidney disease,these substances are not excreted normally,and so they accumulate in the body, thus causing an increase in blood levels of urea.

Serum creatinine is primarily ametabolite of creatine, almost all of which is located in skeletal muscle .The amount of creatine per unit of skeletal mass is consistent and the breakdown rate of creatine is also consistent. Thus ,plasma creatinine concentration is very stable and a direct reflection of skeletal muscle mass [94]. Interestingly, Nobuko Harita etal [95],have been hypothesized that lower serum creatinine is associated with an increased risk of T2DM, which might reflect a lower volume of skeletal muscle. Skeletal muscle would mean fewer target sites for insulin, which causes an increase in insulin resistance. This leads to the development of T2DM [96].

High blood glucose levels damage millions of nephrons resulting in inability of kidneys to maintain fluid and electrolyte homeostasis. Creatinine is filtered by glomerulus and thus, serum creatinine level is considered as an indirect measure of glomerular filtration. Diminishing of glomerluar filtration rate results in rise of plasma concentrations of serum creatinine and urea. This rise indicates progression of kidney disease and thus serum creatinine has greater prognostic ability compared with urea for predicting the adverse outcomes [97].

This work was show that a significant difference in serum urea and creatinine between the T2DM patients with nephropathy as compared without nephropathy and control groups. Urea and creatinine were elevated in patients as the disease progressed to late stages [98]. In this study, the groups of diabetes and diabetes nephropathy previously diagnosed were selected by a physician.

3-4 Serum Albumin

In this study Serum Albumin level in T2DM with nephropathy, T2DM without nephropathy and control groups with highly significant difference P(>0.05) as shown in Table (3-6). whereas there was no significant difference between T2DM without nephropathy and control group P (0.09).

Table 3-6: Serum Albumin in the study groups

Parameter	Study groups	No.	Mean $\pm$ SD	P-value
Serum Albumin (g/dl)	Control	40	43.13 $\pm$ 4.74	**0.001**
	Diabetes mellitus	40	42.62 $\pm$3.63	
	Diabetic Nephropathy	40	26.34 $\pm$ 3.77	

Human serum albumin, as the most represented plasma protein, is synthesized in the liver and secreted into the vascular space to distribute in all body tissues [99]. It plays a decisive role in the maintenance of homeostasis and makes a balance between hydrostatic and colloid osmotic pressure within vessels [100].

Growing evidence has proven that the hypoalbuminemia was caused by an inadequate energy or protein intake, impaired liver synthesis, decreased intestinal absorption, increased tissue catabolism, or increased loss and is an important risk factor and predictor of increased morbidity/mortality despite of the implicated diseases [101].

In this study, the results strongly suggested that the serum levels of albumin related to the renal prognosis. Moreover, the serum albumin level had a significant inverse correlation with proteinuria and glomerular lesions, two well-known risk factors for DN progression, which might give some explanation for the association between the hypoalbuminemia and renal outcome. The results of this study suggested that low levels of serum albumin might have prognostic utility in DN. Our observations that patients with lower levels of serum albumin were more prone to progress to ESRD [102].

3-5 Measurement of Lipid profile levels

The results of the current study found the mean of serum (TC, TG, HDLc, LDLc, and VLDLc) levels were a significantly increase in patient T2DM and T2DMNP (p value <0.005) as compared with control group, at mean ± SD as like in Table (3-7).

Table 3-7: Comparison of Lipid profile level in patients (T2DM ,T2DMNP) with control groups.

Paremeters	Study group	No.	Mean ± SD	P-value
T.Cho (mmol/L)	Control	40	3.98 ± 0.46	0.025
	Diabetes Mellitus	40	4.45 ± 0.58	
	Diabetic Nephropathy	40	4.97 ± 0.68	
TG (mmol/L)	Control	40	1.81 ± 0.48	<0.05
	Diabetes Mellitus	40	3.02 ± 0.56	
	Diabetic Nephropathy	40	3.69 ± 0.84	
HDL-c (mmol/L)	Control	40	0.84 ± 0.14	<0.05
	Diabetes Mellitus	40	0.38 ± 0.13	
	Diabetic Nephropathy	40	0.27 ± 0.11	
LDL-c (mmol/L)	Control	40	2.74 ± 0.44	0.008
	Diabetes Mellitus	40	3.47 ± 0.58	
	Diabetic Nephropathy	40	4.2 ± 0.49	

VLDL-c (mmol/L)	Control	40	0.4 ± 0.08	0.001
	Diabetes Mellitus	40	0.6 ± 0.11	
	Diabetic Nephropathy	40	0.72 ± 0.16	

Dyslipidemia is an important clinical abnormality have seen in patients with diabetes mellitus. The characteristic features of diabetic dyslipidemia are high plasma triglycerides (TG) concentration, low high density lipoprotein cholesterol (HDL-C) concentration and increased concentration of small, dense, low density lipoprotein (LDL-c) particles. Insulin resistance leads to mobilization of free fatty acid from adipose tissue to liver, which causes increased TG production[103].

Dysfunction of lipoprotein lipase due to long-standing diabetes would further increase TG level causing accumulation of large TG-rich very low density lipoprotein particles, which in turn generate sdLDL particles [104]. Thus, even when low density lipoprotein cholesterol (LDL-c) level in type 2 diabetes mellitus (T2DM) patients is not elevated, they still have a preponderance of atherogenic LDL-c particles [105].

These results are consistent with the findings of this study .The lipid alterations stem from insulin resistance and a defective insulin action in the metabolism of lipoproteins.The net result consists of enhanced lipolysis with a subsequent increase in free fatty acids and VLDLc synthesis, a defect in LPL activity leading to the increased life span of chylomicrons and VLDLc in circulation, an increased transfer of cholesterol esters resulting in triglyceride-rich LDLc, and finally the elevation of plasma triglycerides and the reduced ratio of LPL to hepatic lipase causing the accelerated breakdown of HDLc [106].

3-6 Serum Asprosin concentration

The results of the current study found the serum Asprosin levels in the T2DM with nephropathy, T2DM without nephropathy, and control groups were significant difference P=(0.002), as shown in table (3-8).

Table 3-8: Asprosin level in patients (T2DM ,T2DMNP) with control groups.

Parameter	Study groups	No.	Mean $\pm$ SD	P-value
Serum Asprosin (ng/ml)	Control	40	12.12 $\pm$ 1.24	**0.002**
	Diabetes Mellitus	40	15.95 $\pm$1.23	
	Nephropathy Diabetes	40	18.20 $\pm$ 0.97	

Asprosin, a recently discovered hormone, is synthesized and released primarily from white adipose tissues and transported to the liver. Asprosin promotes hepatic gluconeogenesis, which subsequently leads to increased levels of glucose and insulin concentrations. Moreover, Asprosin across the blood-brain barrier and induces orexigenic neurons directly through a cAMP-dependent signals, triggering appetite stimulation and weight gain [63].

Recent studies have documented that the role of a Asprosin in diabetes mellitus and insulin resistance. serum Asprosin was elevated in T2DM patients as comperd with control. Furthermore, serum Asprosin levels were elevated in the impaired glucose regulation compared with healthy controls [107]. However, the current study was to investigate the possible role of Asprosin in T2DM patients with early-stage renal diseases.

The results of the present study demonstrated that serum Asprosin levels significantly higher in T2DM patients with nephropathy as compared to T2DM without nephropathy and normal control groups. These results suggesting that Asprosin may be involved in the development of DN. The current results are in agreement with previous studies[108].

The potential mechanism underlying elevated Asprosin levels in DN may be that Asprosin plays an important role in regulating blood glucose (induce hepatic glucose release) and insulin sensitivity. So, hyperglycemia and IR are two major causes of DN. Therefore, targeting Asprosin therapeutically may be beneficial for the treatment of diabetes and diabetic complications. On the other hand, Asprosin may promote inflammatory responses in multiple organs. In pancreatic beta cells, Asprosin able to bind to Toll-like receptor 4 (TLR4) through TLR4/JNK mediate pathway to increase ROS production and pro-inflammatory cytokines and thus promotes inflammation and apoptosis of B-cells and reduced insulin secretion [66].

Furthermore, Asprosin impair insulin sensitivity in muscles due to PKC-mediated ER stress/ inflammation route [109]. In other words, Asprosin may trigger inflammatory responses in the kidney, and this inflammation plays an important role in the occurrence and development of DN.

3-7 Serum Vitamin D3 concentration

Results of the present study reveal that there is a significant difference in serum concentration of calcitriol patients (T2DM, T2DMNP) and control group (P=0.000).Vitamin D_3 concentration was much lower in the patients group than control group as shown in Table (3-9). And a significant difference in serum concentration of calcitriol patients (T2DM) and patients (T2DMNP) (P=0.001). as shown in Table (3-9).

Table 3-9: Comparison of Vit D3 level in patients (T2DM ,T2DMNP) and control groups.

Parameter	Study groups	No.	Mean $\pm$ SD	P-value
Vit D_3 (ng/ml)	Control	40	23.16 $\pm$ 4.28	0.001
	Diabets mellitus	40	17.9 $\pm$ 2.54	
	Diabetic Nephropathy	40	15.5 $\pm$ 4.28	

Vitamin D3 (cholecalciferol) that presents in human body, full human vitamin D3 requirement can tak from either exposure to sunlight for enough time or by supplementation [73].

Numerous previous studies have been reported the association between vitamin D and blood glucose metabolism, and some are consistent with our findings [110]. For instance, several cross-sectional studies proposed the modest negative correlation between total 25(OH)D and T2DM [111]. Additionally, a series of prospective studies also reported that low levels of serum total 25(OH)D increased the risk of T2DM [112]. In addition, Danting Li et al. advocated that elevated total serum 25(OH)D levels were related to a reduced risk of impaired glucose homeostasis among adults without diabetes [113].

As known, high lipid levels were the potential risk factors for diabetes [114]. These results have been indicated that the lipid profile might mediate the role of vitamin D in diabetes status.
This means that when vitamin D levels were high, the chance of developing T2DM could be decreased by reducing TC and TG levels.
The possible mechanisms were that low vitamin D status induced secondary hyperparathyroidism, which caused calcium to spill out into fat cells and thus increased lipogenesis[115]. and the increase in calcium ions in adipocytes increased the expression of fatty acid synthase, which was a key regulator of lipid deposition and reduced lipolysis[116].

Some studies indicate that 25(OH) D levels decrease in patients with CKD [117]. Also The same results have found out in the current study. There are several theories about the pathogenesis of vitamin D deficiency in CKD. Megalin, which is present in endocytic receptors in proximal tubule cells, is involved in the reabsorption of DBP from glomerular ultrafiltrates [118].

In addition, megalin also mediates the subsequent intracellular conversion of 25(OH)D to its active form. As kidney function declines, megalin expression in the proximal tubule decreases [119]. Megalin function is also attenuated with reduced kidney function, because of damages from low molecular weight proteinuria. The activity of CYP27B1 is also associated with decreasing kidney function [120].

3-8 Diagnostic values for serum Asprosin and serum Vit D3

Receiver Operating Characteristic curve (ROC) was used to assess the diagnostic values of Vit D3 and Asprosin in identifying Nephropathy from diabetic patients, and which of them is more specific or sensitive in the diagnosis of diabetic nephropathy.

3-8-1 Serum Asprosin values:

The Area Under the Curve (AUC) was 0.92, P-value=0.001. The sensitivity and specificity of the test at the cut-off value of Asprosin ≥ 17.5 ng/ml were 86.7% and 80%, respectively . As shown in

Figure (3-2).

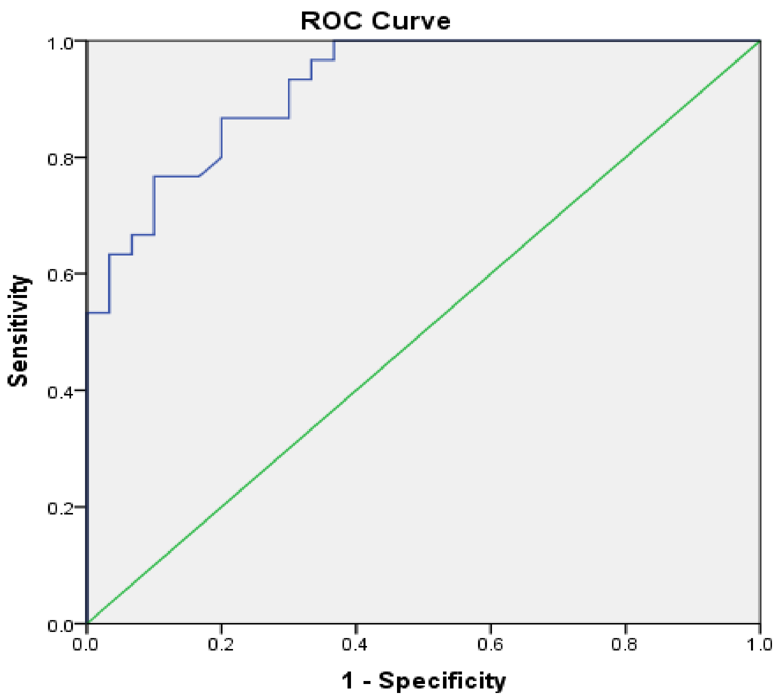


Figure (3-2) :ROC curve for sensitivity and specificity of Asprosion to predict Diabetes Nephropathy

3-8-2 Serum Vitamin D3 values:

The area under the curve (AUC) was 0.72, P-value=0.002. The sensitivity and specificity of the test at the cut-off value of Vit D3 ≤ 16.0 ng/ml were 76.7% and 60%, respectively . As shown in figure (3-3).

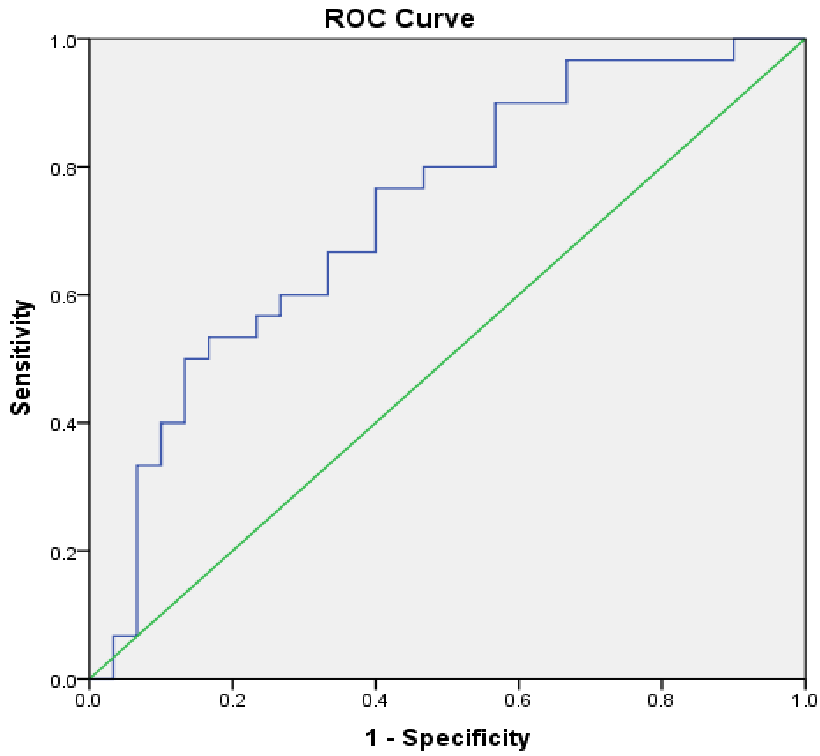


Figure 3-3 :ROC curve for sensitivity and specificity of Vitamin D3 to predict Diabetes Nephropathy

The present results showed that a good diagnostic profile of serum Asprosin at optimal cut off value for early detection of DN is 17.5 ng/ml with sensitivity 86.7 %, specificity 80%. For Vit D3 our results stated that weak diagnostic profile at cut off value 16.0 ng/ml with sensitivity 76%, specificity 60 %, as illustrated in Table (3-7). These results illustrate that the Asprosin is more specific and sensitive than Vitamin D3 in the early prediction of nephropathy.

3-9 The effect of serum Asprosin on other Variables in T2DM patients and T2DMNP:

Serum Asprosin showed appositive correlation with HbA1c (r=0.63, P=0.02), urea (r=0.562, P=0.003) and creatinine (r=0.468, P=0.009), TG (r= 0.33, P=0.02) and a negative correlation with Albumin (r= -0.49, P=0.008). As shown in Table (3-11).

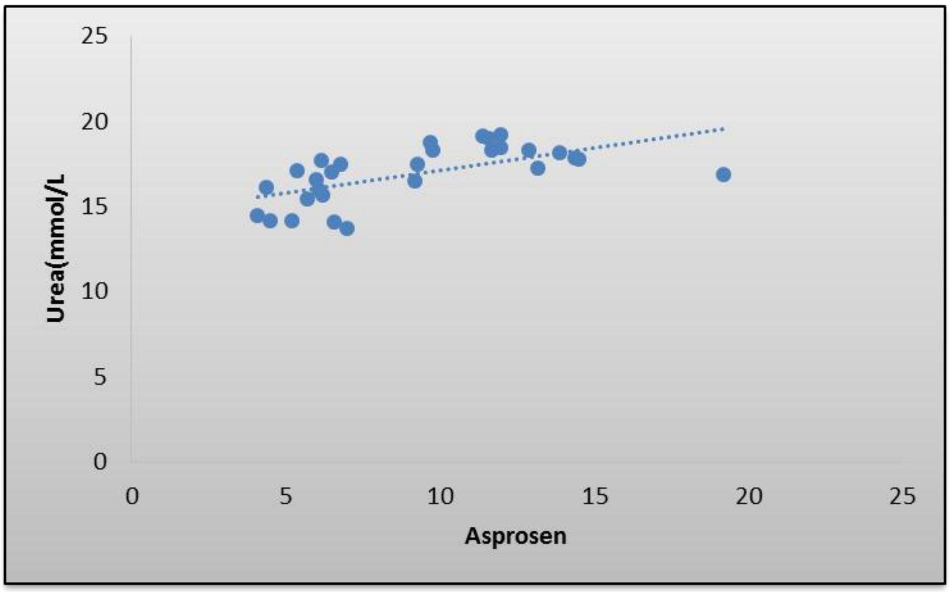
Table 3-11: The effect of serum Asprosin on other Variables in T2DM patients and T2DMNP

Study variables	Asprosin (ng/ml)	
	R	P-value
BMI	0.271	0.062
Age	0.168	0.347
FBG(mmol/L)	0.048	0.801
HbA1C	0.63	0.002*
serum urea(mmol/L)	0.562	0.003*
Creatinine(mg/dL)	0.468	0.009*
TG(mmol/L)	0.33	0.04*
TC(mmol/L)	0.131	0.397
HDL-c(mmol/L)	-0.265	0.071
LDL-c(mmol/L)	0.22	0.081
VLDL-c(mmol/L)	0.207	0.11
Albumin(g/dL)	-0.493	0.008*
Vitamin D_3(ng/ml)	-0.19	0.311

(P-value <0.05* is considered to be statically significant) r = cercelation coficient

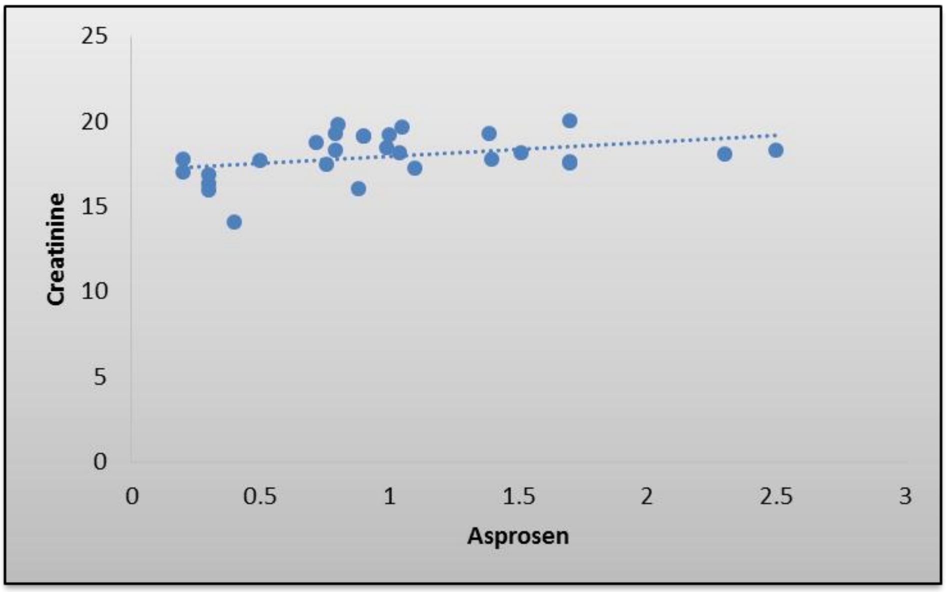
The results of the present study found that Asprosin was correlated positively with urea, ceratinine,TG and HbA1c and correlated inversely with Albumin in T2DM patients, which implies circulating Asprosin may participate in the pathogenesis of DN due to affecting blood glucose concentration and IR, as well as inflammatory responses in the kidney. as shown in Figures (3-4),(3-5),(3-6),(3-7) for blood urea, creatinine, Albumin,and TG**.**

T2DM patients with Nephropathy



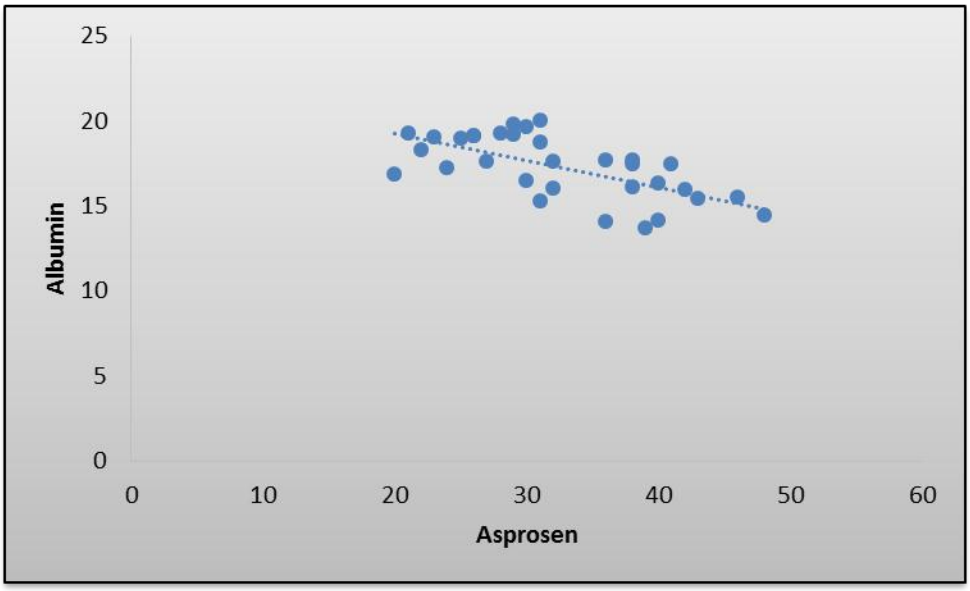
Figure(3-4): Correlation between Asprosin (ng/ml) and urea (mmol/L) (r= 0.562 P=0.003).

T2DM patients with Nephropathy



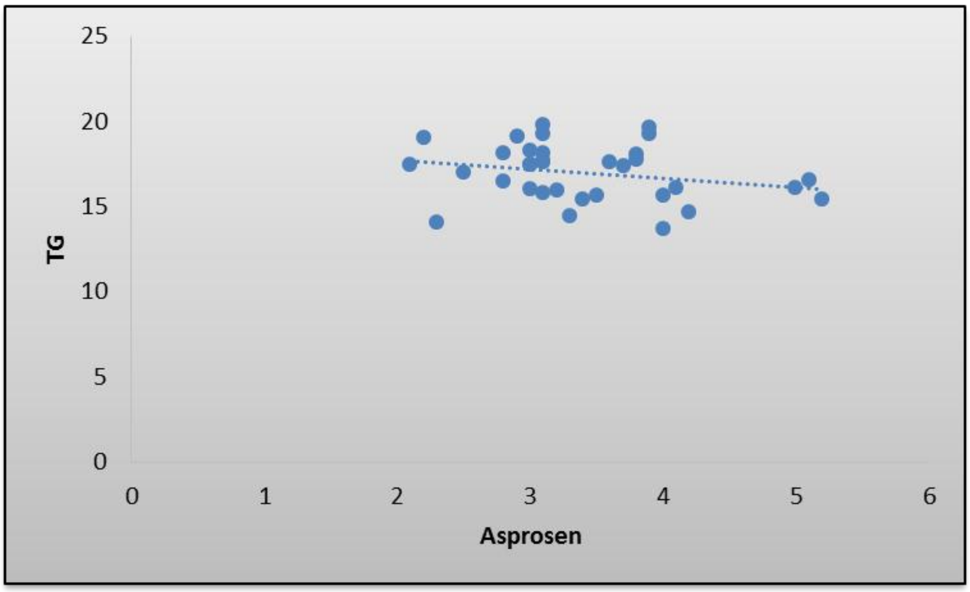
Figure(3-5): Correlation between Asprosin (ng/ml) and creatinine (mmol/L) (r= 0.468 P=0.009).

T2DM patients with Nephropathy



Figure(3-6): Correlation between Asprosin (ng/ml) and Albumin (mmol/l) (r=-0.493 P=0.008)

T2DM patients with Nephropathy



Figure(3-7): Correlation between Asprosin (ng/ml) and TG (mmol/L) (r=0.33 P=0.04)

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References

Conclusions:-

1- Serum Asprosin level positively correlated with blood urea, TG and creatinine, and negatively correlated with Albumin in T2DM patients, which implies circulating Asprosin may participate in the pathogenesis of DN due to affecting blood glucose concentration and insulin resistance, as well as inflammatory responses in the kidney.

2- A good diagnostic profile of serum asprosin at optimal cut off value for early detection of DN is ≥17.5ng/ml with sensitivity 86.7 %, specificity 80%. For Vit D3, a weak diagnostic profile at cut off value ≤ 16.0 ng/ml with sensitivity 76.0%, specificity 60.0 %, This indicated that serum Asprosin is more specific and sensitive than Vit D3 in the early prediction of nephropathy.

Recommendations:-

1- Introduce Asprosin clinical practice as a routine test besides serum albumin to early detection of renal impairment in diabetic patients.

2- Assessment of reactive oxygen species (ROS) and antioxidants and their relation to Asprosin levels in DN.

3- More focus is needed on good control of the patients' blood sugar and HbA1c to prevent diabetic nephropathy.

3- Estimate KB (NF-KB) and MAPK level and its correlation with Asprosin level in DN patients .

Summary

Diabetes Mellitus is a series of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes mellitus is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. diabetic Nephropathy is one of the most common micro vascular complications of diabetes affecting up to 20% to 40% of type 2 diabetes mellitus patients and may lead to End-Stage Renal Disease .Asprosin is primarily produced by white adipocytes, Asprosin promotes hepatic gluconeogenesis,which subsequently leads to increased levels of glucose and insulin concentrations. HbA1c was presented into clinical use in the 1980s and then has become a basis of clinical practice. It exposes an average of serum glucose over the prior 8 to 12 weeks. Serum albumin,as the most represented plasma protein, is synthesized in the liver and secreted into the vascular space to distribute in all body tissues. Urea is byproduct of protein breakdown in kidney disease these substances are not excreted normally,and so they accumulate in the body. Serum creatinine is primarily ametabolite of creatine, almost all of which is located in skeletal muscle. Dyslipidemia is an important clinical abnormality seen in patients with diabetes mellitus. The characteristic features of diabetic dyslipidemia are high plasma triglycerides concentration, low high density lipoprotein cholesterol concentration and increased concentration of small, dense, low density lipoprotein particles. Insulin resistance leads to mobilization of free fatty acid from adipose tissue to liver, which causes increased TG production. Vitamin D_3 synthesizes in the skin by exposure to the ultraviolet Brays

The current study was designed as a case control study, involved 120 individuals, 80 patients with type 2 diabetes mellitus group and 40 participants who are apparently healthy was used as control group. The patients were subdivided into two equal groups 40 diabetes without nephropathy,40diabetes with nephropathy. The concentration of glucose, HbA1c, blood urea, serum creatinine, Albumin, lipid profile, Vit D3 and Asprosin were estimated for all participants. The results of the present study found that the mean concentration of FBG and HbA1c levels were significantly increased in type 2 diabetes mellitus patients with nephropathy and those without nephropathy compared with the control group,in addition, serum albumin recorded lower levels in type 2 diabetes mellitus patients with nephropathy as compared without nephropathy and the control group. In contrast, Vit D3 was significantly lower in type 2 diabetes mellitus patients with nephropathy as compared with those without nephropathy and the control group.Serum Asprosin levels in type 2 diabetes mellitus with nephropathy (18.20 ± 3.21) were much higher than type 2 diabetes mellitus without nephropathy and control groups (15.95 ± 1.23),(12.12 ± 1.24), respectively with statistically significant difference (P-value=0.002). In addition , Asprosin showed a positive correlation with urea (r=0.526, P=0.003) and creatinine (r=0.468, P=0.009) and TG (r=0.33, p=0.04). And a negative correlation with serum Albumin (r=-0.493, P=0.008). Serum Vit D3 levels in T2DM with Nephropathy (15.68 ± 2.98) were much lower than T2DM without Nephropathy and control groups (17.82 ± 2.54), (23.16 ± 4.28), respectively with statistically significant difference (P-value <0.001). Receiver Operating Characteristic curves revealed that for early detection of DN, the best cut-off values to type 2 diabetes mellitus with nephropathy than those without nephropathy were ≥17.5 ng/ml for Asprosin with 86% sensitivity, 80% specificity, and area under the curve

(AUC) =0.92; and ≤ 16.0 ng/ml for Vit D3 with 76.7% sensitivity, 60.0% specificity, and area under the curve (AUC) = 0.72. So, serum Asprosin level may be considered as an independent and reliable biomarker for early detection of DN because its sensitivity and specificity is more than 80% in T2DM patients with nephropathy .

Contents

List of Figures

List of Table

List of Abbreviation

Abbreviation	Description
4-AA	4-aminoantipyrine
ADA	American Diabetes Association
AGEs	Advanced glycation end-products
AgRP	Agouti-related peptide
AR	Aldose reductase
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
BCG	Bromo Cresol Green
DBP	vitamin D-binding protein
CAMP	Cyclic Adenosine Mono Phosphate
CVD	Cardiovascular disease
CE	Cholesterol Esterase
CO	Cholesterol Oxidase
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DN	Diabetic nephropathy
EDTA	Ethylene di amine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
ESRD	End-Stage Renal Disease
FBN 1	Fibrillin 1
FBG	Fasting Blood Glucose
FPOX	Fructosyl – Peptide Oxidase

FFA	Free Fatty Acids
GABA	Gamma-Aminobutyric Acid
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GBM	Glomerular Basement Membrane
GDM	Gestational Diabetes mellitus
GFR	Glomerular Filteration Rate
GOD	Glucose Oxidase
G.3.P	Glycerol.3.Phosphate
GPO	Glycerol Phosphate Oxidase
GK	Glycerol Kinase
HDL	High Density Lipoprotein
HRP	Horseradish Peroxidase
IGT	Impaired Glucose Tolerance
IR	Insulin Resistance
LDL	Low Density Lipoprotein
LPL	Lipo protien Lipase
Ml	Milliliter
MODY	Maturity Onset Diabetes of the Young
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-Kb	Nuclear factor-Kb
NPS	Neonatal Progeroid Syndrome
OGTT	Oral Glucose Tolerance Test
OLFR734	Olfactory 734 receptor
PAI-1	plasminogen Activator Inhibitor-1
PARP	polymers of ADP-ribose polymerase
PKA	Protein kinase A

PKC	Protein kinase C
POD	Peroxidase
POMC	Proopiomelanocortin
RAGE	Receptor for advanced glycation end-products
ROC	Receiver Operating Characteristic curve
ROS	Reactive Oxygen Species
sdLDL	small,dense,Low Densty Lipoprotein
SD	Standard Deviation
SDH	Sorbitol Dehydrogenase
SN	Sensitivity
SP	Specificity
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGF-β1	Transforming growth factor-β1
T2DMNP	Type 2 Diabetes Mellitus Nephropathy
TG	Triglycerides
TC	Total Cholesterol
UVB	ultraviolet B
VEGF	Vascular endothelial growth factor
VLDL	Very Low Density Lipoprotein
VDR	vitamin D receptor
WHO	World Health Organization
Ml	Microliter

بسم الله الرحمن الرحيم

م/اعتذار

الى السيد رئيس اللجنة المحترم

الى اعضاء اللجنة المحترمين اعتذر عن الاخطاء المطبعية التي وردت في الرسالة

page	The error	The correct
57	Table(3-4) FSG(mmol/L)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.84)
	HbA1c	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.245)
59	Table (3-5) Serum urea (mmol/L)	Diabetes VS Control → p(0.01) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.001)
	Serum creatinine (mg/dl)	Diabetes VS Control → p(0.62) Diabetic Nephropathy VS Control → P (0.011) Diabetes VS Diabetic Nephropathy → p(0.001)

60	Table (3-6) Serum Albumin (g/dl)	Diabetes VS Control → p(0.052) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.001)
62	T Cho (mmol/L)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.002)
	TG(mmol/L)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.004)
	HDL-c (mmol/L)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.003)
	LDL-c(mmol/L)	Diabetes VS Control → p(0.005) Diabetic Nephropathy VS Control → P (0.002) Diabetes VS Diabetic Nephropathy → p(0.001)
	VLDL-c(mmol/L)	Diabetes VS Control → p(0.011) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.001)
64	Table (3-8) Serum Asprosin (ng/ml)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.002) Diabetes VS Diabetic Nephropathy → p(0.001)
66	Table (3-9) Serum Vitamin D3(ng/ml)	Diabetes VS Control → p(0.001) Diabetic Nephropathy VS Control → P (0.001) Diabetes VS Diabetic Nephropathy → p(0.002)

46	Calculation of Results The amount of the measured biomarker in the serum was determined by plotting the standard curve between the absorbance and standard concentrations in a logarithmic regression type using Excel software (Figure). Then, the straight-line equation was obtained in the following form: Absorbance=Slope*Ln(Concentration)+Intercept Then Ln(Concentration)=(Absorbance-Intercept)/Slope Then the unknown concentration is obtained from the following equation: Concentration of biomarker=2.718^((Absorbance-Intercept)/Slope))	
62	Table (3-7) Nephropathy	Nephropathy Diabetes
70	Albumin (g/L)	Albumin (g/dl)

الخلاصة

داء السكري (DM) هو مرض ناتج عن فرط في سكر الدم بسبب مشاكل في إفراز الأنسولين أو بسبب الية عمل الأنسولين أو كليهما معا.يسبب مرض السكري بالضرر طويل الأمد والخلل الوظيفي ويسبب فشل الأعضاء المختلفة بسبب ارتفاع السكر في الدم، وخصوصا العين والكلى والأعصاب والقلب والأوعية الدموية.

يعتبر اعتلال الكلية السكري (DN) الاكثر شيوعا في مضاعفات الأوعية الدموية الدقيقة لمرض السكري الذي يؤثر على ما يصل إلى 20٪ إلى 40٪ من مرضى السكري من النوع الثاني (T2DM) وبالتالي قد يصل إلى مرض الكلى في نهاية المرحلة الى الفشل الكلوي وبالتالي يؤثر بمعدل الوفيات للمرضى الذين يعانون من T2DM. ومع ذلك فإن الكشف المبكر عن DN أمر بالغ الأهمية في تحسين الإدارة السريرية وتقدم التدخل العلاجي إلى الداء الكلوي بمراحله الأخيرة وغسيل الكلى. وفقًا للدراسات الحديثة فإن الإصابة الأنبوبية الكلوية لها دور مهم في التسبب في DN المبكر وقد تسبق الإصابة الأنبوبية بداية الإصابة الكبيبية. يعزز Asprosin استحداث السكر في الكبد مما يؤدي لاحقًا إلى زيادة مستويات الجلوكوز وتركيزات الأنسولين.

صممت الدراسة الحالية للتحقيق في الفائدة التشخيصية للمعدلات Asprosin وفيتامين D3 كمؤشرات حيوية للكشف المبكر عن اعتلال الكلية في مرضى السكري من النوع الثاني وكذلك لدراسة العلاقة بين هذه المؤشرات الحيوية مع معايير وظائف الكلى التقليدية.

أجريت الدراسة الحالية على (٦٠) مريضا يعانون من النوع الثاني من مرض السكري.و تم اختيار ٣٠ شخصًا يبدو انهم يتمتعون بصحة جيدة يتطابقون مع نفس العمر والحالة الاجتماعية والديموغرافية مع مرضى السكري كمجموعة ضابطة. تم تقسيم المرضى إلى مجموعتين متساويتين 30 مرض السكري بدون اعتلال الكلية ، 30 مرض السكري مع اعتلال الكلية. المرضى (DM، DMNP) التشخيص المسبق من قبل الطبيب (عن طريق اختبار الكيمياء الحيوية (FBS، HbA1c، RFT ، الألبومين) تم تقدير تركيز الجلوكوز ، HbA1c ، اليوريا في الدم ، الكرياتينين في الدم ، الألبومين ، الدهون ، فيتامين D3 والأسبروسين لجميع المشاركين.

وجدت نتائج الدراسة الحالية أن متوسط تركيز مستويات FBG و HbA1c قد زاد بشكل كبير في مرضى T2DM المصابين باعتلال الكلية والذين لا يعانون من اعتلال الكلية مقارنة مع مجموعة التحكم ،قيمة ($P<0.001$). بالإضافة إلى ذلك ، سجل ألبومين المصل مستويات أقل في مرضى T2DM الذين يعانون من اعتلال الكلية مقارنة بغير اعتلال الكلية والمجموعة الضابطة. في المقابل ، كان فيتامين D3 أقل بشكل ملحوظ في مرضى T2DM المصابين بمرض اعتلال الكلية مقارنة مع أولئك الغير مصابين باعتلال الكلية والمجموعة الضابطة ، (قيمة ($P<0.001$).

كانت مستويات Asprosin المصل في T2DM مع اعتلال الكلية (18.20 ± 3.21) أعلى بكثير من T2DM بدون مجموعات اعتلال الكلية ومجموعات التحكم (15.95 ± 1.23) ، (12.12 ± 1.24) ، على التوالي مع فرق معتد به إحصائيًا (قيمة $P = 0.002$). بالإضافة إلى ذلك ، أظهر Asprosin ارتباطًا إيجابيًا مع اليوريا ($r = 0.526$ ،$P = 0.003$) والكرياتينين ($r = 0.468$ ،$P = 0.009$) ، ($r =0.33$ ، $P = 0.04$) T G. وارتباطًا سلبيًا مع الألبومين المصل ($r=-493$,$p=0.008$).

كانت مستويات مصل فيتامين D3 في T2DM مع اعتلال الكلية (15.68 ± 2.98) أقل بكثير من T2DM بدون مجموعات اعتلال الكلية ومجموعات التحكم (17.82 ± 2.54) ، (23.16 ± 4.28) ، على التوالي مع فرق معتمد به إحصائيًا (قيمة $p=0.001$).

كشفت منحنيات تشغيل جهاز الاستقبال (ROC) أنه بالنسبة للاكتشاف المبكر لـ DN ، فإن أفضل قيم القطع لتمييز T2DM مع اعتلال الكلية من تلك التي لا تحتوي على اعتلال الكلية كانت ≥ 17.5 نانوغرام / مل لـ Asprosin مع حساسية 86 ٪ ، ونوعية 80 ٪ ، ومنطقة تحت المنحنى $AUC = 0.92$ ؛ و ≤ 16.0 نانوغرام / مل لفيتامين D3 مع حساسية 76.7٪ ونوعية 60٪ ومنطقة تحت المنحنى ($AUC = 0.72$). لذلك ، يمكن اعتبار مستوى Asprosin في المصل علامة حيوية مستقلة وموثوقة للكشف المبكر عن DN لأن حساسيته ونوعية تزيد عن 80٪ في مرضى T2DM المصابين باعتلال الكلية.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل /كلية العلوم للبنات /قسم الكيمياء

دراسة المؤشرات البايوكيميائية في النوع الثاني من داء السكري

رسالة

مقدمة الى مجلس كلية علوم البنات في جامعة بابل

كجزء من متطلبات نيل شهادة الماجستير

في العلوم الكيمياء

من قبل

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٢٠١٠-٢٠١١

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