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ESTIMATION OF HUMAN ERYTHROPOIETIN AND VISFATIN SERUM LEVELS AND THEIR RELATION WITH NEPHROPATHY DEVELOPMENT IN TYPE 2 DIABETIC PATIENTS

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Farah Abdulkhaleq Khattab ALHADDAD

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ESTİMATİON OF HUMAN ERYTHROPOİETIN AND VİSFATİN SERUM LEVELS AND THEİR RELATİON WİTH NEPHROPATHY DEVELOPMENT IN TYPE 2 DIABETİC PATİENTS

By Farah Abdulkhaleq Khattab ALHADDAD

May 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Farah Abdulkhaleq Khattab ALHADDAD

ABSTRACT

ESTIMATION OF HUMAN ERYTHROPOIETIN AND VISFATIN SERUM LEVELS AND THEIR RELATION WITH NEPHROPATHY DEVELOPMENT IN TYPE 2 DIABETIC PATIENTS

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Master of Science in Chemistry

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May 2022

The much more frequent cause of diabetic nephropathy is type 2 diabetes. The glomerular filtration rate (GFR) decreases due to type 2 diabetic nephropathy, and protein excretion in the urine (especially albumin) increases. As a result, end-stage renal failure takes hold. Microalbuminuria is present in people with diabetes, those with a GFR less than 60 (mL/min/1.73m^2), and those with severe DKD. The case-control study included 180 volunteers undergoing medical follow-up at Salah El-Din General Hospital; they have divided into two groups, the foremost group of 60 T2DM patients without nephropathy and the second group of 60 T2DM patients with nephropathy. In the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients, the results of EPO, VF, and other biomarkers showed a statistically significant difference was found between control vs. each patients groups and between the first patients group vs. second patients group (p-value $\leq$ 0.001) for each variable separately, and Erythropoietin correlated negatively with microalbuminuria, ACR, and HbA1c, respectively, and VS correlated positively with HbA1c. In conclusion, Erythropoietin & Visfatin rises in patients with T2DM without nephropathy, and Visfatin increases in T2DM with nephropathy patients while EPO starts to decrease in the same category.

2022, 43 pages

Keywords: Type 2 diabetes mellitus, Diabetic nephropathy, Erythropoietin, Visfatin

ÖZET

TİP 2 DİYABETİK HASTALARDA İNSAN ERİTROPOİETİN VE VİSFATİN SERUM DÜZEYLERİNİN TAHMİNİ VE NEFROPATİ GELİŞİMİ İLE İLİŞKİSİ

Farah Abdulkhaleq Khattab ALHADDAD
Kimya, Yüksek Lisans
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Mayıs 2022

Diyabetik nefropatinin çok daha sık görülen nedeni tip 2 diyabettir. Tip 2 diyabetik nefropati nedeniyle glomerüler filtrasyon hızı (GFR) azalır ve idrarda protein atılımı (özellikle albümin) artar. Sonuç olarak, son dönem böbrek yetmezliği tutulur. Mikroalbüminüri diyabetli kişilerde, GFR'si 60'ın altında (mL/dk/1.73m2) ve şiddetli DKD'li kişilerde bulunur. Vaka-kontrol çalışmasına Salah El-Din General Hospital'da tıbbi takipte olan 180 gönüllü dahil edildi; nefropatisi olmayan 60 T2DM hastasının en önde gelen grubu ve nefropatisi olan 60 T2DM hastasının ikinci grubu olmak üzere iki gruba ayrıldılar. Kontrol grubu, nefropatisi olmayan T2DM hastaları ve nefropatili hastaları olan T2DM'de, EPO, VF ve diğer biyobelirteçlerin sonuçları, kontrol ile her hasta grubu arasında ve birinci hasta grubu ile ikinci hastalar arasında istatistiksel olarak anlamlı bir fark bulundu. grubu (p değeri ≤ 0,001) her değişken için ayrı ayrı ve Eritropoietin sırasıyla mikroalbüminüri, ACR ve HbA1c ile negatif korelasyon gösterdi ve VS HbA1c ile pozitif korelasyon gösterdi. Sonuç olarak, nefropatisi olmayan T2DM'li hastalarda Eritropoietin & Visfatin yükselirken, nefropatili T2DM'li hastalarda Visfatin yükselirken, aynı kategoride EPO azalmaya başlar.

2022, 43 sayfa

Anahtar Kelimeler: Tip 2 diabetes mellitus, Diyabetik nefropati, Eritropoietin, Visfatin

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Farah Abdulkhaleq Khattab ALHADDAD

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LIST OF SYMBOLS

-	Minus
%	Percent
**	Significant
/	Divide
+	Plus
<	Greater than
=	Equel
>	Less than
±	Plus-minus
≤	Greater or equal to
≥	Less or equal to
dL	Deciliter
g	Gram
gm	Gram
kg	Kilogram
L	Liter
m^2	Square meter
mcg	Microgram
mg	Microgram
mIU	Milli-international units
min	Minute
mL	Milliliter
mmol	Milli mole
mol	Mole
ng	Nanogram
nm	Nanometer
NS	Non-significant
rpm	Revolutions per minute
µL	Microliter

LIST OF ABBREVIATIONS

ACR	Albumin Creatinine Ratio
CD38/157	Cluster of Differentiation 38/157
CDC	Center for Diseases Control
CDK	Chronic Kidney Disease
CVD	Cardiovascular Disease
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
eNAMPT	extracellular Nicotinamide Phosphoribosyl Transferase
EPO	Erythropoietin
ESRD	End Stage of Renal Disease
FBG	Fasting Blood Glucose
FPG	Fasting Plasma Glucose
GFR	Glomerular Filtration Rate
Hb	Hemoglobin
HbA1c	Hemoglobin A1c
IADPSG	International Society of Diabetes in Pregnancy Study Group
iNAMPT	intracellular Nicotinamide Phosphoribosyl Transferase
MA	Microalbuminuria
MARTs	Mono(ADP-Ribosyl) Transferases
mGFR	measured Glomerular Filtration Rate
NAD	Nicotinamide Adenine Dinucleotide
NAD+	Nicotinamide Adenine Dinucleotide Plus
OGTT	Oral Glucose Tolerance Test
PARPs	Poly (Adenosine Diphosphate-Ribose) Polymerases
ROS	Reactive Oxygen Species
SCr	Serum Creatinine
SD	Standard Deviation
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
UACR	Urinary Albumin to Creatinine Ratio
VF	Vistatin
WHO	World Health Organization

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1. INTRODUCTION

Type two diabetes mellitus (T2DM) is one of the most familiar metabolic illnesses worldwide. It is pushed by two factors: insulin secretion drop by pancreatic beta-cells & imperfect insulin response by insulin-sensitive tissues (like the pancreas). To fulfill metabolic needs, insulin secretion and action must be precisely timed. Thus, the molecular mechanisms involved in insulin production, release, and tissue insulin response must be tightly controlled (Galicia-Garcia *et al.* 2020, Kaneto 2015). T2DM causes chronic kidney failure in the developed & growing world. Diabetic nephropathy is an established consequence of both T1 and T2 diabetes. There are two types of diabetic nephropathy: Diabetes, commonly known as diabetic nephropathy is the foremost causality of the end-stage renal disease (ESRD) and death in both developed and developing world. Notably, renal dysfunction increases the chance of death from any cause in diabetics. Microvascular problems in diabetes are linked to poor glycemic control. The proof for an explicit causal association between hyperglycemia and renal illness in animals is stronger than in humans. Diabetic nephropathy is marked by increased albumin excretion, a reduction in glomerular function, and a rise in blood pressure. Diabetes patients are more prone to having hyperlipidemia and hypertension due to related variables (Bosman *et al.* 2001, Sulaiman 2019). Erythropoietin (EPO) is a glycated protein consisting of 165 amino acids that circulate in a concentration of approximately one-hundredth of other main hormones. Erythropoietin is a 40 percent carbohydrate connected to sialic acid in the blood. Glycosylation, which increases a substance's carbohydrate content, decreases hepatic clearance. A severe drop in erythropoietin can result in hemoglobin levels as low as 7.0 g/dL. This activation of the nuclear transcription factors and Ras/mitogen-activated kinase pathway is hypothesized to occur when erythropoietin interacts with its receptors on erythroid progenitor cells. Erythropoietin has been demonstrated to prevent erythrocyte apoptosis as well as promote red blood cell proliferation and differentiation. Unlike other hormones regulated by environmental cues, erythropoietin is regulated by tissue hypoxia and vasoconstriction rather than haemoglobin (Babitt and Lin 2012). Low haemoglobin (Hb) levels are linked to the progression of DKD, whether or not albuminuria is present. While many variables

contribute to anemia in diabetes, a shortage of erythropoietin (EPO) appears to be the most important. Although elevated EPO levels have been linked to poor survival in patients with heart failure, diabetic CKD, and kidney transplant recipients, the influence of elevated EPO levels on renal outcomes remains unknown (Babitt and Lin 2012, McGill and Bell 2006). Visfatin (VF)is a 52 kDa multifunctional protein. In addition to these functions, it is adipokine-like. Visfatin was initially described by Fukuhara et al. in 2005. They found visfatin, an insulin-like protein expressed in visceral adipose tissue (Kang and Cha 2011). Visfatin is a multi-adipokine involved in obesity, inflammation, cardiovascular diseases, and metabolic syndrome, including diabetic nephropathy. Several investigations imply that visfatin may be a replacement marker for type 2 diabetic nephropathy in people and animals (Kalantarhormozi *et al.* 2020, Sakin *et al.* 2020). Urinary albumin is the multiple often lost protein in the urine; it is more standardized compared to total urinary protein. Healthful adults excrete smaller than 150 mg protein and 30 mg albumin every day. Increased glomerular permeability allows macromolecules to be filtered out of the circulation that should have remained in it. Albuminuria can be caused by urinary tract infection, contamination with menstrual blood, hard activity, orthostatic Proteinuria, or other circumstances that enhance vascular permeability, like sepsis. Proteinuria raises the risk of ESRD and mortality. Diverse methods for calculating total albumin loss or total protein loss from urine protein. A timed 24-hour urine albumin collection is used to monitor urinary protein loss. Urinary dipsticks and assays of albumin or entire protein content in a site urine specimen are more convenient. Using dipsticks to evaluate renal protein loss has been normal for over 50 years (Ullah *et al.* 2020).

1.1 The Study Objectives

1. Evaluation of Erythropoietin and Visfatin role in the development of nephropathy.
2. Study the possibility of using EPO & VF as biomarkers in the early diagnosis of nephropathy.
3. Using EPO & VF as biomarkers in type 2 Diabetes Mellitus development monitoring.
4. Explanation of influence of the consequences of diabetic nephropathy on EPO and VF levels and Microalbuminuria.

2. LITERATURE REVIEW

2.1 Diabetes Mellitus Overview

Diabetes mellitus (DM) is one of the serious diseases that accompanies the patient throughout his life and is of a chronic nature, after the inability of pancreatic beta-cell to produce an adequate amount of the hormone insulin responsible for regulating the amount of glucose in the blood. Uncontrolled glucose level, a common side effect of this disease is causing physiological problems for most of the body's organs, especially the nerves, circulatory system, and blood vessels. World Health Organization (WHO) data shows that in 2014, only 8.5% of adults over the age of 18 were documented to have complications from diabetes. In another statistic released in 2019, more than 1.5 million people died due to diabetes as the main cause of death (Hemayati *et al.* 2020).

2.1.1 Epidemiology & pathogenesis of diabetes mellitus

Diabetes mellitus is a complex syndrome related to glucose and carbohydrate metabolism that is always characterized by hyperglycemia. About T1DM diabetes is considered an immune disease because the immune system attacks the beta cells in the islets of Langerhans and destroys them permanently, causing the inability to completely secrete insulin, the hormone responsible for reducing the amount of circulating sugar in the blood by binding to it and entering it into cells for metabolism. T2DM has a distinctive feature, insulin resistance, which is offset by a gradual decrease in the production and secretion of insulin, and the inability to control the high blood sugar level is noted. According to statistics over the past thirty years, more than 400 million people had diabetes all over the world in 2017, where T1DM represents 5%, and T2DM patients account for more than 89% and up to 5% for some other classifications of diabetes, which Refers to an increase in the spread of the disease globally during the past three decades and is subject to increase. Genetic predisposition is one of the most dangerous causes of the first and second types of this syndrome, which warn of high rates of obesity globally and the occurrence of metabolic problems that threaten life directly and have effects on all body systems such as ketoacidosis and others that resulted in a threat to life and permanent

damage to the nerves, eyes, cardiovascular system, kidneys, etc. compared to healthy people in the same age group (Mekala and Bertoni 2019).

2.1.2 Pathophysiology of diabetes mellitus

One of the most important diagnostic characteristics in T2DM is insulin resistance, especially in peripheral tissues such as liver, fat and muscle, accompanied by a gradual loss of beta-cell function with a clear deficiency in insulin secretion as a functional response to glucose stimulation, and it is noted in the production of glucose from the liver, all of this occurs without any signs of autoimmunity in the pancreas. Its rate ranges between 20 - 30% of young people, and they notice a faster cellular functional deterioration if compared to adults, as the percentage ranges between 7 - 11% despite undergoing medical treatment (Standl *et al.* 2019).

2.1.3 Type 2 diabetes mellitus

T2DM is the most common type of DM, accounting for 90% of people with diabetes around the world. We also mentioned that the most characteristic of this type is insulin resistance, where the response to this hormone decreases and becomes less effective, which stimulates the pancreas to produce more insulin, but as a result of the feed-back feature, it begins to gradually decrease, resulting in T2DM, which appears suddenly, especially after the fourth decade of life. Unfortunately, it began to increase in the lower age groups and even in children, due to the modern lifestyle of lack of physical activity and the type of food and its nature full of energy, which increased the rates of obesity (Lende and Rijhsinghani 2020).

2.1.4 Diagnostic criteria for diabetes mellitus

It is possible to diagnose diabetes through certain criteria and tests that show the incidence of this chronic disease, the most important of which is measuring the value of glucose in the blood plasma (FPG) during fasting FPG ≥126 mg/dL equal to (7.0 mmol/L), fasting

for at least 8 hours or through a glucose tolerance test (OGTT) by taking glucose load suspension having equal to 75 gram anhydrous glucose is liquefied in drinking water and measuring plasma glucose every two hours (2-h PG), 2-h PG ≥200 mg/dL equal to (11.1 mmol/L) OGTT, according to WHO, or measuring the value of hemoglobin A1c (HbA1c) in the blood A1c ≥ 6.5% equal to (48 mmol/mol) (Care and Suppl 2020).

2.2 Diabetic Nephropathy

Diabetic nephropathy (DN), or the so-called diabetic kidney disease (DKD), is a chronic disease that affects diabetic patients, especially T2DM patients, resulting in serious complications in the small blood vessels. It is noted through several tests such as serum creatinine, cystatin C, albumin urea, and others. Excessive filtration of albuminuria and a clear functional decrease in glomerular filtration lead to renal function collapse. The overlap of severe consequences of this disease, such as peripheral microvascular problems and renal glomerulosclerosis, leads to an increase in mortality rates to more than 30 times compared to diabetic patients without DN, where patients are accompanied by physiological and dynamic problems in the heart and blood vessels that gradually increase until they reach renal disability (Sagoo and Gnudi 2020).

2.2.1 Epidemiology of diabetic nephropathy

Many risk factors increase the prevalence and severity of diabetic nephropathies, such as elevated albuminuria, Hyperglycemia, Hypertension, Dyslipidemia, Obesity, and Smoking; as a result, it is widely spread over the world. A study in China uncovered a 38.8% prevalence of DN in 15856 diabetic patients. A futuristic effect study in the United Kingdom diabetes study including over 4000 patients showed that 28% of T2DM patients evolve renal impairment after 15 years. DN's prevalence in the United States is rising in lockstep with diabetes, according to data collected between 1988 and 2008. DN prevalence in the USA population was discovered at 2.2%, according to a study of the third national health and nutrition examination survey. The study in India found 34.4% suffering from DN from diabetic patients. A study in Japan Diabetes clinical data management showed 15.3% had low GFR in T2DM patients (Hussain *et al.,* 2021).

2.2.2 Pathophysiology of diabetic nephropathy

Different mechanisms, such as metabolic, hemodynamic, and inflammatory, play a role in the DN pathophysiology. Advanced glycation end products, hexosamine, protein kinase, and polyol pathways are all part of the metabolic process. An increase in glycosylated end products, activation of protein kinase C, and an increase in the synthesis of diacylglycerol all contribute to hyperglycemia-induced kidney injury. The stimulation of the electron transport chain by hyperglycemia results in an upsurge in reactive oxygen species (ROS) appearance, which is thought to be the underlying cause of diabetic complications (Hussain *et al.,* 2021).

2.2.3 Determination of kidney damage and function

The kidney is an important organ in the body that has the ability to excrete toxins and waste products such as uric acid, creatinine, and urea and works effectively to regulate the osmolarity and electrolytes in the blood and the volume of extracellular fluids and has the ability to produce important hormones such as renin and erythropoietin. The kidney is made up of approximately one million nephrons, which is the kidney functional unit, which in turn contains the glomeruli, distal tubules that meet in the collecting duct. When signs of kidney disease appear, we need to assess the damage and functional capacity of the kidneys, and there are several tests to ensure these functions to evaluate the condition and prescribe the necessary treatment with an evaluation of the response to treatment. The most important vital indicators for assessing kidney disease are albuminuria, serum creatinine, glomerular filtration ratio, albumin to creatinine ratio, etc. (Kalantarhormozi *et al.* 2020).

2.2.4 Microalbuminuria (MA)

Microalbuminuria (MA) is defined as a concentration above the normal value in the patient's urine. It is considered an indicator of primary nephropathy. The higher the concentration indicates the increase in kidney disease, and the increase in its

concentration is directly proportional to the age of diabetes. It is reported that the reference value of urinary albumin excretion is less than 30 mg/day, similar to (20 mcg/min); The term microalbuminuria is a term used to describe sustained albumin excretion between 30 to 300 mg/day, similar to (20 to 200 mcg/min). Suppose albumin excretion exceeds 300 mg/day (200 mcg/min). In that case, it is macroalbuminuria (Prasad & Tikaria, 2022; Sana *et al.,* 2020).

2.2.5 Serum creatinine (SCr)

Serum creatinine (SCr) is a breakdown product of creatine with a molecular mass of 113.12 g/mol. It is not absorbed or metabolized and is derived from the proximal tubular secretion. In the case of stable kidney function, creatinine-based estimation equations are used. However, changes in muscle mass and extra-renal creatinine concentration associated with kidney disease and diet type must be considered. SCr can be used to determine the GFR, as the examination cost is low. The glomeruli easily filter creatinine, but it is also secreted by 10-30% from the proximal tubules. Normal values during the tests for men range between 0.7 - 1.3 mg/dL (equivalent to (61.9 - 114.9 μmol/L) and women between 0.6 to 1.1 mg/dL (53 - 97.2 μg/L). In general, the creatinine level in men is higher than in women because of the difference in muscle mass (Lopez-Giacoman, 2015).

2.2.6 Albumin to creatinine ratio

The urinary albumin / creatinine ratio (UACR) is a new innovative method designed to determine the amount of urinary protein. This test is considered a clinical and diagnostic biomarker quantitatively and qualitatively for albuminuria and can replace the traditional tests to check urine protein within 24 hours. Within 24 hours, the urinary albumin to creatinine ratio can be estimated with high accuracy, as the test is characterized by its simplicity and reliability. The increased cardiovascular mortality associated with urinary albumin excretion with elevated levels is observed, especially in patients with diabetes and hypertension and those with a history of cardiovascular disease. Generally speaking,

in healthy subjects (UACR <30 mg/g), (UACR) is considered an alarming indicator for most causes of death in healthy subjects (Zhang *et al.,* 2022).

2.2.7 Glomerular filtration rate (GFR)

Determining glomerular filtration rate (GFR) is critical in research, medical practice, and public health for determining kidney function. Measured GFR (mGFR) is still the gold standard. However, estimates of GFR have advanced significantly during the past two decades (eGFR). Either the eGFR or MGFR is prone to inaccuracy compared to a person's GFR. Estimated GFR is currently advised by clinical approaches, public health agencies, and state agencies for the initial evaluation of GFR, with mGFR generally assumed as a necessary laboratory test based on how accurate the assessment of GFR needs to be research, clinical, or public health applications. Our strategy is to conduct initial and follow-up testing to determine the genuine GFR (Levey *et al.,* 2020).

2.3 Overview of Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone with a regulatory and physiological ability responsible for stimulating red blood cells production. Its molecular weight is 30.4 kDa. Produced in the fetus in the liver cells and adults in the kidneys, red blood cells' progenitor cells bind to bilateral receptors. This connection promotes cell permanence, proliferation, and differentiation into mature cells. It has protective physiological functions in the nerves, kidneys, heart, and wound healing. The mechanism of stimulating its production is through the amount of oxygen reaching the kidneys through blood cells moving through the bloodstream, where a decrease in oxygen leads to the loss of red blood cells or their decomposition and decreased production, which leads to the secretion of this hormone and thus the production of new red blood cells (Kato, 2021).

2.3.1 Relationship between erythropoietin and diabetic nephropathy

Anemia is caused by a lack of erythropoietin (EPO), when somebody has chronic kidney disease (CKD), they will be more likely to develop anemia, leading to cardiovascular disease. An increase in erythropoiesis was once reported to be associated with insulin resistance; however, this may be a non-issue. As a result of more complex mechanisms such as greater bleeding tendency, renin-angiotensin-aldosterone system inhibitor use or less oxygen sensing due to autonomic neuropathy, the inhibitory influence of inflammation cytokines, loss of EPO in urine, and insufficient response to EPO, DKD anemia developed earlier than non-DKD anemia and was more severe. Anemia treatment in DKD patients had the same encouraging effect on cardiovascular outcomes as anemia correction in CKD patients. The EPO renal benefit was less obvious in individuals with CKD and DKD despite animal studies showing that EPO has a pleiotropic influence on renal protection. Nevertheless, current evidence suggests that anemia treatments in DKD patients should avoid high hemoglobin (Hb) level. Greater EPO levels and higher Hb levels may indeed reduce renal ischemia (Tsai & Tarng, 2019).

2.4 Overview of Visfatin

Visfatin/ NAMPT (nicotinamide phosphoribosyltransferase) is one of the more interesting adipocytokines. It is first thought to be a precursor to B cell colony-enhancing factor; it was later found to be involved in the manufacture of nicotinamide-adenine dinucleotide (NAD). Both an intracellular and an external version of visfatin can be found. Visfatin/iNAMPT regulates NAD+ production, affecting several cytoplasmic NAD-dependent proteins, like MARTs, sirtuins, PARPs, and CD38/157 (Dakroub *et al.,* 2020).

2.4.1 Relationship between visfatin and diabetic nephropathy

Diabetes, dyslipidemia, hypertension, and atherosclerosis are all symptoms of metabolic syndrome that result from obesity's energy overload. An interesting finding is that being

overweight or obese causes an increase in the production of adipokines, including visfatin, resistatin, and leptin, all of which have been correlated to the diabetic nephropathy negative progress. Renal damage linked with insulin resistance appears to be mediated by adipokines produced in the kidney. Patients with type 2 diabetes have elevated visfatin levels, a biomarker of systemic inflammation. However, glucose uptake into kidney cells, which activates the internal insulin signaling pathway and pro-inflammatory processes, also increases its expression in diabetic kidneys. Visfatin appears to have a positive influence on T2DM. Diabetic nephropathy was improved in vivo by visfatin injection (Kang & Cha, 2011).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagent

Analytical reagents include human erythrobiotein ELISA Kit, human visfatin ELISA Kit, HbA1c, Microalbuminuria, Blood glucose, Creatinine, various substrates and all others materials were purchased from (BT LAB/China, BT LAB/China, BODITCH/S. Korea, BODITCH/ S. Korea, AGAPPE/ Spain & BIOLABO/ France) respectively.

3.1.2 Patients groups

The case-control study included 180 volunteers who were undergoing medical follow-up at Salah El-Din General Hospital, about 60 of whom were healthy individuals as a standard group for the study, and the remaining 120 patients were diagnosed clinically with T2DM; they have divided into two groups, the foremost group 60 T2DM patients without nephropathy and the second group 60 T2DM patients with nephropathy, the patients groups are illustrated in Figure 3.1. As an ethical statement, the study was performed according to the Helsinki Declaration of Human Rights and Medical Ethics; specimens will be collected after informing the patients and obtaining their express consent to participate in this study as full-fledged volunteers.

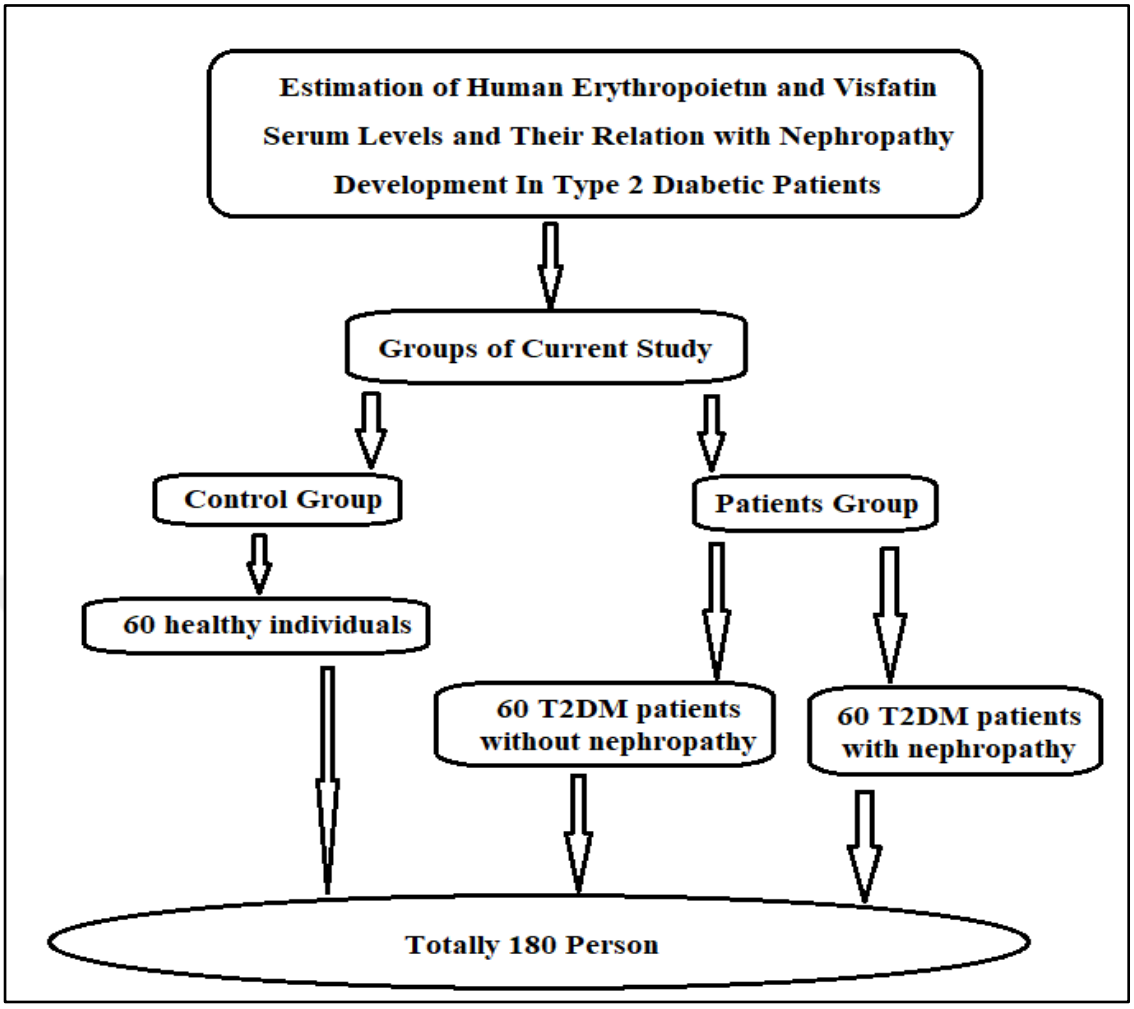

Figure 3.1 Groups of the current study

3.1.3 Instruments

Some of important instruments, equipments which are used to complete the current study are illustrated in Table 3.1.

Table 3.1 The instruments and equipment which used in the current study

No.	Instrument and Equipment	Manufacturer	Country of Origin
1	ELISA device & plate washer	BioTek	United States
2	CBC Analyzer	Medonic	Sweden
3	Spectrophotometer	Jenway	Italy
4	Centrifuge	Hettich	Germany
5	Incubator	Thermo Fisher	USA
6	Refrigerator	Hitachi	Japan
7	Shaking Water Bath	Stuart	UK
8	Plain Tube & Gel Tube	AFCO	Jordan
9	Eppendorf Tube 5ml	AFCO	Jordan
10	micropipette	Accumax	Europe Union
11	Disposable syringe	TERUMO	Turkey

3.1.4 Blood and urine sample collection

Five milliliters (5 mL) of venous blood from each participating subject were obtained and distributed into two tubes, the first 3mL was dispensed into a gel tube containing a clot activator, left at room temperature to clot for about (10) minutes, and then centrifuged for (10 min at 4000 rpm). The other 2mL was transmitted to the EDTA tube to assess HbA1c level, and hemoglobin and urine were collected from each subject in the study using a disposable urine cup to measure urinary albumin/creatinine ratio UACR by testing micro-albumin in urine. The separated serum was distributed into 4 Eppendorf tubes frozen at -20 until the assessment of Erythropiotein, Visfatin, Serum creatinine, and fasting blood sugar.

3.2 Methods

3.2.1 Erythrobiotein Concentration: Lot No. (20220110)

Principle

The kit employs the Sandwich-ELISA principle. This kit's micro ELISA plate is pre-coated with an anti EPO antibody. Samples (or Standards) are mixed with the particular antibody in micro ELISA plate wells. Then each microplate well gets a biotinylated detection antibody specific for EPO and an Avidin-Horseradish Peroxidase (HRP) conjugate. Free elements wash away. Each well gets a substrate solution. EPO,

biotinylated detection antibody, and Avidin-HRP conjugate wells appear blue. The stop solution stops the enzyme-substrate reaction, and the color turns yellow. Spirophotometrically, the optical density is 450nm + 2nm. The OD value relates to the EPO concentration. Comparing the samples' OD to the standard curve yields the concentration of EPO in the samples.

Procedure

1. The recommended guidelines for preparing all reagents, stock solutions, and test specimens are followed. Before utilizing any reagent, to be brought ambient temperature. The assay is carried out at ambient temperature during the procedure.
2. The needed test strips are to be calculated. Place the strips into the frames to be used. ' Keep the unused strips between two to eight °C.
3. 50 µL of Std. to be put into the standard well. The standard solution contains biotin - conjugated antibodies, so do not add antibodies into the standard well.
4. In the sample wells, add 40 uL of the specimen, followed by 10 µL of Human EPO antibody, and then 50 µL of streptavidin-HRP (Not blank control well). Make sure everything is well-combined. Seal the plate in place with a sealant. Incubate for 1 hour in a warm (37°C).
5. The plate to be washed five times by wash buffer after removing the sealant. For each wash, soak the wells for a half minute to one minute in 300 µL wash buffer. Wash each well five times with wash buffer for automated washing.
6. In each well, first 50 µL to be added of substrate solution (A) & 50 uL of substrate solution (B). Incubate a new sealer-coated plate at 37°C in the dark for 10 minutes.
7. The blue color will turn yellow as soon as you add 50 µL Stop Solution for every well.
8. After ten minutes of adding the stopping sol., the optical density to be measured for all wells using only a microplate reader was adjusted to four hundred fifty nanometers.

3.2.2 Visfatin Concentration: Lot No. (20220114)

Principle

The Sandwich-ELISA method is employed in this ELISA kit. This kit includes a micro ELISA plate that has been pre-coated with a Human VF antibody. In the micro ELISA

plate wells, samples (or standards) are mixed with antibodies specific to the assay. Biotinylated detection antibodies specific to human FV, Avidin-Horseradish Peroxidase (HRP), and incubated microplate wells are added to each. Water washes away any free components. Each well is then filled with the substrate solution. Biotinylated detection antibodies and Avidin-HRP conjugate are required to produce a blue color in the wells. Color changes to yellow when the stop solution is added to the enzyme-substrate reaction. An optical density (OD) of 450nm + 2nm can be determined when using a spectrophotometer. In terms of OD, the Human VF concentration is directly proportional to the OD. The OD of the samples can be used to determine the concentration of Human VF in the samples.

Procedure

1. The recommended guidelines for preparing all reagents, stock solutions, and test specimens are followed. Before utilizing any reagent, to be brought ambient temperature. The assay is carried out at ambient temperature during the procedure.
2. The needed test strips are to be calculated. Place the strips into the frames to be used. ' Keep the unused strips between two to eight °C.
3. 50 µL of Std. to be put into the standard well. The standard solution contains biotin - conjugated antibodies, so do not add antibodies into the standard well.
4. In the sample wells, add 40 uL of the specimen, followed by 10 µL of Human VISFATIN antibody, and then 50 µL of streptavidin-HRP (Not blank control well). Make sure everything is well-combined. Seal the plate in place with a sealant. Incubate for 1 hour in a warm (37°C).
5. The plate to be washed five times by wash buffer after removing the sealant. For each wash, soak the wells for a half minute to one minute in 300 µL wash buffer. Wash each well five times with wash buffer for automated washing.
6. In each well, first 50 µL to be added of substrate solution (A) & 50 uL of substrate solution (B). Incubate a new sealer-coated plate at 37°C in the dark for 10 minutes.
7. The blue color will turn yellow as soon as you add 50 µL stop solution for every well.
8. After ten minutes of adding the stopping sol., the optical density to be measured for all wells using only a microplate reader was adjusted to four hundred fifty nanometers.

3.2.3 Measurement of HbA1c: Lot No. (AAQLF99)

Principle

The sandwich approach is used to detect immunity. Then an immunological complex is formed between the reagent's antigen and the sample's antigen, which is picked up by a second antibody fixed to the test strip's nitrocellulose matrix. This equipment displays the ionized percentage of glycated hemoglobin from total hemoglobin in the blood.

Procedure

(1) Take 100 µL of hemolysis buffer to a detecting buffer tube and mix thoroughly; (2) A capillary tube containing 5 µL of fingertip or tubed blood should be inserted into the detecting buffer tube; (3) Seal the detecting buffer tube lid and shake the sample fifteen times to properly mix it; (4) The i-Chamber slot cassette is partially removed; (5) Pipette 75 µL of the specimen combination into a specimen well of the testing cassette and insert it into the test device; (6) Place the cassette into the i-Chamber device; (7) Remove the cassette after 12 minutes in the i-Chamber; (8) Once the incubation period is over, instantly scan the specimen cassette. If this isn't done, testing results will be off; (9) Insert the loaded cassette into the cassette holder. Before inserting the cassette all the way, be sure it is oriented correctly. The cassette has an arrow printed on it just for this function; (10) To begin scanning, click the 'Select' or 'Start' button on the ichroma^{TM} analyzer; (11) Look & read at the device's digital display to see the results.

3.2.4 Measurement of microalbuminuria: Lot No. (MAQCC65)

Principle

Antigen-antibody response and fluorescence technology immunoassay system for microalbumin. The complexes of antibody (anti-Albumin)-antigen (Albumin)-antibody (anti-Albumin) produce fluorescence on the test cartridge membrane when a test sample and detection buffer are thoroughly mixed and inserted into the sample well. Complexes were collected on the test cartridge membrane as albumin test sample increased. The intensity of fluorescence created on test cartridge membrane scans and shows albumin concentration on the reader's LCD screen.

Procedure

(1) Add the detection buffer to a pipette and transfer 10 µL (Human urine/control) of the sample; (2) After securing the detecting buffer tube's top, shake the sample for about ten times to ensure that it is properly mixed; (3) Fill (75 µL) the sample well on the cassette with the mixture you've just pipetted out; (4) For 12 minutes, keep the sample-loaded cartridge at room temperature. When the incubation period is over, immediately scan the specimen cassette. If you don't, you'll get erroneous results from the tests; (5) Insert the loaded cassette into the cassette holder. Before inserting the cassette all the way, be sure it is oriented correctly. The cassette has an arrow printed on it just for this function; (6) To begin scanning, click the 'Select' or 'Start' button on the ichroma™ analyzer; (7) Look & read at the device's digital display to see the results.

3.2.5 Measurement of creatinine: Lot No. (061824A)

Principle

The colorimetric reaction between alkaline picrate and creatinine will be measured at 490 nm with no pre-treatment stage. This reaction's quickness, flexibility, and specificity have been improved by improving a primary-speed approach.

Procedure

Added 500 µL of reagents (1) to 500µL from reagent (2), and added 100 µL from the specimen then mixed then Read A1 absorbance after 30 seconds & A2 after 120 seconds at 490 nm against distilled water.

3.2.6 Measurement of blood glucose: Lot No. (30090389)

Principle

Trinder's method: A red quinoneimine is produced by reacting peroxidase with glucose oxidase, which produces gluconic acid and hydrogen peroxide. The hue of the colored chemical at 500 nm is directly related to the amount of sugar in the specimen, according to the results of the experiment.

Procedure

Added 1000µL of reagents and 10µL from standard, control, then sample and Mixed then, waitting for 10 min at 37°C or 20°C min at Room temperature, then read absorbance at 500 nm, this procedure is illustrated in Equation (3.1).

$$\text{Glucose concentration } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Std. concentration} \quad (3.1)$$

3.2.7 Urine albumin / creatinine ratio calculator

The ratio of albumin to creatinine is calculated using the Equation (3.2):

$$\text{ACR } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{Albumin}}{\text{Creatinine}} \quad (3.2)$$

3.2.8 Estimation of GFR

The eGFR is a beneficial and accurate sign of renal function and is calculated using serum creatinine and age, race, and gender variables. The most often utilized evaluation is Equation (3.3), it is the shortened renal disease modified diet (MDRD).

$$\text{GFR } \left(\frac{\frac{\text{mL}}{\text{min}}}{1.73}\text{m2}\right) = 175 \times (\text{Serum creatinine}) - 1.154 \times (\text{Age}) - 0.203 \times (0.742\ if\ female) \quad (3.3)$$

3.3 Statistical Analysis

It is important to evaluate analytic results in terms of how accurate they are in terms of their sensitivity and specificity and positive and negative predictive values. Mean, and standard deviation are two statistical concepts used to describe analysis results. Mann-Whitney tests are used to compare groups that are abnormally distributed. To be considered statistically significant, the P-value had to be less than 0.01. SPSS 20.0 was used to review and approve all results (SPSS Inc, Chicago, Illinois, USA).

4. RESULTS

4.1 Age in Study Groups

The statistical analysis has been shown the mean and standard deviation (SD) in the age of the Control group, T2DM without, and with nephropathy patients (57.85 ± 8.76, 56.42 ± 8.71, and 56.80 ± 8.25 year) respectively. There were non-significant differences among all groups under study, as represented in Table 4.1 and Figure 4.1.

Table 4.1 Age in study groups

Age (years)	Mean	Std. Deviation
Controls	57.85	8.76
T2DM without nephropathy	56.42	8.71
T2DM with nephropathy	56.80	8.25
p value	0.855^{NS}	
Controls vs T2DM without nephropathy	0.361^{NS}	
Controls vs T2DM with nephropathy	0.503^{NS}	
T2DM without nephropathy vs T2DM with nephropathy	0.807^{NS}	

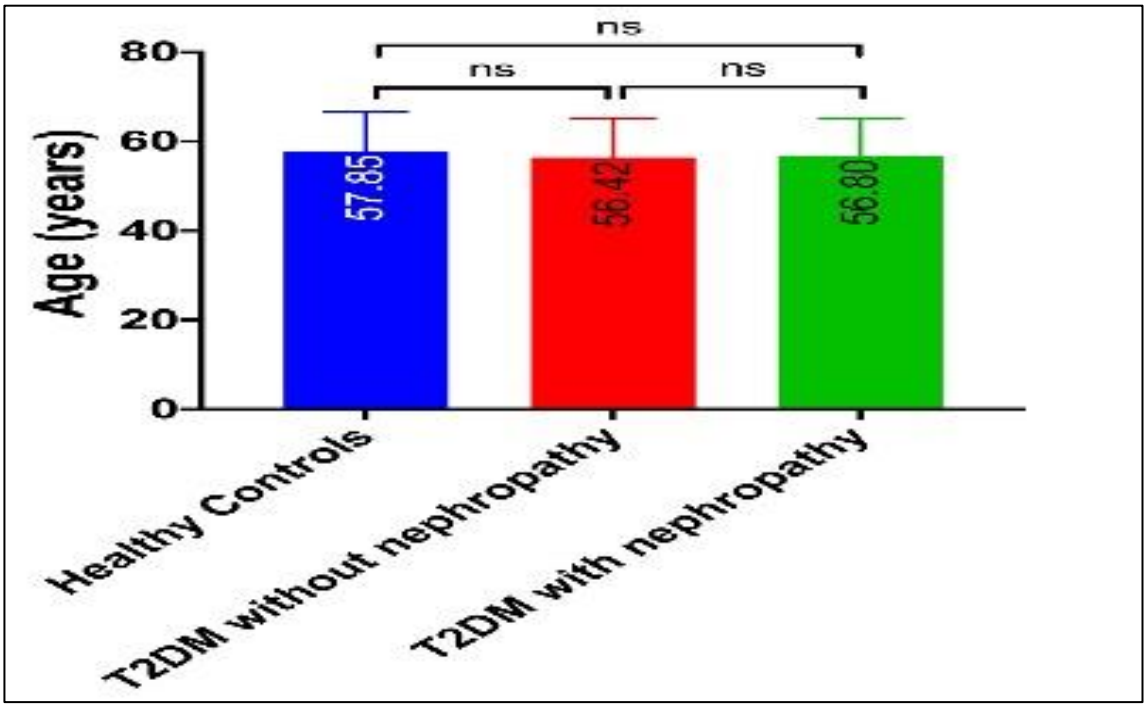

Figure 4.1 Age in study groups

4.2 Erythropoietin in Study Groups

The results of Erythropoietin (EPO) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the EPO levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (7.90 ± 0.91, 14.43 ± 1.27, and 4.56 ± 0.62 mLU/mL) respectively, There were significant differences among all groups under study, as represented in Table 4.2 and Figure 4.2.

Table 4.2 Erythropoietin in stury groups

Erythropoietin (mIU/mL)	Mean	Std. Deviation
Controls	7.90	0.91
T2DM without nephropathy	14.43	1.27
T2DM with nephropathy	4.56	0.62
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant **: significant

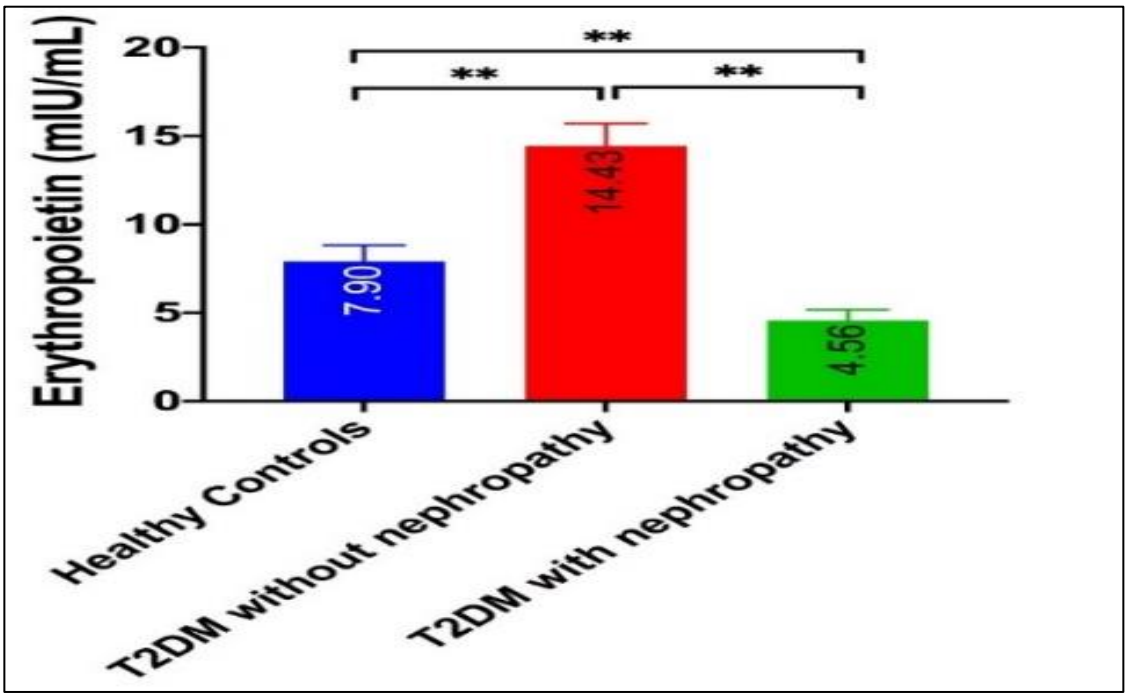

Figure 4.2 Erythropoietin in study group

4.3 Visfatin in Study Groups

The results of Visfatin (VF) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the VF levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (0.78 ± 0.13, 2.92 ± 0.50, and 5.06 ± 0.67 ng/mL) respectively, there were significant differences among all groups under study, as represented in Table 4.3 and Figure 4.3.

Table 4.3 Visfatin in study groups

Visfatin (ng/mL)	Mean	Std. Deviation
Controls	0.78	0.13
T2DM without nephropathy	2.92	0.50
T2DM with nephropathy	5.06	0.67
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

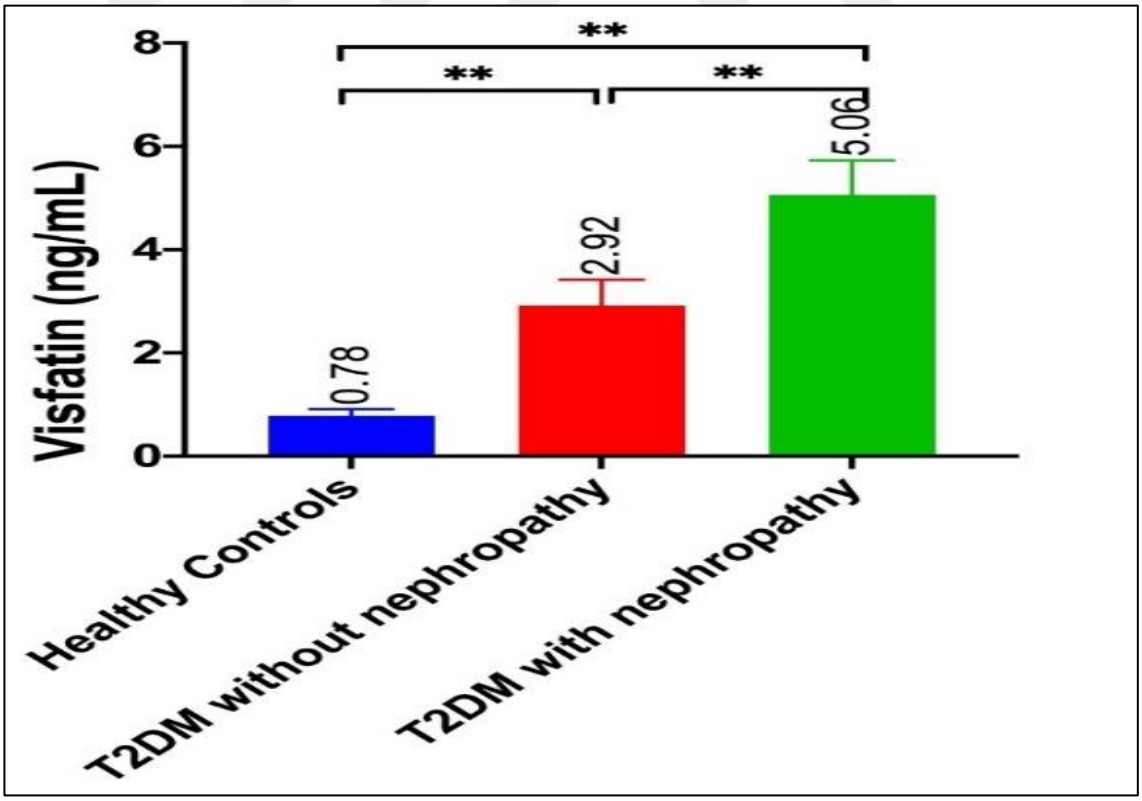

Figure 4.3 Visfatin in study groups

4.4 Microalbuminurea in Study Groups

The results of Microalbuminurea (MA) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the MA levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (9.05 ± 0.63, 39.17 ± 5.10, and 83.20 ± 7.75 mg/L) respectively, there were significant differences among all groups under study, as represented in Table 4.4 and Figure 4.4.

Table 4.4 Microalbuminurea in study groups

Microalbuminuria (mg/L)	Mean	Std. Deviation
Controls	9.05	0.63
T2DM without nephropathy	39.17	5.10
T2DM with nephropathy	83.20	7.75
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

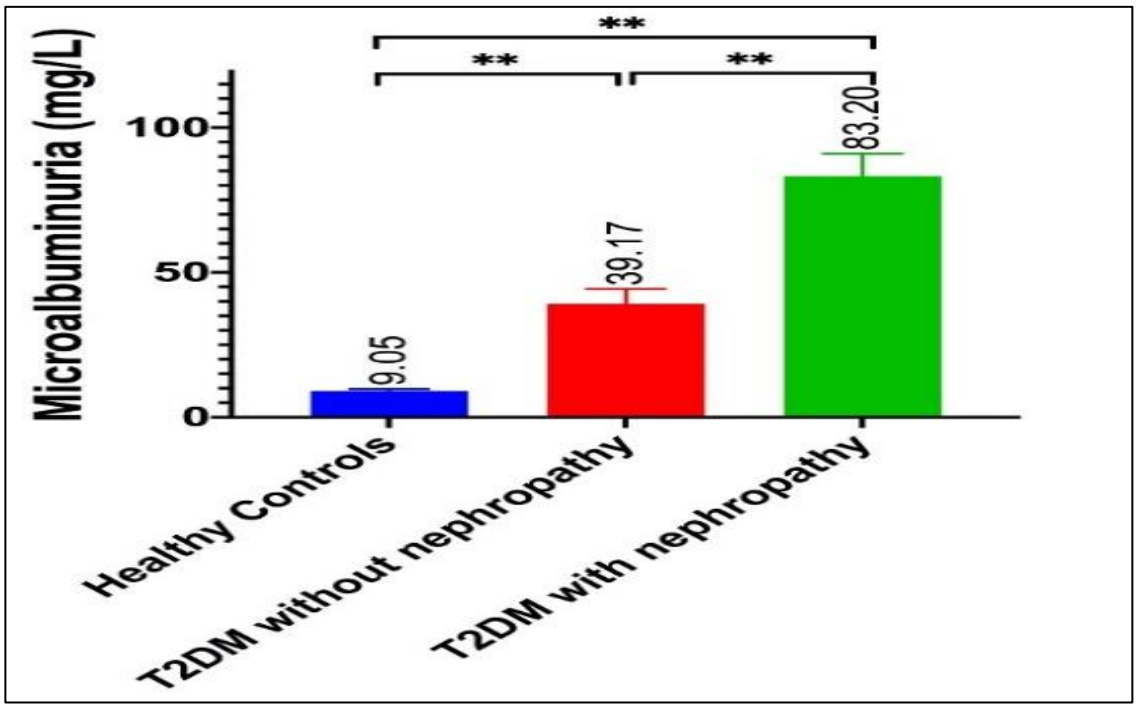

Figure 4.4 Microalbuminurea in study groups

4.5 Hemoglobin in Study Groups

The results of Hemoglobin (Hb) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the Hb levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (13.78 ± 0.73, 12.78 ± 0.73, and 10.78 ± 0.73 gm/dL) respectively, there were significant differences among all groups under study, as represented in Table 4.5 and Figure 4.5.

Table 4.5 Hemoglobin in study groups

Hemoglobin (gm/dL)	Mean	Std. Deviation
Controls	13.78	0.73
T2DM without nephropathy	12.78	0.73
T2DM with nephropathy	10.78	0.73
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

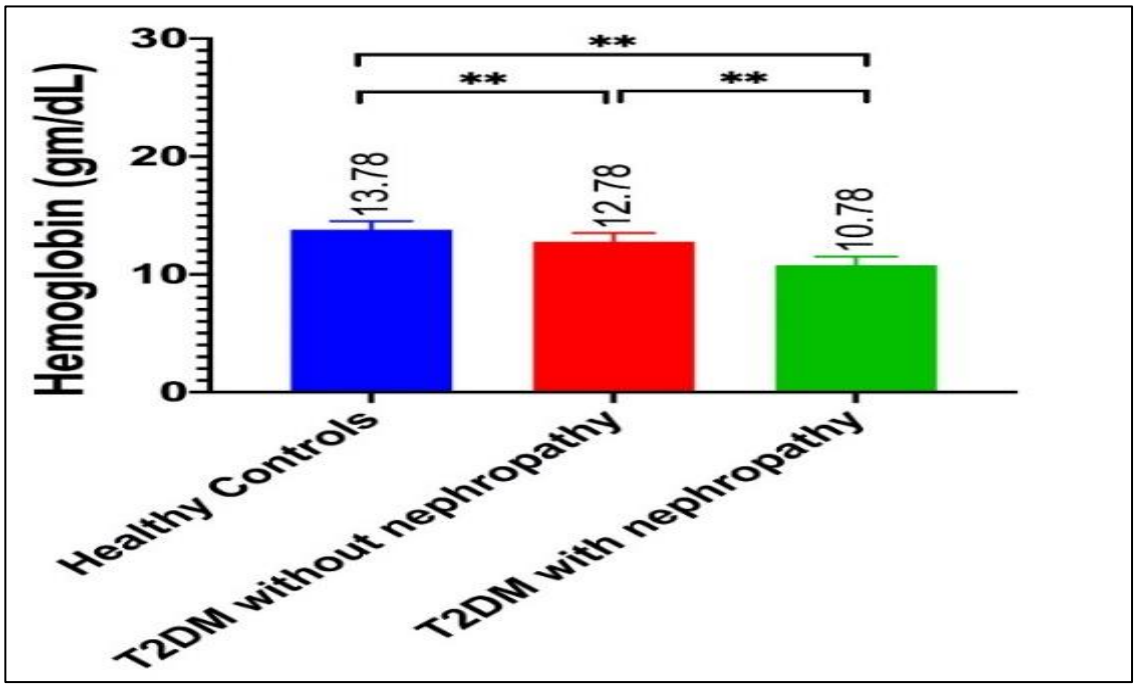

Figure 4.5 Hemoglobin in study groups

4.6 Hemoglobin A1c in Study Groups

The results of Hemoglobin A1c (HbA1c) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the HbA1c levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (5.01 ± 0.34, 7.51 ± 0.34, and 9.01 ± 0.34 %) respectively, there were significant differences among all groups under study, as represented in Table 4.6 and Figure 4.6.

Table 4.6 Hemoglobin A1c in study groups

Hemoglobin A1c (%)	Mean	Std. Deviation
Controls	5.01	0.34
T2DM without nephropathy	7.51	0.34
T2DM with nephropathy	9.01	0.34
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

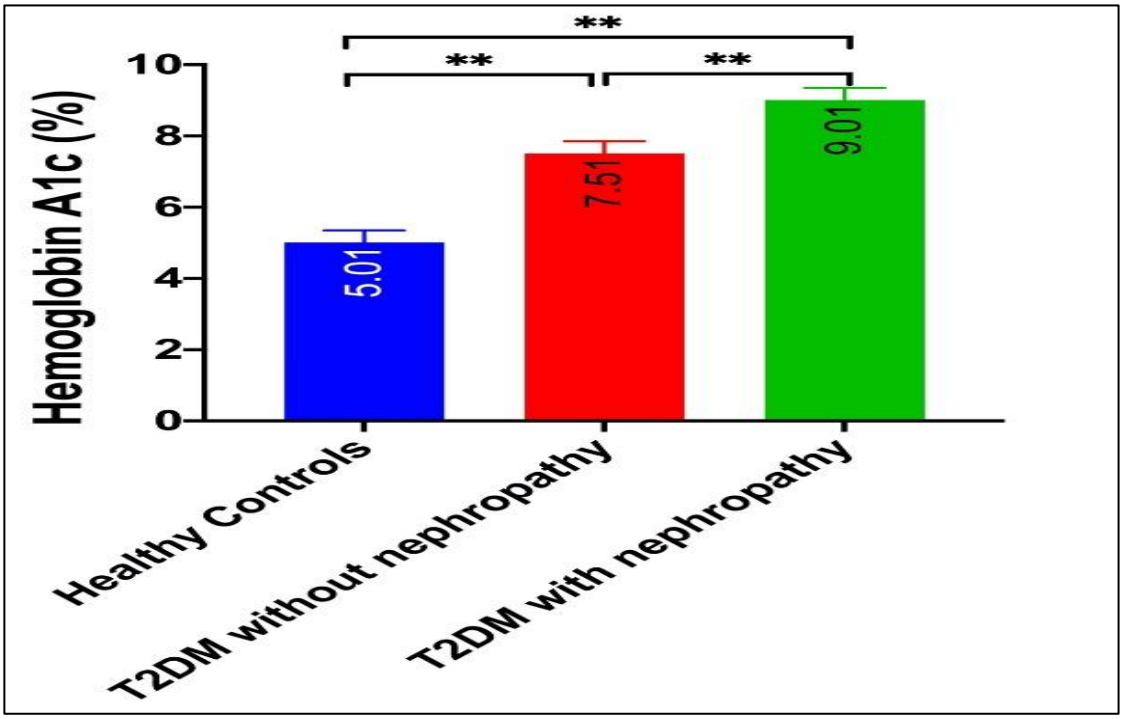

Figure 4.6 Hemoglobin A1c in study groups

4.7 Fast Blood Glucose in Study Groups

The results of fast blood glucose (FBG) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the FBG levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (87.03 ± 5.76, 159.30 ± 5.69, and 227.30 ± 7.33 mg/dL) respectively, there were significant differences among all groups under study, as represented in Table 4.7 and Figure 4.7.

Table 4.7 Fast blood glucose in study groups

Fast Blood Glucose (mg/dL)	Mean	Std. Deviation
Controls	87.03	5.76
T2DM without nephropathy	159.30	5.69
T2DM with nephropathy	227.30	7.33
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

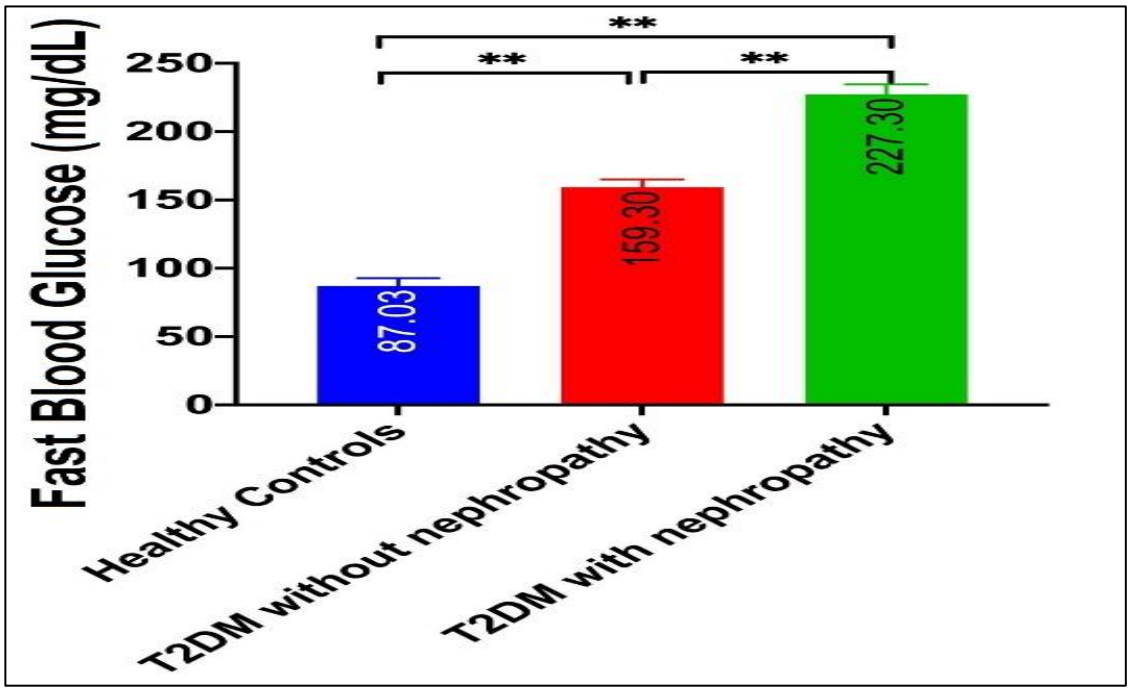

Figure 4.7 Fast blood glucose in study groups

4.8 Serum Creatinine in Study Groups

The results of serum creatinine (SCr) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the SCr levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (0.68 ± 0.13, 1.31 ± 0.05, and 2.97 ± 0.16 mg/dL) respectively, there were significant differences among all groups under study, as represented in Table 4.8 and Figure 4.8.

Table 4.8 Serum Creatinine in study groups

Serum Creatinine (mg/dL)	Mean	Std. Deviation
Controls	0.68	0.13
T2DM without nephropathy	1.31	0.05
T2DM with nephropathy	2.97	0.16
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

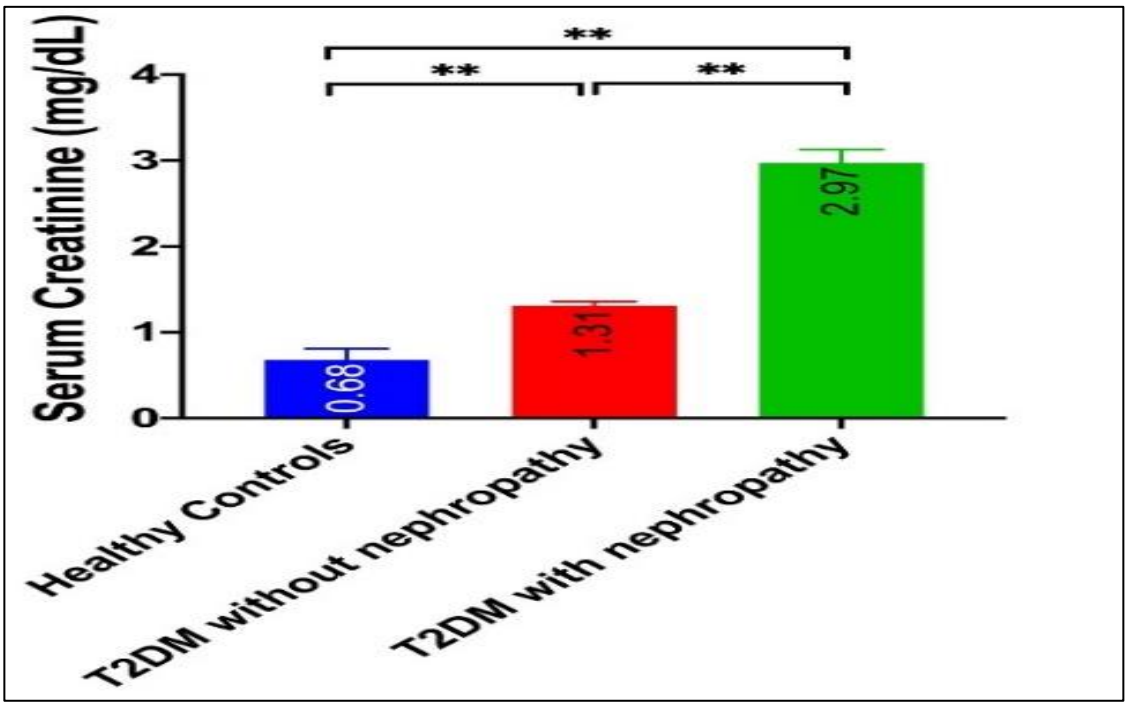

Figure 4.8 Serum Creatinine in study groups

4.9 Glomeruler Filtration Rate in Study Groups

The results of glomeruler filtration rate (GFR) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the GFR of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (101.77 ± 22.78, 49.32 ± 7.56, and 19.09 ± 1.97 mL/min/1.73m^2) respectively, there were significant differences among all groups under study, as represented in Table 4.9 and Figure 4.9.

Table 4.9 Glomeruler filtration rate in stury groups

Glomerular filtration rate (mL/min/1.73m^2)	Mean	Std. Deviation
Controls	101.77	22.78
T2DM without nephropathy	49.32	7.56
T2DM with nephropathy	19.09	1.97
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

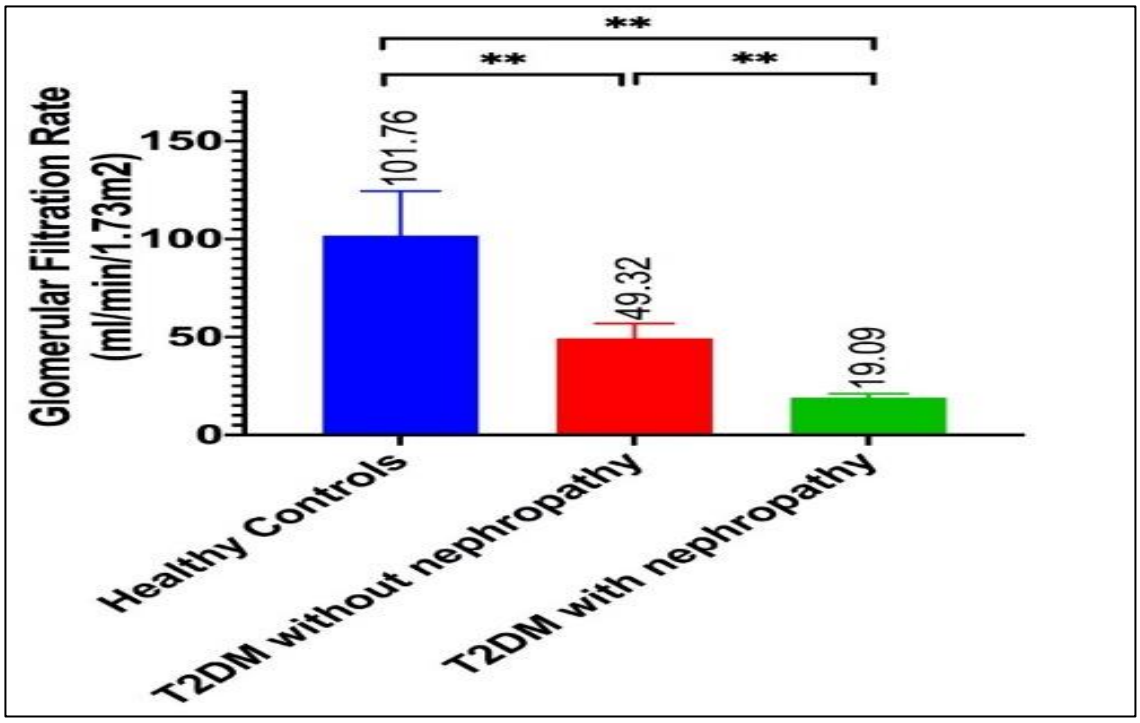

Figure 4.9 Glomeruler filtration rate in study groups

4.10 Albumin Creatinine Ratio in Study Groups

The results of albumin creatinine ratio (ACR) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the ACR of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (13.74 ± 2.71, 29.90 ± 3.93, and 28.10 ± 2.66 mg/g) respectivel, there were significant differences among all groups under study, as represented in Table 4.10 and Figure 4.10.

Table 4.10 Albumin creatinine ratio in study groups

Albumin creatinine ratio (mg/g)	Mean	Std. Deviation
Controls	13.74	2.71
T2DM without nephropathy	29.90	3.93
T2DM with nephropathy	28.10	2.66
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

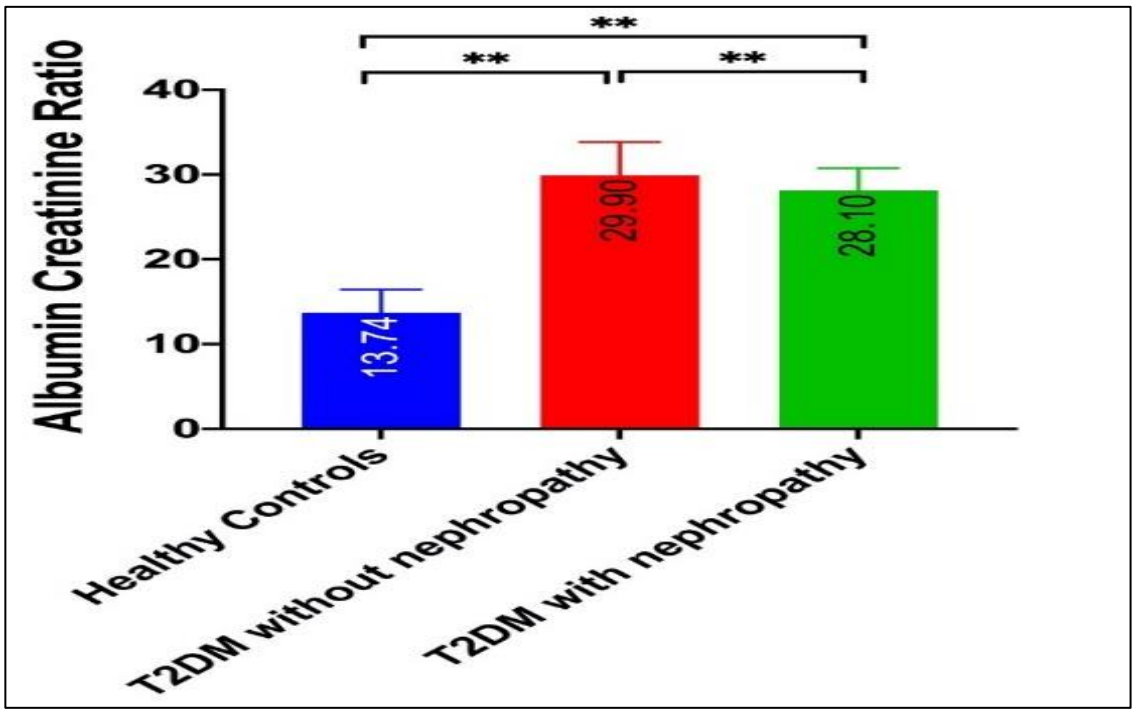

Figure 4.10 Albumin creatinine ratio in study groups

4.11 Body Mass İndex in Study Groups

The results of body mass ındex (BMI) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the BMI of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (21.01 ± 1.76, 24.01 ± 1.75, and 19.01 ± 1.75 kg/m^2) respectively, there were significant differences among all groups under study, as represented in Table 4.11 and Figure 4.11.

Table 4.11 Body mass index in stury groups

Body mass ındex (kg/m^2)	Mean	Std. Deviation
Controls	21.01	1.76
T2DM without nephropathy	24.01	1.75
T2DM with nephropathy	19.01	1.75
p value	≤0.001**	
Controls vs T2DM without nephropathy	≤0.001**	
Controls vs T2DM with nephropathy	≤0.001**	
T2DM without nephropathy vs T2DM with nephropathy	≤0.001**	

NS: non-significant, **: significant

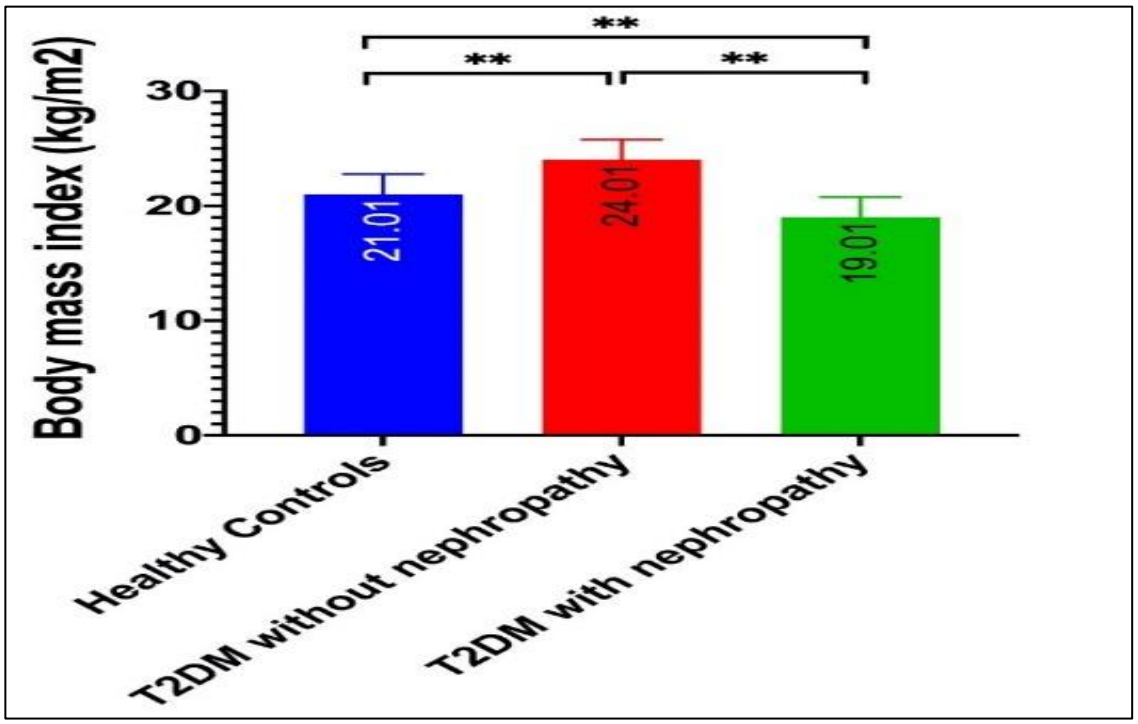

Figure 4.11 Body mass index in study groups

4.12 The correlation between erythropoietin and visfatin with the study markers

When the correlation between erythropoietin and visfatin was compared with the measured markers in the study, it was found that there was a negative correlation between erythropoietin and microalbuminuria, HbA1c, and ACR where P= 0.023, P= 0.026, and P = 0.022 respectively. In addition, it was found that there was a positive correlation between visfatin and HbA1c, where P= 0.006, as presented in Table 4.12.

Table 4.12 The correlation between EPO and VF with the study markers

Marker		Erythropoietin (mIU/mL)	Visfatin (ng/mL)
Erythropoietin (mIU/mL)	r	1	0.200
	p		0.126
Visfatin (ng/mL)	r	0.200	1
	p	0.126	
Microalbuminuria (mg/L)	r	-0.293*	0.185
	p	0.023	0.157
Hemoglobin (gm/dL)	r	-0.006	-0.022
	p	0.962	0.869
Fast Blood Glucose (mg/dL)	r	0.002	-0.038
	p	0.987	0.771
Hemoglobin A1c (%)	r	-0.294*	0.351**
	p	0.022	0.006
Serum Creatinine (mg/dL)	r	0.010	0.060
	p	0.942	0.649
Glomerular Filtration Rate (ml/min/1.73m2)	r	-0.007	0.097
	p	0.958	0.459
Albumin Creatinine Ratio	r	-0.287*	0.152
	p	0.026	0.246
Body mass index (kg/m2)	r	0.027	-0.098
	p	0.839	0.457

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

5. DISCUSSION

5.2 Age in Study Groups

The study included 180 volunteers who are undergoing medical follow-up at Salah El-Din General Hospital, about 60 of whom are healthy individuals as a standard group for study, and the remaining 120 patients are diagnosed clinically with T2DM; they have divided into two groups, the first group 60 T2DM patients without nephropathy and the second group 60 T2DM patients with nephropathy. The statistical analysis has been shown the mean and standard deviation (SD) in the age of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (57.85 ± 8.76, 56.42 ± 8.71, and 56.80 ± 8.25 year) respectively. In another study conducted in 2013, the mean and standard deviation (SD) in the age of men was (64.0 ± 12.6) and the average age of women was (62.6 ± 14.0) (Yu *et al.,* 2012).

5.2 Erythropoietin in Study Groups

The results of Erythropoietin (EPO) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the EPO levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (7.90 ± 0.91, 14.43 ± 1.27, and 4.56 ± 0.62 mIU/mL) respectively, there were significant differences among all groups under study. In another study performed in 2018, EPO levels were elevated compared to healthy controls (1618 ± 535 pg/mL) in diabetic patients without nephropathy (Jwad *et al.,* 2018). In another study conducted in 2012, the mean of EPO levels were decreased compared to healthy controls (9.1 ± 2.60 IU/L) in diabetic patients with nephropathy progression (Mercadal *et al.,* 2012). Another research executed on patients with T2 diabetes without nephropathy found that EPO levels were higher than normal and reached (24-42) against 13 (9-15) IU/L in control vs patients, respectively (Craig *et al.,* 2005).

5.3 Visfatin in Study Groups

The results of Visfatin (VF) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the VF levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (0.78 ± 0.13, 2.92 ± 0.50, and 5.06 ± 0.67 ng/mL) respectively, There were significant differences among all groups under study. A study performed in 2010 showed elevated visfatin levels in the control group compared with T2DM with nephropathy patients and agreed with my results (5.2 ± 3.3, 9.2 ± 5.5 ng /mL), respectively (Mahmood *et al.,* 2010). An unpublished master's thesis at Tikrit University reached in 2020 that Visfatin levels for T2DM with CKD were (5.99 ± 1.19 ng/mL), and T2DM without CKD were (3.02 ± 0.49 ng/mL). The control group were (0.77 ± 0.14 ng/mL); these results are consistent with the results of my study, which means that visfatin levels increase with the severity of diabetic kidney disease (Ali, 2020).

5.4 Microalbuminurea in Study Groups

The results of Microalbuminurea (MA) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the MA levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (9.05 ± 0.63, 39.17 ± 5.10, and 83.20 ± 7.75 mg/L) respectively, there were significant differences among all groups under study. A published article in 2018 showed that Microalbuminurea levels for the control group, T2DM without & with nephropathy cases, were (5.99 ± 1.19 ng/mL), and T2DM without CKD were (11.90 ± 4.47, 43.33 ± 29.43, and 80.0 ± 62.6 mg/L) respectively; these results are consistent with the findings of my study, which means that Microalbuminurea levels increase with the severity of diabetic nephropathy (Jwad *et al.,* 2018).

5.5 Hemoglobin in Study Groups

The results of hemoglobin (Hb) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the Hb levels of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (13.78 ± 0.73, 12.78 ± 0.73, and 10.78 ± 0.73 gm/dL) respectively, There were significant differences among all groups under study. In another research executed on patients with T2 diabetes without nephropathy, it was found that haemoglobin levels were lower than normal and reached Hb < or = 11.5, and this decrease if compared to normal rates (Craig *et al.,* 2005). Research published in 2020 agreed with our conclusion, as it was found that the haemoglobin level reached anaemic patients were 12.2 ± 0.73 g/dL in males and 11.4 ± 0.58 g/dl in females, that the risk of anaemia and low haemoglobin is linked with the increasing severity of kidney disease (Taderegew *et al.,* 2020).

5.6 Hemoglobin A1c in Study Groups

The results of Hemoglobin A1c levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the HbA1c levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (5.01 ± 0.34, 7.51 ± 0.34, and 9.01 ± 0.34 %) respectively, There were significant differences among all groups under study. The research published in 2018 agreed with our conclusion as that the level of HbA1c reached in patients was 6.6 ± 0.9, 7.4 ± 1.0, and 8.6 ± 1.4%, respectively and that the increase in the level of HbA1c is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increase in the severity of diabetic kidney disease (Lee *et al.,* 2018). The investigation published in 2013 agreed with our conclusion that the level of HbA1c reached in patients was (7.3 ± 1.4%), and it was shown in its conclusion that the increase in the level of HbA1c is associated with the risk of worsening the severity of T2DM and the severity of DKD (Hernandez *et al.,* 2013).

5.7 Fast Blood Glucose in Study Groups

The results of fast blood glucose levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the FBG levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (87.03 ± 5.76, 159.30 ± 5.69, and 227.30 ± 7.33 mg/dL) respectively, there were significant differences among all groups under study. The research published in 2021 agreed with our conclusion that the level of FBG reached in diabetic patients without and with DKD was 140 ± 44.6 and 156 ± 47.6 mg/dL, respectively, and that the increase in the level of FBG is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increase in the severity of diabetic kidney disease (Jung, 2021).

5.8 Serum Creatinine in Study Groups

The results of serum creatinine (SCr) levels in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the SCr levels of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (0.68 ± 0.13, 1.31 ± 0.05, and 2.97 ± 0.16 mg/dL) respectively, there were significant differences among all groups under study. The same finding concluded in the unpublished thesis in 2020 agreed with our conclusion that the level of SCr reached in control vs diabetic patients without and with DKD was 0.54 ± 0.08, 0.45 ± 0.0803, and 5.82 ± 1.76 mg/dL, respectively, and that the increase in the level of SCr is associated with the risk of worsening the severity of T2DM, which leads to an increase in the severity of diabetic kidney disease (Ali, 2020). The same finding concluded in the published article in 2020 agreed with our conclusion that the level of SCr reached in control vs diabetic patients was 10.63 ± 0.27 and 0.87 ± 0.44 mg/dL, respectively, and that the increase in the level of SCr is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increase in the severity of diabetic kidney disease (Asmamaw *et al.,* 2020).

5.9 Glomerular Filtration Rate in Study Groups

The results of glomeruler filtration rate (GFR) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the GFR of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (101.77 ± 22.78, 49.32 ± 7.56, and 19.09 ± 1.97 mL/min/1.73m^2) respectively, there were significant differences among all groups under study. The same finding concluded in the unpublished thesis in 2020 agreed with our conclusion that the level of GFR reached in control vs diabetic patients without and with DKD was 127.9 ± 13.9, 121.2 ± 7.8, and 9.9 ± 3.3 mL/min/1.73m^2, respectively, and that the decrease in the level of GFR is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increase in the severity of diabetic kidney disease (Ali, 2020).

5.10 Albumin Creatinine Ratio in Study Groups

The results of albumin creatinine ratio (ACR) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the ACR of the Control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (13.74 ± 2.71, 29.90 ± 3.93, and 28.10 ± 2.66 mg/g) respectivel, there were significant differences among all groups under study. The same finding concluded in the published article in 2015 agreed with our conclusion that the level of ACR reached in control vs diabetic patients was less than 2.5 and 82 ± 34 mg/g, respectively, and that the increase in the level of ACR is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increase in the severity of diabetic kidney disease (Karar *et al.* 2015).

5.11 Body Mass Index in Study Groups

The results of body mass ındex (BMI) in study groups and the statistical analysis have shown the mean and standard deviation (SD) in the BMI of the control group, T2DM without nephropathy patients, and T2DM with nephropathy patients (21.01 ± 1.76, 24.01 ± 1.75, and 19.01 ± 1.75 kg/m^2) respectively, there were significant differences among

all groups under study. The same finding concluded in the published article in 2006 agreed with our conclusion that the level of BMI reached in diabetic nephropathy patients was 27.9 ± 1.0 kg/m^2, compared to the normal criteria, and that the increase in the level of BMI is associated with the risk of worsening the severity of type 2 diabetes, which leads to an increse in the severity of diabetic kidney disease (Idogun *et al.* 2006).

5.12 The correlation between erythropoietin and visfatin with the study markers

T2DM patients with nephropathy have an erythropoietin concentration less than healthy individuals and a high concentration of microalbuminuria and HbA1c compared with healthy individuals where erythropoietin correlated negatively with microalbuminuria and ACR and HbA1c with a p-value (0.023, 0.026, and 0.022) respectively. T2DM patients with nephropathy have visfatin concentration more than healthy individuals, where visfatin correlated positively with HbA1c with a p-value (0.006).

In earlier research, a correlation was found between the intensity of the association between erythropoietin and microalbuminuria and ACR (Mojiminiyi *et al.,* 2006). Another study showed an inverse association between HbA1c and erythropoietin levels at $p<0.001$ (Rafiu *et al.,* 2019). On the other hand, a published article found a correlation between visfatin and HbA1c at $p = 0.005$ (Toruner *et al.,* 2009).

6. CONCLUSIONS AND RECOMMENDATION

6.1 Conclusions

1. Age is not a determining factor in patients with T2DM without or with nephropathy.
2. Through the final results of the study, it was found that Erythropoietin rises in patients with T2DM without nephropathy, but it begins to decline with the development of nephropathy.
3. Depending on the study's final results, it was found that Visfatin rises in patients with/without T2DM nephropathy and continues rising with the development of nephropathy.
4. Erythropoietin correlated negatively with microalbuminuria, ACR, and HbA1c, respectively, and Visfatin correlated positively with HbA1c.
5. The study concluded that it is possible to use erythropoietin and visfatin as biomarkers in monitoring the development of T2DM, as it was observed that erythropoietin rises in patients with T2DM without renal complications and begins to gradually decline with the development of kidney disease; As for visfatin, it begins to gradually rise to reach its highest levels with a decrease in the glomerular filtration of the kidney.
6. According to the results obtained from the study, there is the possibility of using Erythropoietin and Visfatin as a biomarker to monitor the progression of nephropathy.
7. Through the study's final results, I found that Microalbuminuria rises in patients with T2DM without/with nephropathy, in ascending order with the development of nephropathy.
8. The study found that FBG, HbA1c, and SCr rise in patients with T2DM without/with nephropathy, in ascending order with the development of nephropathy.
9. The study found that Hb and GFR decreased in patients with T2DM without/with nephropathy, in descending order with progression of nephropathy.
10. An increase in BMI increases the risk of developing T2DM and leads to nephropathy as a final result.
11. The ACR increases in patients with T2DM without or with nephropathy.

6.2 Recommendations

1. To reduce the prevalence of renal impairment, efforts should be directed to improve disease initiation and progression prediction. However, due to its limitations, more research is recommended to identify new biomarkers to improve risk classification.
2. Although the most commonly used markers for DKD are still being studied, further research is recommended to examine new biomarkers that can improve risk classification.
3. Dig into more detailed studies based on the correlation studies between the vital indicators included in this study to see the effect of one on the other and nephropathy.
4. Interest in studying recombinant erythropoietin and the possibility of using it as a compensatory treatment for anaemic patients suffering from nephropathy.
5. Several new biomarkers have been established to capture a unique pathophysiological mechanism of the disease, such as inflammation caused by hyperglycemia, oxidative stress and tubular damage, which ultimately leads to kidney damage and fibrosis.
6. It is necessary to focus on analyzing biological samples that include proteins, such as haptoglobin, metabolites, phospholipid metabolism, and transcriptomics, such as TGF-1-regulated miRNAs, which have emerged as a powerful candidate tool in the field of biomarker discovery.

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