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Assessment of SMAD2, SMAD3 and Their Correlation with TGF-BETA in Type 2 Diabetic Patients with Early and Chronic Diabetic Nephropathy

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, as Partial Fulfillment for the Requirements of the Degree of Master in Clinical chemistry and

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Dedication

TO MY PRECIOUS BELOVED HOMELAND, IRAQ

TO MY FATHER AND MY MOTHER FOR THEIR KINDNESS AND SUPPORT

TO MY Lovely Wife

TO MY BROTHER AND MY SISTERS

TO MY LOVELY DAUGHTERS

"jumana AND jana"

TO ALL PEOPLES WHO SUPPORT ME

WITH LOVE

Hussein 2022

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List of Abbreviations

Abbreviation	Details
A	Absorbance
A.D	After date
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AGAE	Advanced glycation end products
AGE	Advanced glycation end-products
AHA	American Heart Association
ANOVA	Analysis of variance
AR	Aldose reductase
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
BMP	Bone morphogenic protein
CKD	Chronic kidney disease
Co-SMADs	Common-partner SMADs
CVD	Cardiovascular disease
CVD	Cerebrovascular disease
Da	Dalton
DAG	Diacylglycerol
DCCT	Diabetes Control and Complications Trial
DHAP	Dihydroxyacetone phosphate
DKA	Diabetic ketoacidosis

DM	Diabetes Mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
DW	Deionized water
EASD	European Association for the Study of Diabetes
ECM	Extra cellular matrix
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked Immuno-Sorbent Assay
ENOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
FOXO1	Factor fork head box protein O1
FPG	fasting plasma glucose
GAPDH	Glyceraldehyde-3-phosphate dehydrogenas2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GBM	Glomerular basement membrane
GDM	Gestational diabetes mellitus
GFAT	Fructose-6-phosphate amidotransferase
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GLUT4	Glucose transporter type 4
GOD	Glucose oxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HbA1c	Glycosylated hemoglobin

HDL	High-density lipoprotein
HDL-C	High-density lipoprotein-cholesterol
HHS	Hyperosmolar Hyperglycaemic State
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
HONK	Hyperosmolar non ketonic
HRP	Horse radish Peroxidase
IADPSG	International Association of Diabetes and Pregnancy Study Group
IDDM	Insulin-dependent Diabetes Mellitus
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein-cholesterol
IFG	Impaired fasting glucose
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin Resistance
IRSs	Insulin receptor substrates
I-SMADs	Inhibitory SMADs
LAP	Latency-associated protein
LDL	Low-density lipoprotein
LDLc	Low-density lipoprotein -cholesterol
LPL	Lipoprotein lipase
Min.	Minute
ML	Milliliter
NAD	Nicotinic acid adenine dinucleotide
NADPH	Nicotinic acid adenine dinucleotide phosphate

NEFA	Non-esterified fatty acid
NKHS	Non-ketotic hyperosmolar syndrom
NO	Nitric Oxide
NPV	Negative predictive value
OGTT	Oral glucose tolerance test
PAD	Peripheral artery disease
PG	Plasma glucose
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase c
POD	Peroxidase
PPV	Positive predictive value
RAAS	Renin–angiotensin II–aldosterone system
RAS	Renin-angiotensin system
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
RPG	Random plasma glucose
R-SMADs	Receptor-regulated SMADs
SD	Standard deviation
SDH	Sorbitol dehydrogenase
SMAD	Small Mothers Against Decapentaplegic
Smurf1	SMAD Ubiquitination Regulatory Factor 1
Smurf2	SMAD Ubiquitination Regulatory Factor 2
SN	Sensitivity
SP	Specificity
SPSS	Software package for social science

St.	Standard
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Total cholesterol
Temp.	Temperature
TG	Triglycerides
TGF	Transforming growth factor
TTR	Transthyretin
TβRI	Type I TGF-β receptor
UACR	Urine albumin-to-creatinine ratio
UDP-GlcNAc	Uridine diphosphate N-acetyl glucosamine
VEGF	Vascular endothelial growth factor
VLDL	Very Low Density Lipoprotein
VLDLc	Very Low-Density Lipoprotein Cholesterol
WHO	World Health Organization

Summary

Diabetes Mellitus (DM) is a metabolic disease 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. Diabetic nephropathy (DN) is one of the most frequent and severe complications of diabetes mellitus and is associated with increased morbidity and mortality in diabetic patients. DN represents the leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD). Early detection of DN is critical in improving clinical management and therapeutic intervention progression to ESRD and dialysis. Diabetic kidney disease is characterized by progressive kidney damage reflected by increasing albuminuria (urine albumin-to-creatinine ratio (ACR) exceeding 30 mg/g CR) together with impairment in renal function (decline in glomerular filtration rate (GFR), elevated blood pressure, and excess morbidity and mortality due to cardiovascular complications.

Transforming growth factor (TGF-β) is recognized as a key profibrotic cytokine involved in fibrotic disease, particularly in the vasculature and kidney. The TGF-β signaling pathway is activated in DN. Increased extracellular glucose levels, mesangial cell stretch, activation of renin-angiotensin system, reactive oxidant species (ROS), and advanced glycation end products (AGEs) activate TGF-β synthesis via protein kinase C. TGF-β stimulates its pathway through autocrine or paracrine action. Transcription factor small mothers against decapentaplegic (Smad) family has key roles in cell fate decision in transmitting extracellular signals to the nucleus through transforming growth factor-beta(TGF-β) receptors to activate downstream target gene transcription. For TGF-β signaling, phosphorylation and activation by the active type II and type I receptor complex causes Smad2 and Smad3 to form heterooligomers with Smad4 and translocate the entire complex into the nucleus. There they interact with different cellular partners, bind to DNA, and regulate the transcription of various downstream response genes.

The current study aims to identify the role of SMAD2, and SMAD3 and their Correlation with TGF-β in Type 2 Diabetic Patients with Early and Chronic Diabetic

Nephropathy in a sample of the Iraqi population, as well as to study the correlation between these biomarkers with traditional renal function biochemical parameters.

A case-control study was conducted in Hilla city, from October 2021 to May 2022. The samples were collected from Diabetes and Endocrinology Center in Marjan Teaching Hospital in Babylon / Hilla city.

The current study involved 120 individuals, 60 patients with type 2 diabetes mellitus (T2DM) group and 60 participants who are healthy was used as a control group. The patients were subdivided into three equal groups according to their urinary albumin to creatinine ratio (ACR), including patients with normoalbuminuria (n=20)(ACR < 30 mg/g creatinine) and those with microalbuminuria (n=20)(ACR= 30–299 mg/g creatinine) and macroalbuminuria (n=20)(ACR= more than 300 mg/g creatinine). The levels of glucose, HbA1c, blood urea, serum creatinine, TGF-Beta, SMAD2, and SMAD3 were estimated for all participants, in addition to microalbumin in the urine.

The results of the present study found that the mean concentration of FBG and HbA1c levels were significantly increased in T2DM patients with macroalbuminuria, microalbuminuria, and normoalbuminuria compared with the control group, (P-value<0.001).

Serum TGF-β, SMAD2, and SMAD3 levels in T2DM with macroalbuminuria and in T2DM with microalbuminuria were much higher than in T2DM with normoalbuminuria and control groups with highly statistically significant difference (P-value <0.001). On other hand the TGF-β, SMAD2, and SMAD3 was significantly increased in macroalbuminuria as compared with microalbuminuria with a highly statistically significant difference (P-value <0.001). In addition, serum TGF-β, SMAD2, and SMAD3 showed a positive correlation with ACR, Blood Urea, Serum Creatinine, FBG, HbA1c, Diabetic Duration, and a negative correlation with eGFR.

Receiver operating characteristic curves (ROC) revealed that for early detection of DN, A good diagnostic profile of serum TGF-B for early detection of DN, with a sensitivity of 80%, specificity of 95%. Also, the good diagnostic profile of serum SMAD3 for early detection of DN, with a sensitivity of 78.30%, specificity of 95%. So SMAD2 revealed a good diagnostic profile for early detection of DN, with a sensitivity

of 66.70 %, specificity of 90 %. all of them are considered independent and reliable biomarkers for the early detection of DN.

In conclusion, this study revealed the importance of these markers in DN pathogenesis which is powered by their correlation with albuminuria and high specificity and sensitivity through ROC analysis, and thus the possibility of their use as biochemical markers in DN was suggested.

CHAPTER ONE

INTRODUCTION

And

LITERATURE REVIEW

1. Introduction:

Diabetes mellitus (DM) is a group 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 (ADA, 2021b). The report to the International Diabetes Federation (IDF) 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(Saeedi *et al.*, 2019). Type 2 diabetes (T2DM) is a multifaceted disease involving the pancreatic β-cells, adipose tissue, liver, and other organs. Hyperglycemia, insulin resistance (IR), and obesity are characteristic features of T2DM and play crucial parts in the pathogenesis of the disease(Chatterjee, Khunti and Davies, 2017).

Diabetic nephropathy (DN) is one of the most common diabetic microvascular complications affecting up to 20–40% of patients with Type 2 diabetes mellitus (T2DM) and may lead to end-stage renal disease (ESRD), thus influencing the morbidity and mortality of patients with T2DM(Motawi *et al.*, 2018). The molecular pathophysiology of DN involves complex interactions between hyperglycemia-induced metabolic, hemodynamic and inflammatory factors. These factors alter the function and morphology of blood vessel walls and interact with adjacent cells leading to renal endothelial dysfunction, which plays a crucial role in the development of DN(Motawi *et al.*, 2018). Early changes in DN include increases in kidney size, glomerular volume, and glomerular filtration rate (GFR), followed by the accumulation of glomerular extracellular matrix, increased urinary albumin excretion, and glomerular sclerosis and tubular fibrosis. Late-stage overt DN is clinically characterized by proteinuria, hypertension, and progressive renal insufficiency(Guo *et al.*, 2015).

There are many mediators in DN, including advanced glycation end-products (AGE), intracellular polyols, angiotensin II and inflammatory cytokines. Of these, transforming growth factor (TGF)-beta is a key mediator in DN. Transforming growth factor-beta is a member of the TGF-beta superfamily. There are three TGF-β isoforms, namely TGF-β1, TGF-β2, and TGF-β3. Although all three TGF-β isoforms and receptors are widely expressed in the kidney, TGF-β1 is a key mediator in the

pathogenesis of DN(Ziyadeh, 2004). Transforming growth factor-β1 is secreted as an inactive pro-cytokine, consisting of the mature active TGF-β1 protein non-covalently bound to a dimmer of its NH2-terminal pro-peptide, called latency-associated protein (LAP), and a latent TGF-β binding protein(Lan, 2012). TGF-β1 exerts its major biological effects through its cell surface receptors: TGF-bRI and TGF-bRII. Upon TGF- b1 binding, the TGF-bRI phosphorylates SMAD2 and SMAD3 which then oligomerize with SMAD4 to form a complex that translocates into the nucleus. SMAD2, SMAD3 and SMAD4 can directly bind to DNA sequences or cofactors to regulate transcription of their target genes(Li *et al.*, 2020).

1.1 Diabetes Mellitus

1.1.1 Definition and Description of Diabetes Mellitus

The term "Diabetes" is from the Greek (dia), through, and (bainen) to go or passing through, which depicts the excessive production of urine in this condition. Diabetes Mellitus refers to the sweet taste of urine, which Mellitus means honey (Lakhtakia, 2013). Diabetes mellitus (DM) is an insidious, complex, and chronic condition that is now found to affect almost every population worldwide (Association AD, 2014). Diabetes is a set of metabolic diseases and is characterized by high blood glucose levels caused by the body's inability to produce or use insulin effectively (Sanhueza *et al.*, 2019). The American Diabetes Association (ADA) has defined diabetes as a group 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(Association AD, 2014). Insulin is a hormone that is made by the pancreas, and allows the glucose from the food that is consumed, to pass from the bloodstream into cells that produce energy. If individuals are not capable of producing enough insulin or are not able to use it effectively, this may lead to raised glucose levels in the bloodstream, known as hyperglycemia(Saltiel and Kahn, 2001).

1.1.2 Pathogenesis and symptoms of Diabetes Mellitus

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic beta cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action(Ozougwu *et al.*, 2013). The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is the 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 (ADA, 2013).

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening

consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or a nonketotic hyperosmolar syndrome(Preethikaa and Brundha, 2018).

1.1.3 Epidemiology of Diabetes Mellitus

The number and prevalence of people with diabetes mellitus are rapidly rising. Diabetes is a chronic progressive condition that results in significant morbidity, premature death, and economic burden to any healthcare system(Pinchevsky *et al.*, 2020). According to a World Health Organization (WHO) global report on diabetes released on April 6, 2016, the prevalence of diabetes in the adult population globally was 8.5 percent (422 million) in 2014, which is nearly twofold that (4.7 %) in 1980(WHO, 2016). The report to the International Diabetes Federation (IDF) in 2021, a total of 536.6 million people are estimated to be living with diabetes, representing 10.5% of the global population (20–79 years). This number is expected to increase to 783.2 million (12.2%) in 2045 (Sun *et al.*, 2021).

1.1.4 Classification of Diabetes Mellitus

Diabetes Mellitus is classified based on the pathogenic process that leads to hyperglycemia, as opposed to earlier criteria such as the age of onset or type of therapy(ADA, 2015b). As shown in Table (1.1).

Table 1.1: Classification of Diabetes Mellitus.

I. Type 1 diabetes (β-cell destruction, generally leading to absolute insulin deficiency)
A- Immune-mediated
B- Idiopathic
II. Type2 diabetes (may extent from predominantly insulin resistance(IR) with relative insulin deficiency to prevalently secretory defect with insulin resistance)
III. Other specific types
A-Genetic defects of β-cell function
1. Chromosome12, HNF-1α (MODY3)

2. Chromosome7, glucokinase (MODY2)
3. Chromosome20, HNF-4α (MODY1)
4. Chromosome13, insulin promoter factor-1 (IPF-1; MODY4)
5. Chromosome17, HNF-1β (MODY5)
6. Chromosome2, *NeuroD1* (MODY6)
7. Mitochondrial DNA

B-Genetic defects in insulin action

1. Type An insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipoatrophic diabetes

C-Diseases of the exocrine pancreas like

1. Pancreatitis
2. Trauma/pancreatectomy
3. Neoplasia
4. Cystic fibrosis
5. Hemochromatosis

D-Endocrinopathies like

1. Acromegaly
2. Cushing's syndrome
3. Glucagonoma
4. Pheochromocytoma
5. Hyperthyroidism
6. Somatostatinoma
7. Aldosteronoma

E-Drug or chemical-induced

F-Infections

G-Unusual forms of immune-mediated diabetes-like

Anti-insulin receptor antibodies

H-Other genetic syndromes occasionally related to diabetes like

Down's syndrome

IV.Gestational diabetes mellitus(GDM)

1.1.4.1 Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM), also known as "juvenile DM" or "insulin-dependent DM" (IDDM) is the result of the interaction of genetic, environmental, and immunological factors that ultimately lead to the destruction of Beta cells of the pancreas that characterized by increase blood glucose values (hyperglycemia), which are results from a deficiency of insulin secretion(Katsarou *et al.*, 2017) (Warshauer, Bluestone and Anderson, 2020).

T1DM accounts for just around 10% of all diabetes occurrences worldwide, although it is becoming more common as people get older. T1DM can affect anyone at any age, however, it is more frequent in children and adolescents(Thomas *et al.*, 2018). However, In the vast majority of patients (70–90%), the defect of beta cells is the consequence of T1DM-related autoimmunity due to the production of T1DM-associated autoantibodies; these patients have autoimmune T1DM (also known as type 1a DM). In a smaller subdivision of patients, no immune responses or autoantibodies are preserved, and the cause of β-cell destruction is unknown (idiopathic T1DM or type 1b DM); this type has a high genetic component (Atkinson, Eisenbarth and Michels, 2014).

1.1.4.2 Type 2 Diabetes Mellitus

Type 2 Diabetes (T2DM), also known as "noninsulin-dependent diabetes" or "adult-onset diabetes," is the most common type of diabetes, accounting for 90-95 percent of all diabetes cases(Association, 2015a). Hyperglycemia is present as a result of insulin resistance in peripheral tissues and decreased insulin production by pancreatic

beta cells. T2DM also occurs when beta cells fail to compensate for the increased insulin demand induced by insulin resistance (Dayeh and Ling, 2015).

Type 2 Diabetes may be still undiagnosed for many years because hyperglycemia develops gradually and, at earlier stages, is often not severe enough for the patient to notice the classic symptoms of diabetes. Nevertheless, even undiagnosed patients are at increased risk of developing microvascular and macrovascular complications(Association, 2015a). Most patients with this form of diabetes are obese, which is a major cause of insulin resistance (Zatterale *et al.*, 2020).

1.1.4.3 Gestational Diabetes Mellitus

The American Diabetes Association (ADA) defined it as "Diabetes diagnosed in the second or third trimester of pregnancy that is not overt diabetes"(Association, 2010). GDM is one of the most frequent metabolic diseases during pregnancy and approximately affects 7% (range: 2–18%) of all pregnancies (Marchetti *et al.*, 2017). The global prevalence of GDM is approximately around 17%, with rates varying by geography, race or ethnicity, and socioeconomic status of individuals (Yu *et al.*, 2020). 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 (Chiefari *et al.*, 2017).

The exact threshold for a diagnosis of GDM depends on the criteria used, and therefore, there has been a lack of consensus amongst health professionals. It is now advised by the ADA, the WHO, the International Federation of Gynaecology and Obstetrics, and the Endocrine Society, that the International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria be utilized in the diagnosis of GDM (Plows *et al.*, 2018).

1.1.5 Diagnosis of Diabetes Mellitus

The American Diabetes Association 2021, has issued diabetes mellitus diagnostic criteria, which are based on one of the following four diagnostic tests as shown in Table 1.2.

Table 1.2: Criteria for the diagnosis of Diabetes(ADA, 2021b)

FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 hours.*
OR
2-h PG ≥200 mg/dl (11.1 mmol/l) during OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75g of anhydrous glucose dissolved in water.*
OR
HbA1c ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to DCCT assay.*
OR
Random plasma glucose ≥200 mg/dl (11.1 mmol/l) plus classic symptoms of hyperglycemia.

DCCT, Diabetes Control and Complications Trial; FPG, Fasting Plasma Glucose; OGTT, Oral Glucose Tolerance Test; WHO, World Health Organization; 2-h PG, 2-h Plasma Glucose. *In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

1.1.6 Risk Factors of Diabetes Mellitus

According to American Diabetic Association criteria, The high-risk individual is one who:

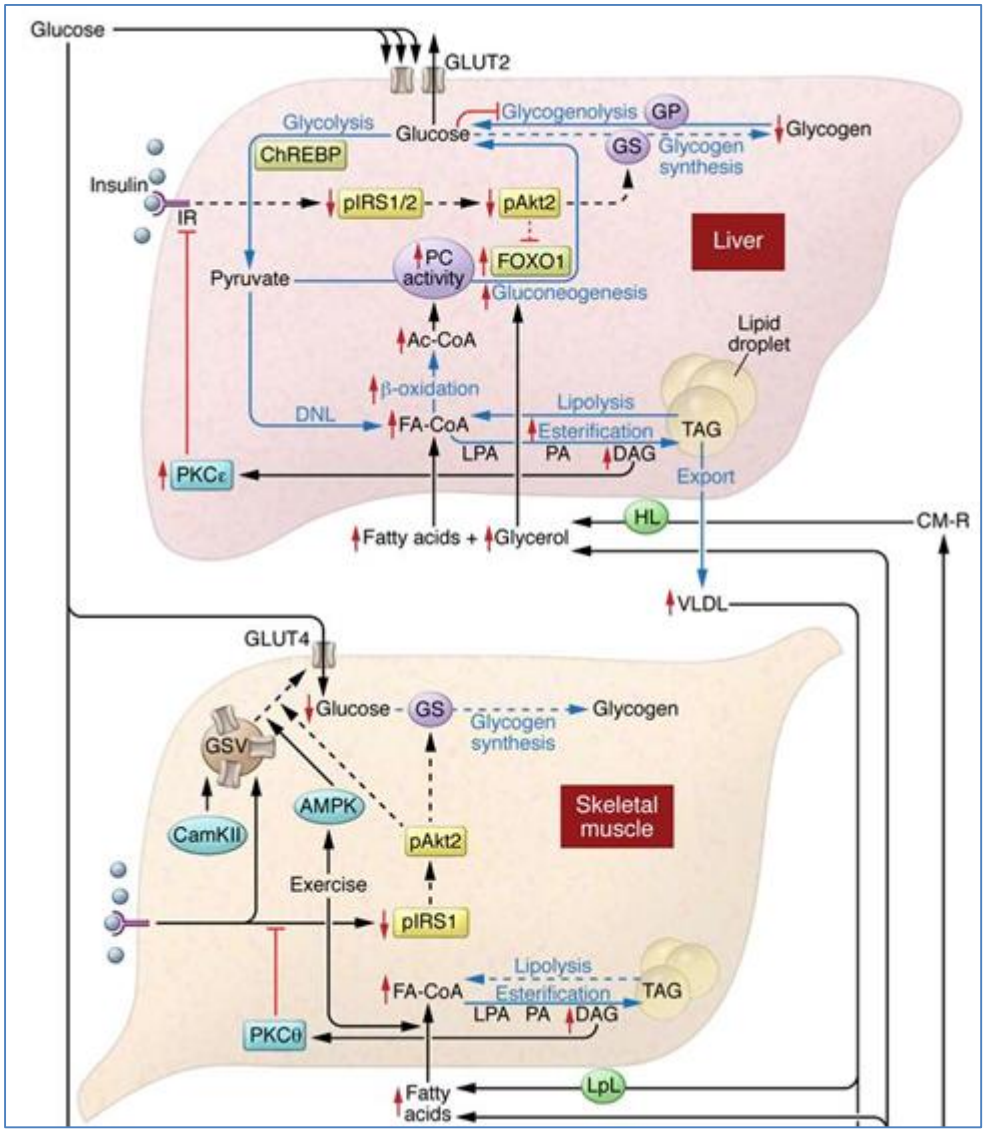
- An obese person (>120% desirable body weight or body mass index >25 kg/m2) who has a first-degree relative with DM.
- Is a member of a high-risk ethnic population (i.e., African American, Hispanic, Native American).
- Has delivered a baby weighing more than 9 pounds or has been GDM
- Is hypertensive (blood pressure >140/90).

- Has an HDL cholesterol level <35 mg/dl and/or a triglyceride level>250 mg/dl.
- Has had Impaired Glucose Tolerance(IGT) or Impaired Fasting Glucose(IFG) on previous testing (Association, 2021b).

1.1.7 Development of Type 2 Diabetes Mellitus:

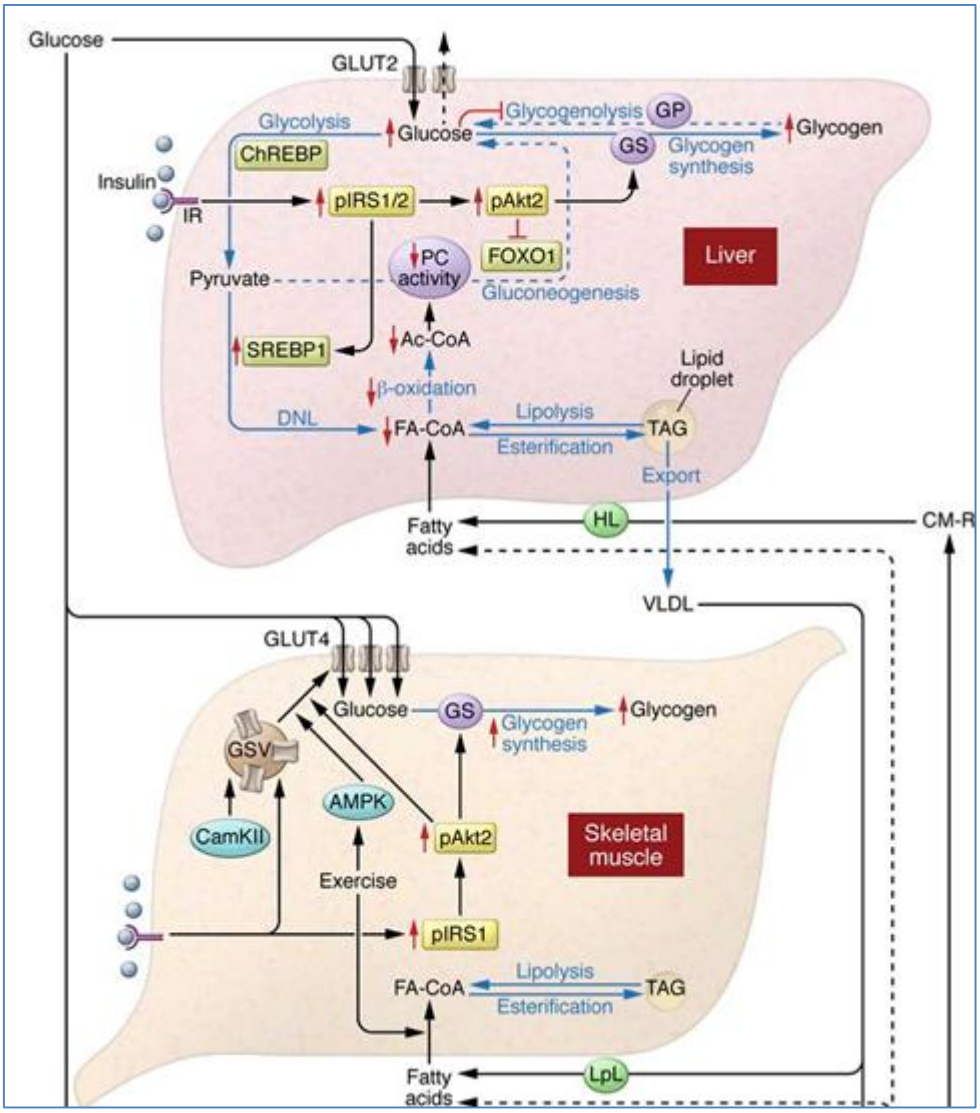
Insulin resistance and abnormal insulin secretion are central to the development of type 2 DM. However, Obesity and physical inactivity lead to insulin resistance, which together with a genetic predisposition, place stress on β-cells, leading to a failure of β-cell function and a progressive decline in insulin secretion(DeFronzo *et al.*, 2015). Insulin resistance precedes T2DM for many years. Insulin resistance 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, brain tissues, and pancreatic β-cells(Muoio and Newgard, 2008).

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 (DeFronzo *et al.*, 2015). In the liver, insulin resistance, together with insulin deficiency, hyperglucagonaemia, enhanced glucagon sensitivity, and increased substrate (fatty acids, lactate, glycerol, and amino acids) delivery, leads to increased gluconeogenesis, which is responsible for the increased basal rate of glucose production and fasting hyperglycemia. Also, insulin resistance in the kidney and augmented renal gluconeogenesis contribute to fasting hyperglycemia(Galicia-Garcia *et al.*, 2020).As shown in Figure(1.1).



Figure(1.1): Mechanisms of insulin resistance in liver and skeletal muscle respectively(Samuel and Shulman, 2016)**.**

Normally, the Binding of insulin to its receptor activates insulin receptor tyrosine kinase and phosphorylation of a family of insulin receptor substrates (IRSs), especially IRS1 and IRS2. These phosphorylated IRS proteins bind to and activate intracellular signaling molecules, the most important of which is phosphatidylinositol 3-kinase (PI3K). PI3K promotes glucose transporter type 4 (GLUT4) translocation to the plasma membrane, resulting in glucose uptake into skeletal muscle, and phosphorylates and inactivates the transcription factor fork head box protein O1 (FOXO1), altering transcription of downstream genes(Czech, 2017). As shown in Figure(1.2).



Figure(1.2): Normal insulin action in liver and skeletal muscle that promote nutrient storage(Samuel and Shulman, 2016)**.**

1.1.8 Complications of Diabetes Mellitus

There are two different types of complications linked to diabetes mellitus: acute (short-term) and chronic (long-term).

1.1.8.1 Acute Complications:

1-Diabetic Ketoacidosis (DKA) is a complication of type 1 diabetes, resulting from extreme insulin insufficiency or insulin abstinence, however, it can also develop in people with type 2 diabetes(Westerberg, 2013).

2- In the absence of ketone bodies, hyperosmolar non-ketosis coma is a life-threatening endocrine emergency characterized by severe hyperglycemia (600 mg/dl), severe hyperosmolarity (> 320 mOsm/l), and dehydration (Pasquel and Umpierrez, 2014).

3-Accidental over administration of insulin, meglitinides, or sulphonylureas is the most common cause of hypoglycemia. A large amount of insulin or a hypoglycemic medicine can precipitate hypoglycemia; on the other hand, the patient may have skipped a meal or done rigorous activity after receiving the typical dose of insulin or oral hypoglycemic drugs(Jensen *et al.*, 2020).

1.1.8.2 Chronic Complications:

Diabetes Mellitus's chronic consequences affect a variety of organ systems and are responsible for the bulk of morbidity and mortality. Micro and macro-vascular problems can be distinguished(Tripathi and Srivastava, 2006).

1.1.8.2.1 Macrovascular Complications of Diabetes:

Cardiovascular disease (CVD), Cerebrovascular disease (CVD), and Peripheral artery disease (PAD) is the macrovascular consequences (PAD). Diabetic Foot Syndrome is characterized as the presence of a foot ulcer in combination with neuropathy, peripheral artery disease, and infection, and it is a leading cause of lower limb amputation (Papatheodorou *et al.*, 2018).

Adults with T2DM have a 2-to-4-fold higher risk of cardiovascular morbidity and mortality than people without diabetes, according to the American Heart Association (AHA). Furthermore, the American Heart Association considers diabetes to be "one of the seven key modifiable risk factors for cardiovascular disease (CVD)." Additional CVD risk factors in T2DM include hypertension, dyslipidemia, obesity, lack of physical exercise, and smoking (Hudspeth, 2018).

The process of atherosclerosis, which causes the narrowing of artery walls throughout the body, is the major pathogenic mechanism in macrovascular disease. Chronic inflammation and damage to the arterial wall in the peripheral or coronary vascular system are thought to cause atherosclerosis. Oxidized lipids from low-density lipoprotein (LDL) particles accumulate in the endothelial wall of arteries in response to endothelial injury and inflammation. The monocytes enter the arterial wall and develop

into macrophages, which collect oxidized lipids and create foam cells. Foam cells, once produced, cause macrophage growth and T-lymphocyte recruitment. T lymphocytes, in turn, stimulate smooth muscle proliferation and collagen buildup in the artery walls. The process results in the creation of a fibrous cap over a lipid-rich atherosclerotic lesion. Acute vascular infarction occurs when this lesion ruptures(Fowler, 2011).

1.1.8.2.2 Microvascular Complications:

Microvascular complications are classified as follows:

1-Diabetic retinopathy

2-Diabetic neuropathy

3- Diabetic nephropathy

1.2 Diabetic Nephropathy

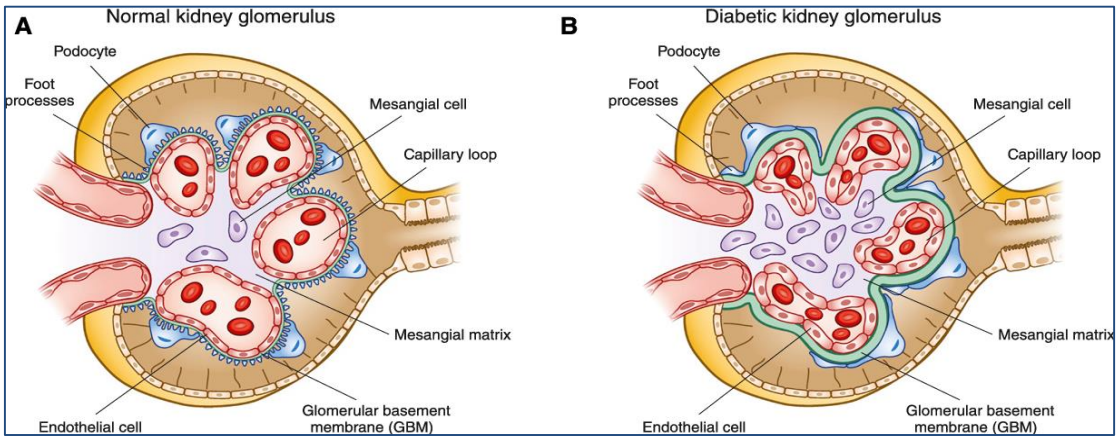
Diabetic Nephropathy (DN) is one of the most frequent and severe complications of diabetes mellitus and is associated with increased morbidity and mortality in diabetic patients(Valencia and Florez, 2017). DN represents the leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) 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(Vallon and Thomson, 2020).

Clinically, diabetic kidney disease is characterized by progressive kidney damage reflected by increasing albuminuria(urine albumin-to-creatinine ratio (ACR) exceeding 30 mg/g creatinine) together with impairment in renal function (decline in glomerular filtration rate (GFR)), elevated blood pressure, and excess morbidity and mortality due to cardiovascular complications(Persson and Rossing, 2018).

Normally, The kidneys produce a virtually protein-free primary urine through a simple process: the blood enters the glomerular tuft via the afferent arteriole and perfused the glomerular capillaries, where filtration across the glomerular filtration barrier into Bowman’s space occurs. The primary urine is drained into the renal tubules, and the blood exits the tuft via the efferent arteriole. However, the success of this filtration process is based on the presence of a highly specialized structure: the glomerular filtration barrier (GFB)(Murray and Paolini, 2020). This filtration barrier

permits highly selective ultrafiltration of the blood plasma: it is freely permeable to water, small- and mid-sized solutes, and low-molecular-weight proteins up to the mass of albumin, but largely precludes the filtration of plasma proteins with a mass of more than 60–70 kDa, especially if they are negatively charged. This filtration apparatus is formed by three layers: the fenestrated endothelium, the glomerular basement membrane (GBM), and the epithelial podocytes with their 'slit diaphragms'(Mora-Fernández *et al.*, 2014). As shown in figure (1.3).

In patients with diabetes, the development of DN is associated with many alterations in the structure of multiple kidney compartments. The major histologic alterations that happen in the glomeruli of persons with diabetic nephropathy include: mesangial expansion is directly influenced by hyperglycemia, may be via increased matrix production or glycation of matrix proteins, increase the thickness of the glomerular basement membrane (GBM) takes place, and glomerular sclerosis is caused by intraglomerular hypertension, which is caused by dilatation of the afferent renal artery or from ischemic injury caused by hyaline narrowing of the vessels providing the glomeruli by blood. As shown in Figure(1.3)(Alicic, Rooney, and Tuttle, 2017). These changes damage the kidney's glomeruli, which leads to the characteristic feature of albumin in the urine (called albuminuria)(Lin *et al.*, 2018).



Figure(1.3): Normal kidney morphology and structural changes in Diabetes Mellitus(Alicic, Rooney, and Tuttle, 2017)**.**

1.2.1 Pathophysiology of Diabetic Nephropathy

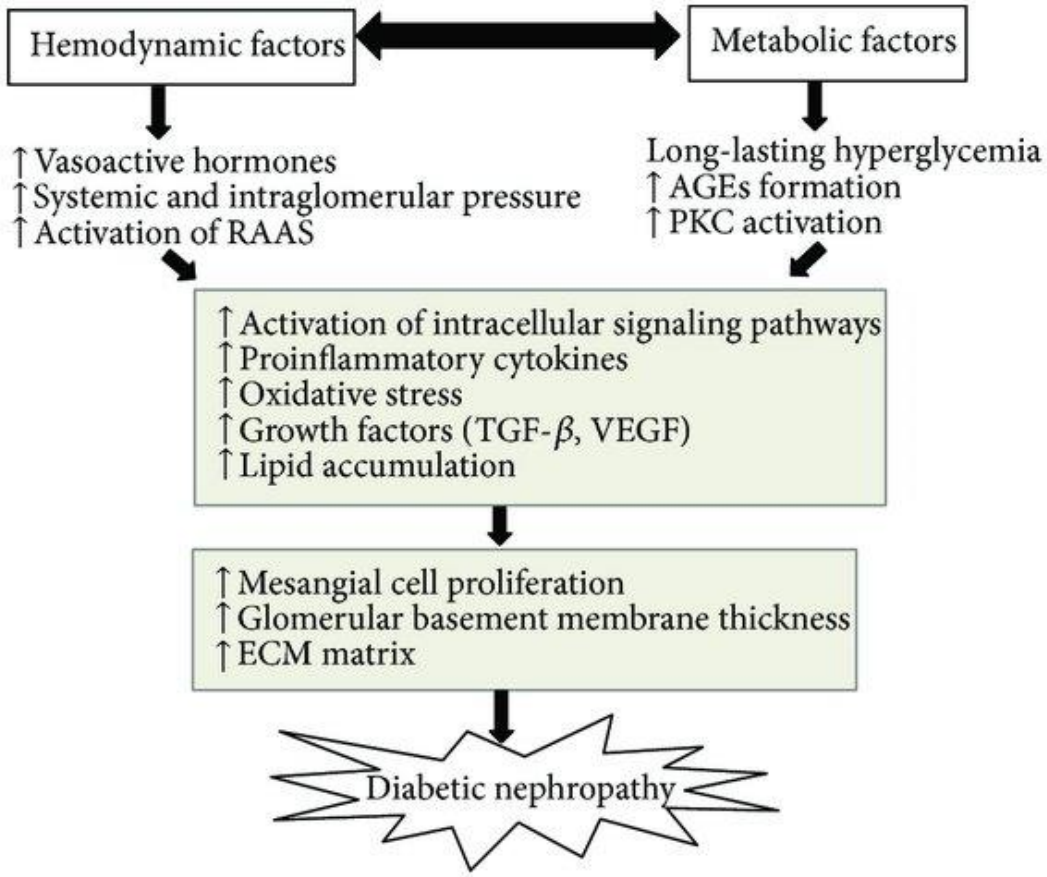
The pathogenesis and progression of diabetic nephropathy are likely to be a result of the interplay between metabolic and hemodynamic pathways, which are often disturbed in the setting of diabetes.

1.2.1.1 Hemodynamic Factors:

In hemodynamic pathways, there is an imbalance in afferent and efferent arteriolar resistance, resulting in increased glomerular hydrostatic pressure and hyperfiltration. Activation of the renin-angiotensin system (RAS) increases angiotensin ıı levels, leading to efferent arteriolar vasoconstriction. In addition, increased levels of endothelin-1 also contribute to vasoconstriction(Lim, 2014).

1.2.1.2 Metabolic Factors

In metabolic pathways, the Activation of glucose-dependent pathways inside the kidney leads to enhanced oxidative stress, renal polyol formation, and accumulation of advanced glycation end products. However, 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(Cao and Cooper, 2011). The interaction of hemodynamic and metabolic pathways in diabetic nephropathy are shown in Figure(1.4).



Figure(1.4): The Interaction of hemodynamic and metabolic pathways in Diabetic Nephropathy(Soetikno *et al.*, 2014)**.**

1.2.2 Biochemistry of Diabetic Nephropathy

A definite biochemical explanation of DN has not been established, but, there are four main pathways for developing DN:

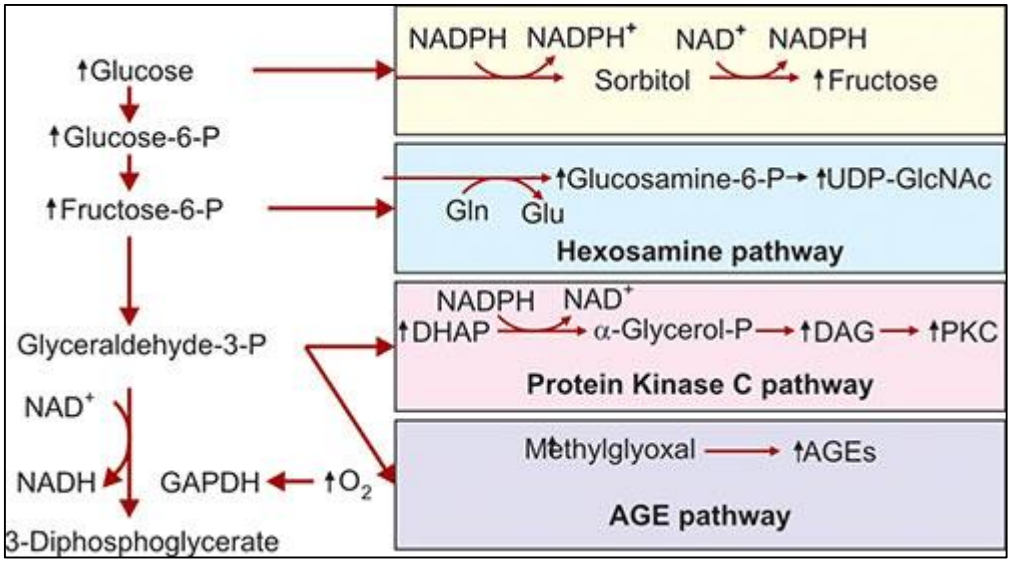
I Polyol / Aldose reductase pathway

II Hexosamine pathway

III Protein kinase C pathway

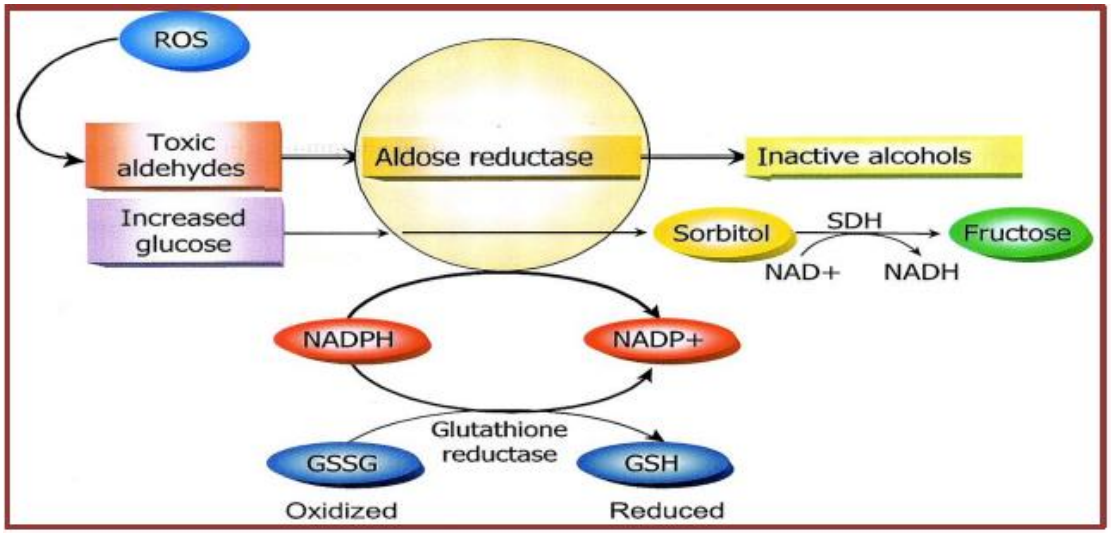
IV Advanced glycation end products(AGAE) pathway(Brownlee, 2005).

The four main candidate mechanisms for the deleterious effect of hyperglycemia are represented in Figure (1.5).



Figure(1.5): Signaling pathways of DN(Lotfy *et al.*, 2017)**. fructose-6-phosphate amidotransferase (GFAT), nicotinic acid adenine dinucleotide phosphate (NADPH), nicotinic acid adenine dinucleotide(NAD), Protein kinase c(PKC). dihydroxyacetone-phosphate (DHAP) glyceraldehyde-3-phosphate dehydrogenas2(GAPDH), uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc), diacylglycerol (DAG).**

I. Increased polyol pathway flux: The polyol pathway was 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 (high Km) 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(Brownlee, 2001). 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 on cells, including osmotic damage. 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) (Vision and Mathebula, 2015).

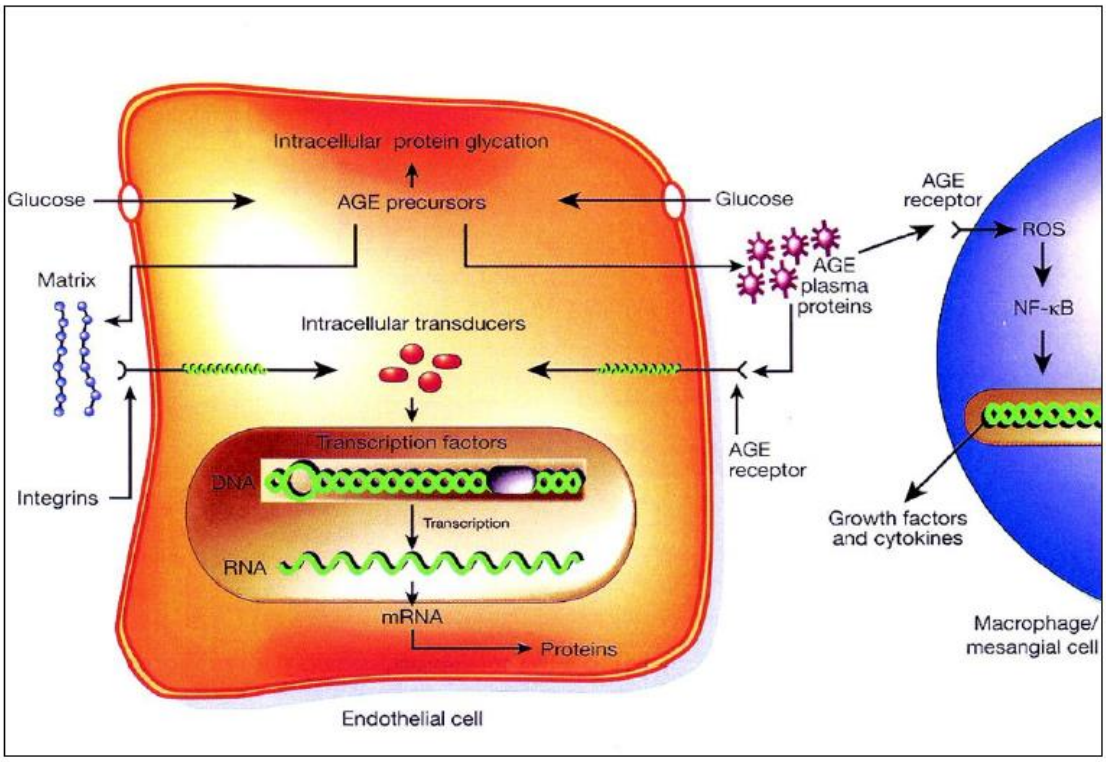


Figure(1.6): Aldose reductase and the polyol pathway (Brownlee, 2005)**. GSH= Reduced glutathione, GSSG= Oxidized glutathione, SDH=Sorbitol dehydrogenase, ROS= Reactive oxygen species.**

II. Increased hexosamine pathway activity: 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 to gene expression modifications that enhance microvascular occlusion/dysfunction, such as increased PAI-1 expression(Altaf and Tahrani, 2015).

III. Increased protein kinase C (PKC) pathway activity: Hyperglycemia increases intracellular content of glyceraldehyde-3-phosphate, which stimulates the synthesis of diacylglycerol (DAG); further activating (PKC). 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(Brownlee, 2005).

IV Increased production of AGE and its precursors appear to cause cellular dysfunction via three routes ,this is demonstrated in Figure(1.7).



Figure(1.7): Production of Advanced Glycation End-Products (AGEs) precursor and their pathological consequence(Brownlee, 2001).

Cellular dysfunction of AGEPs via three routes:

1. By modification of intracellular protein; regulatory proteins of gene(receptor for advanced glycated end products) transcription that promote vascular damage.

2. By modification of intercellular signaling between cell and matrix after diffusion of AGE precursors out of the cell.

3. By modification of extracellular proteins of the bloodstream (e.g. albumin), enabling AGE-receptors interaction and activation. This in turn causes vascular pathology from the production of inflammatory cytokines and growth factors (Brownlee, 2001).

1.2.3 Stages of Nephropathy

There are five stages in the development of diabetic nephropathy(Haneda *et al.*, 2015).

Stage 1: Early hyperfiltration and hypertrophy.

Stage 2: Latent nephropathy: Glomerular lesion without clinical disease.

Stage 3: Incipient diabetic nephropathy/ microalbuminuria stage: urine albumin to creatinine ratio(ACR)= 30-299 mg/g.

Stage 4: Overt diabetic nephropathy/ macroalbuminuria stage: urine albumin to creatinine ratio(ACR) more than 300 mg/g.

Stage 5: End-stage renal disease: Glomerular filtration rate (GFR) less than 15 mL/min/1.73 m2).

Table 1.3: Relationship between the categories of DN stages and the chronic kidney disease severity.

<table>
<tr><td rowspan="2"></td><td rowspan="2">Albuminuria category (mg/g Cr)</td><td>A1</td><td>A2</td><td>A3</td></tr>
<tr><td>Normoalbuminuria
≤30</td><td>Microalbuminuria
30-299</td><td>Macroalbuminuria
≥300</td></tr>
<tr><td rowspan="6">GFR category (ml/min/1.73m^2)</td><td>≥90</td><td>Stage1
Prenephropathy</td><td></td><td></td></tr>
<tr><td>60-89</td><td></td><td>Stage2
Incipient-nephropathy</td><td></td></tr>
<tr><td>45-59</td><td colspan="2" rowspan="2"></td><td rowspan="2">Stage3
Overt nephropathy</td></tr>
<tr><td>30-44</td></tr>
<tr><td>15-29</td><td colspan="3">Stage4 (kidney failure)</td></tr>
<tr><td><15</td><td colspan="3">Stage5 (dialysis therapy)</td></tr>
</table>

1.2.4 Screening of Diabetic Nephropathy

The American Diabetic Association, 2021 guidelines for screening of chronic kidney disease (CKD) in diabetic patients recommends annually least, assess urinary albumin(spot urinary albumin/ creatinine ratio (UACR)) and estimated glomerular filtration rate (eGFR) in patients with type 1 DM with duration ≥5 years, in all patients with type 2 DM and hypertension. Although microalbuminuria(30–300 mg/g

Creatinine) serves as an early indicator of glomerular disease and can predict progression to overt nephropathy and ESRD in diabetic patients(Association, 2020).

1.2.5 Diagnosis of Diabetic Nephropathy

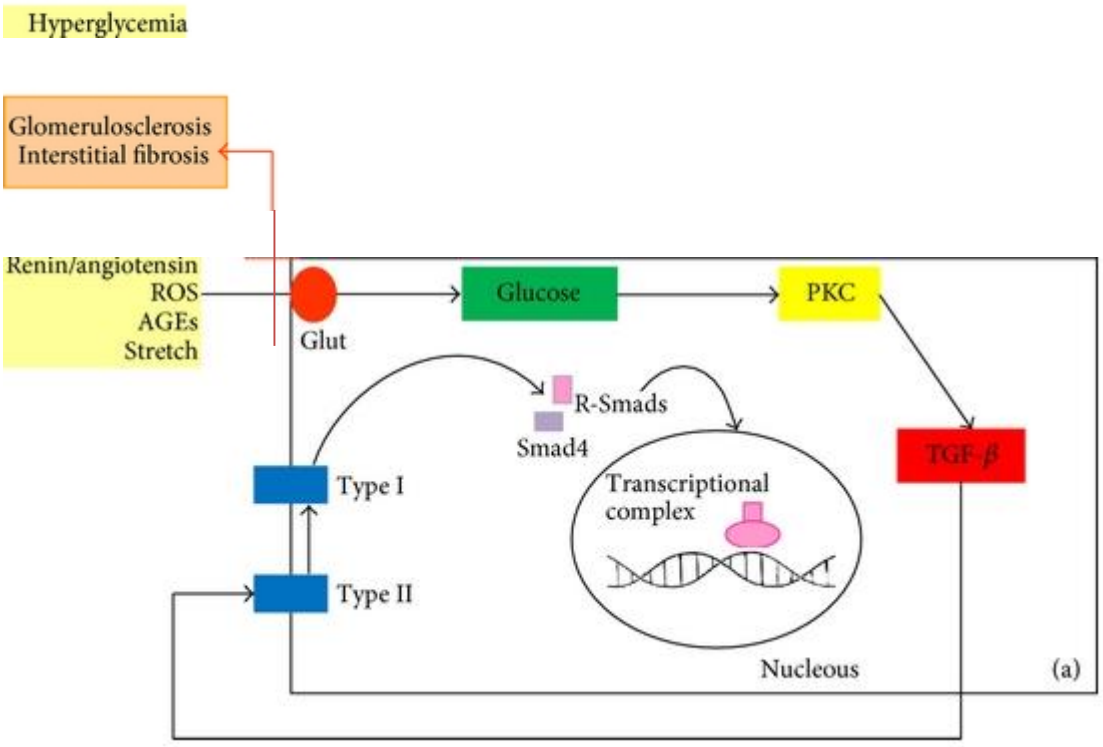
The diagnosis of DN based on ADA 2021 guidelines includes: First, The clinical diagnosis of DN is made based on the presence of albuminuria and /or decreased eGFR in the absence of signs or symptoms of other primary causes of kidney disease. Second, The typical presentation of DN is considered to include a long-standing duration of diabetes, retinopathy, albuminuria without gross hematuria, and gradually progressive loss of eGFR. However, a sign of DN may be present at diagnosis or without retinopathy in T2DM, and reduced eGFR without albuminuria. And finally, An active urinary sediment (white or red blood cells or cellular casts), rapidly elevated albuminuria or nephrotic syndrome, rapidly reduced eGFR, or absence of retinopathy(in T1DM) suggests alternative or additional causes of kidney damage(Association, 2021a).

1.3 Transforming Growth Factor-beta (TGF-β) Signaling Pathway in Diabetic Nephropathy

In the occurrence and development of Diabetic Nephropathy, many factors promote the progression of renal damage by stimulating the TGF-β production, such as hyperglycemia, advanced glycation end products (AGEs), reactive oxygen species (ROS), and renin-angiotensin II–aldosterone system (RAAS) (Y. Zhang *et al.*, 2021). The TGF-β signaling pathway could be activated by high-glucose conditions (Tuleta and Frangogiannis, 2021), which significantly increases the expression of TGF-β1 mRNA and induces the synthetic phenotype of mesangial cells (Ziyadeh, 2004). The injury of mesangial cells and podocytes caused by diabetic nephropathy can activate the signal transduction cascade of the TGF-β/SMAD signaling pathway and lead to increased production of the extra cellular matrix (ECM)(Lan, 2012). As shown in Figure(1.8).

Transforming growth factor-beta is a member of the TGF-beta superfamily. There are three TGF-β isoforms, namely TGF-β1, TGF-β2, and TGF-β3. Although all three TGF-β isoforms and receptors are widely expressed in the kidney, TGF-β1 is a key mediator in the pathogenesis of DN(Li *et al.*, 2020). Transforming growth factor-

β1 is secreted as an inactive procytokine, consisting of the mature active TGF-β1 protein non-covalently bound to a dimmer of its NH2-terminal propeptide, called latency-associated protein (LAP), and a latent TGF-β binding protein. TGF-β1 exerts its major biological effects through its cell surface receptors: TGF-BRI and TGF-BRII. Upon TGF-β1 binding, the TGF-BRI phosphorylates SMAD2 and SMAD3 which then oligomerize with SMAD4 to form a complex that translocates into the nucleus. SMAD2, SMAD3 and SMAD4 can directly bind to DNA sequences or cofactors to regulate the transcription of their target genes(Braga Gomes, Fontana Rodrigues, and Fernandes, 2014).



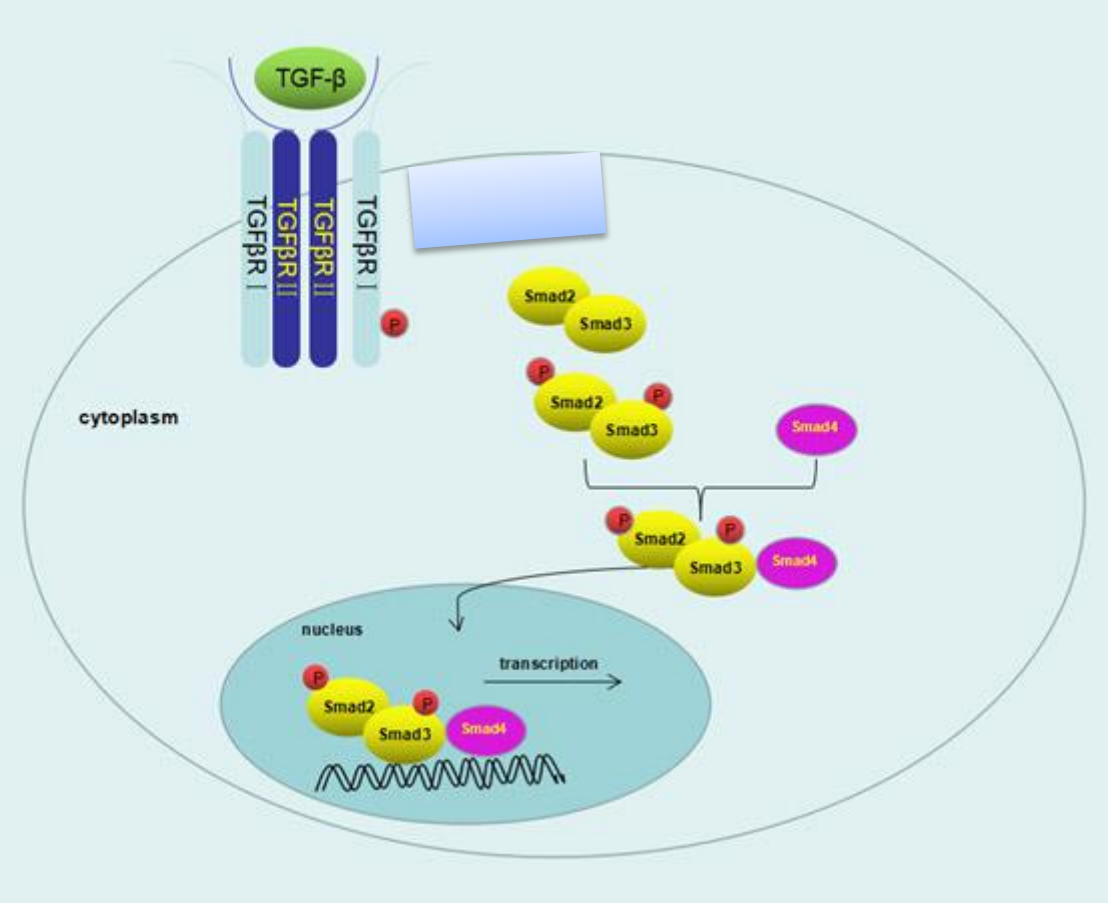
Figure(1.8): Activation of TGF-β synthesis and its role in proinflammatory mechanisms in T2DM Nephropathy (Braga Gomes, Fontana Rodrigues, and Fernandes, 2014)**. Increased extracellular glucose levels, mesangial cell stretch, activation of renin-angiotensin system, reactive oxidant species (ROS), and advanced glycation end products (AGEs) activate TGF-β synthesis via protein kinase C. TGF-β stimulates its pathway through autocrine or paracrine action. TGF-β assembles a receptor complex that activates Smads that regulate nuclear transcription.**

1.3.1 SMAD/TGF-BETA signaling pathway

Transcription factor Small Mothers Against Decapentaplegic (SMAD) family has key roles in cell fate decision in transmitting extracellular signals to the nucleus through transforming growth factor-beta(TGF-β) receptors to activate downstream target gene transcription(Gomes *et al.*, 2021). The TGF-β is widely distributed in various systems of mammalian cells and exerts a various spectrum of cellular functions such as immune response, embryo construction, tissue repair, cell proliferation, differentiation, migration, and apoptosis(Xu *et al.*, 2018). TGF-beta and related proteins initiate cellular responses by binding to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding and initiates specific intracellular signals by SMAD proteins(Liang *et al.*, 2021). SMAD proteins are a group of molecules that function as intracellular signal transducers downstream of the receptors of the TGF-β superfamily. Eight different SMAD proteins have been identified in mammals, divided into three subfamilies based upon their function: receptor-regulated SMADs (R-SMADs), common-partner SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs)(Zhu *et al.*, 2004). R-SMADs are activated by the type I receptor serine kinase through phosphorylation. This family consists of SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8. SMAD1, SMAD5, and SMAD8 mediate signaling for bone morphogenetic proteins (BMP) and anti-Mu¨ Mullerian hormone pathways, while SMAD2 and SMAD3 act in the TGF-β and Activin pathways. SMAD4 is the Co-SMAD, which positively regulates all the above pathways(Zhong *et al.*, 2021). In contrast to R-SMADs and Co-SMADs, I-SMADs, including SMAD6 and SMAD7, bind to the intracellular domain of type I receptors. They comp ete with R-SMADs for activation by the type I receptors, resulting in inhibition of TGF-β superfamily signaling. SMAD6 inhibits BMP signaling, while SMAD7 inhibits TGF-β and Activin signaling(Zhong *et al.*, 2021).

For TGF-β and Activin signaling, phosphorylation and activation by the active type II and type I receptor complex causes SMAD2 and SMAD3 to form heterooligomers with SMAD4 and translocate the entire complex into the nucleus. There they interact with different cellular partners, bind to DNA, and regulate the transcription of various downstream response genes(Figure 1.9). SMAD7 also inhibits

this signaling by forming a complex with SMAD2/3 and thereby interfering with the complex formation between SMAD2, SMAD3 and SMAD4, and preventing further signal propagation(Zhu *et al.*, 2004)(Figure 1-9). Transcription of SMAD7 is initiated by both TGF-β and Activin stimulation, providing a regulatory feedback mechanism to terminate signaling through the activated receptors(Hanna, Humeres, and Frangogiannis, 2021).



Figure(1.9): SMAD/TGF-BETA signaling pathway(Hanna, Humeres, and Frangogiannis, 2021).

TGF-β1 signaling is the prototype of TGF-β superfamily signaling, which also contains Bone Morphogenic Protein(BMP), and Growth and Differentiation Factor (GDF) signaling (Weiss and Attisano, 2013). TGF-β signaling plays pleiotropic roles in various biological processes including cell growth and differentiation, development, apoptosis, cancer, fibrosis, immunity, and so on (Meng *et al.*, 2015). In this review, the role of TGF-β signaling in the pathogenesis of diabetic nephropathy will be discussed.

TGF-β1 has long been known as a key mediator in the pathogenesis of renal fibrosis by activating the downstream SMAD proteins, especially SMAD2/3. Once SMAD3 becomes activated in response to TGF-β1, it can translocate to the nucleus to directly bind to DNA sequences and regulate the target genes (Figure1.10)(Verrecchia, Chu and Mauviel, 2001). Thus, SMAD3 plays a critical role in the development of renal fibrosis in many kidney diseases. An essential role for Smad3 in fibrogenesis is confirmed by the findings that deletion of SMAD3 from mice can suppress renal fibrosis in several rodent models, including diabetic nephropathy, obstructive kidney diseases, and hypertensive nephropathy, and drug-associated nephropathy(Liu, Huang and Lan, 2012).

SMAD3 can also regulate SMAD7 to play a role in renal fibrosis. It is well established that SMAD7 is an inhibitory SMAD that is induced by SMAD2/3 transcriptionally but exerts its negative feedback mechanism to maintain the homeostasis of TGF-β/SMAD signaling (Lan, 2011). In normal situations, renal SMAD7 is abundant and exerts its negative feedback mechanism by causing degradation of Type I TGF-β receptor (TβRI) via a ubiquitin-proteasome degradation mechanism, thereby preventing the recruitment and phosphorylation of SMAD2/3. Under disease conditions, SMAD3 is over-reactive and can also induce several E3 ubiquitin ligases such as the SMAD Ubiquitination Regulatory Factor 1 (Smurf1), SMAD Ubiquitination Regulatory Factor2 (Smurf2), which physically interact with SMAD7 and cause a ubiquitin-dependent degradation of renal SMAD7 protein, resulting in enhanced TGF-β/SMAD2/3 signaling and progressive renal fibrosis(Inoue and Imamura, 2008). This is further supported by the findings that mice lacking SMAD7 largely promote activation of SMAD3 signaling and progressive renal fibrosis in both obstructive nephropathy and diabetic kidney disease (Chen *et al.*, 2011).

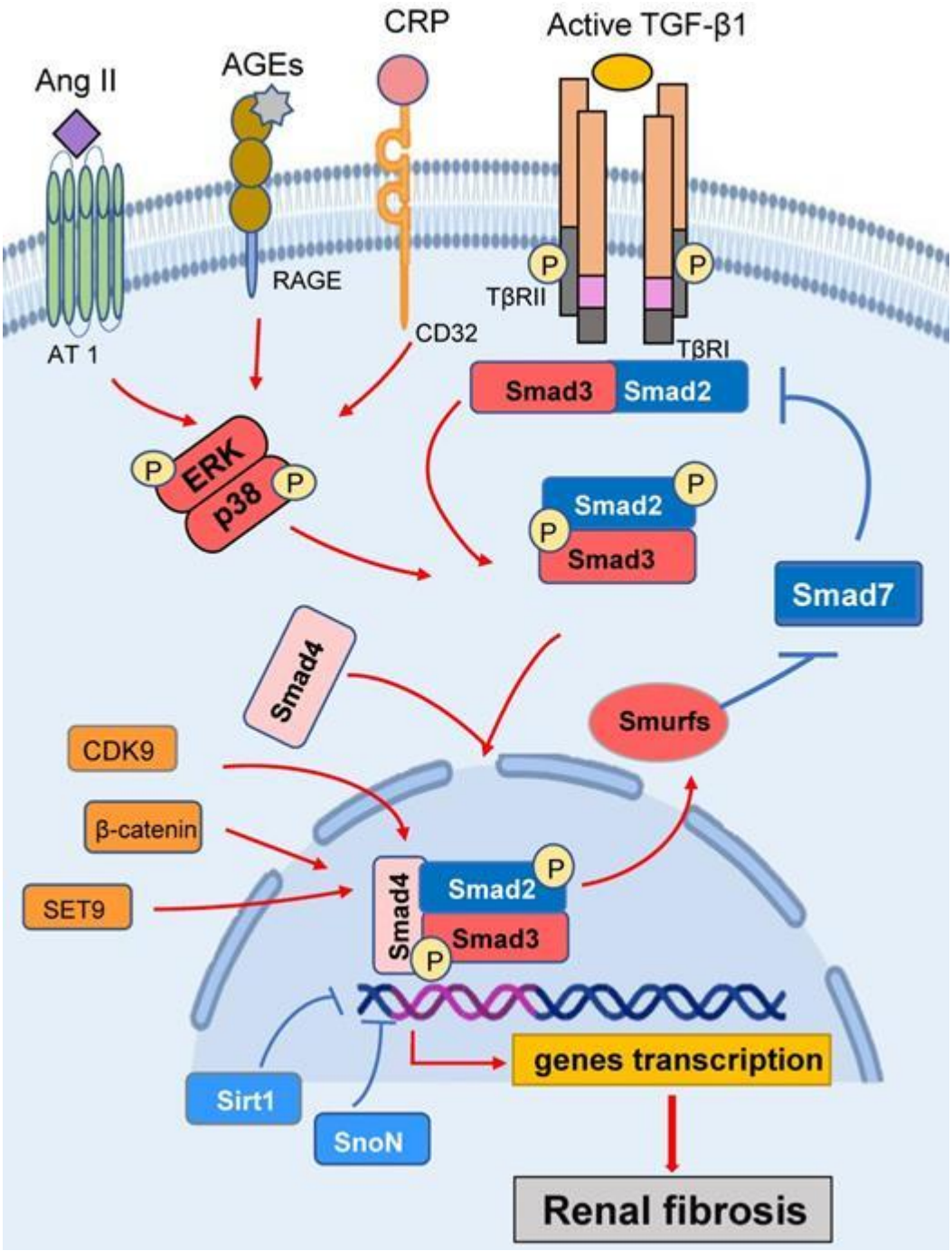


Figure (1.10): SMAD3 signaling and crosstalk pathways in renal fibrosis(Wu *et al.*, 2022)**. SMAD3 signaling and crosstalk pathways in renal fibrosis. After binding to TβRII, TGF-β1 activates the TβRI-kinase which phosphorylates Smad3. The phosphorylated SMAD3 translocates into the nucleus and regulates the target gene transcription. SMAD7 is an inhibitory SMAD that functions to block Smad3 activation by degrading the TβRI and preventing phosphorylation of Smad3.Ang II, AGEs and CRP can activate TGF-β1-independent signaling via the ERK/p38/ MAPK crosstalk pathway. Red arrows/ symbols represent pathogenic or positive regulation pathway, while blue lines/ symbols indicate protective or negative regulation pathways in fibrosis.**

1.4 Aims of the study:

1-To evaluate the diagnostic utility of TGF-BETA, SMAD2, and SMAD3 as a biomarkers for early detection of Nephropathy in type 2 Diabetes Mellitus.

2-To investigate the role of SMAD2 and SMAD3 and their Correlation with TGF-BETA in Type 2 Diabetic Patients with Early and Chronic Diabetic Nephropathy.

3-To study the correlation between serum TGF-BETA, SMAD2, and SMAD3 with other traditional renal function parameters(Albuminuria and eGFR).

4-To determine specificity, sensitivity, and cutoff value for serum TGF-BETA, SMAD2, and SMAD3 through ROC analysis to see whether act as early detectors of Nephropathy in T2DM.

CHAPTER TWO

MATERIALS AND METHODS

2-Materials and Methods

2.1. Subjects:

This study was conducted by sample collection in the outpatient clinic at Diabetes and Endocrine Center in Marjan Medical City in Hilla city, Babylon province from October 2021 until May 2022. The part that deals with the practical aspect of the study were accomplished at the laboratory of the department of biochemistry, College of Medicine, University of Kerbala.

2.1.1 Study design :

The current study was designed as a case-control study and involved 120 individuals, 60 patients with type 2 diabetes mellitus (T2DM) group and 60 participants who are healthy was used as a control group. All patients were diagnosed by physicians according to American Diabetic Association criteria(Association, 2015b). They were enrolled in Diabetes and Endocrine Center in Marjan Medical City in Babylon / Hilla city. As shown in Figure (2.1).

2.1.1.1 Control Group

Control group involved (sixty) healthy subjects. All subjects do not appear any signs and symptoms of diseases. Control subjects consisting of males and females were matched with patients in sex and age to increase the accuracy of the results. The blood samples were collected from relatives and medical staff of Marjan Teaching Hospital.

2.1.1.2 Patient Group

The patient's group was subdivided into three equal groups according to their urinary albumin to creatinine ratio (ACR):

Group one: 20 Type 2 Diabetic patients with normoalbuminuria; included patients with urinary albumin to creatinine ratio (ACR) <30 mg/g creatinine.

Group two: 20 Type 2 Diabetic patients with microalbuminuria; including patients with urinary albumin to creatinine ratio (ACR) = 30 – 299 mg/g creatinine.

Group three: 20 Type 2 Diabetic patients with macroalbuminuria; included patients with urinary albumin to creatinine ratio (ACR) of more than 300 mg/g.

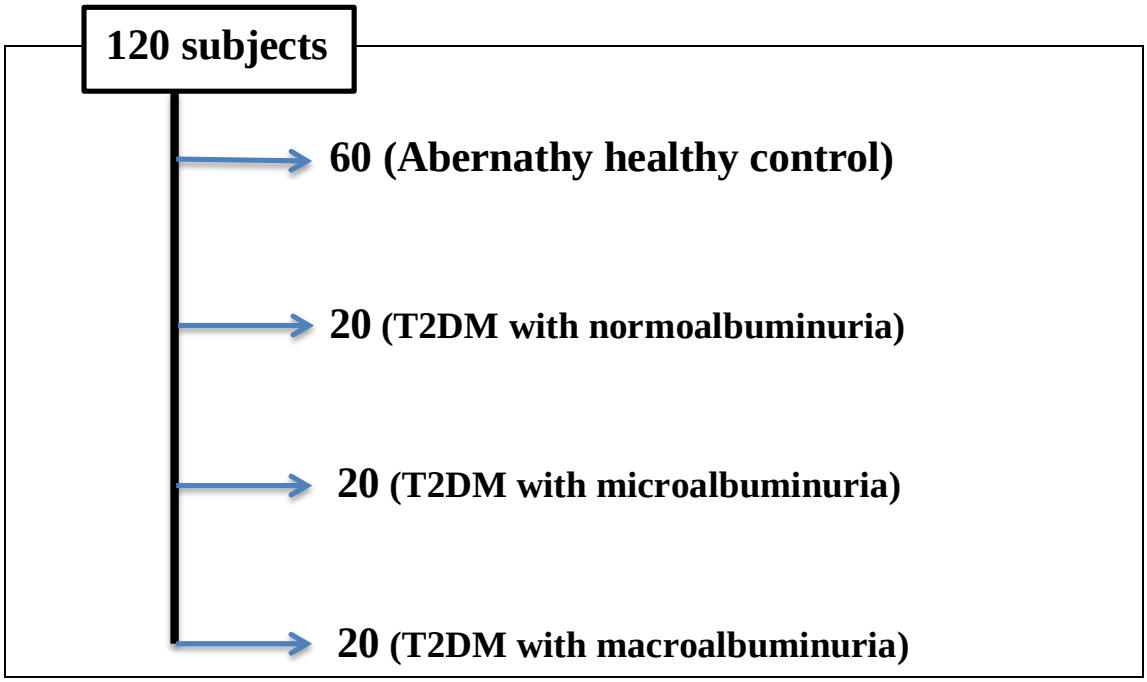


Figure (2.1): Simplified scheme represents the study groups.

A detailed questionnaire (Appendix A) was completed for each participant. Information obtained included age, gender, duration of DM, history of the other complications of DM, and history of hypertension or cardiovascular diseases. Ethical approval was taken for each person involved in this study.

2.1.1.3 Exclusion Criteria

1. Type 1 diabetic patients.
2. Fever
3. Urinary tract infection
4. Chronic Hypertension
5. History of cardiovascular diseases (coronary artery disease, cerebrovascular or peripheral artery diseases).
6. Acute and chronic inflammatory diseases.
7. Pregnant women.
8. Women taking an oral contraceptive.
9. Persons on glucocorticoid medications.
10. Surgery or trauma within 6 months.

11. Malignancies.
12. Proteinuria due to causes other than DM.

2.2. Materials

2.2.1 Diagnostic kits

The diagnostic kits in the present study were used as supplied from purchases without additional purification. Diagnostic kits used in the present study are shown in Table (2-1).

Table 2-1: Diagnostic kits used in the study.

No.	Chemicals	Manufacture	Country
1	A1Care™ HbA1c cartridge	i-sens	**Korea**
2	Combina 13 Urine Test Strips	HUMAN	**Germany**
3	Creatinine Kit	LINEAR	**SPAIN**
4	Glucose Kit	LINEAR	**SPAIN**
5	Human Mother Against Decapentaplegic Homolog 2 ELISA Kit.	Bioassay Technology Laboratory.	**China**
6	Human Mother Against Decapentaplegic Homolog 3 ELISA Kit.	Bioassay Technology Laboratory.	**China**
7	Human Transforming Growth Factor β (TGF-β) ELISA Kit	Bioassay Technology Laboratory.	**China**
8	Microalbumin in Human Urine Kit	HUMAN	**Germany**
9	Urea Kit	LINEAR	**SPAIN**

2.2.2 Apparatus and Equipment:

Standard laboratory equipment's were used in this study as shown the Table 2-2.

Table 2-2 : Apparatus and equipments used in this study.

No.	Apparatus and Equipment's	Origin
1	A1Care™ Analyzer	Korea
2	Blue and yellow tips	China
3	Centrifuge EBA 20	Hettich/ Germany
4	Deep Freeze -40 ^{o}C	GFL / Germany
5	Disposable syringes (5 mL)	Medical jet / Syria
6	Distiller	GFL / Germany
7	EDTA tube (5ml)	AFCO / Jordan
8	ELISA Reader	BioTek USA
9	ELISA Washer	BioTek USA
10	Eppendorf tube (1.5ml)	China
11	Filter papers	China
12	Glass slides and cover slides.	China
13	Incubator 37 ^{o}C	Fisher Scient./ Germany
14	Micropipettes (10,50, 1000µL)	Germany
15	Multichannel micropipette (0-250 µl)	Germany
16	Plain tube	China
17	Spectrophotometer CECIL 7200	Cecil /UK
18	Test tube with Separating gel	AFCO , Jordan
19	Water bath	GFL / Germany

2.3 Methods:

2.3.1 Sample Collection

The study samples include: Whole blood, Serum, and Fresh urine

2.3.1.1 Blood Samples:

Five milliliters of fasting blood samples were obtained from each participant in this study. Each blood sample is 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 were left for 20 minutes in 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, and urea. This analysis was measured by the colorimetric method.

Aliquot 2: Stored at -40Co for the subsequent assay of TGF-β, SMAD2, and SMAD3 which were measured by using an ELISA technique.

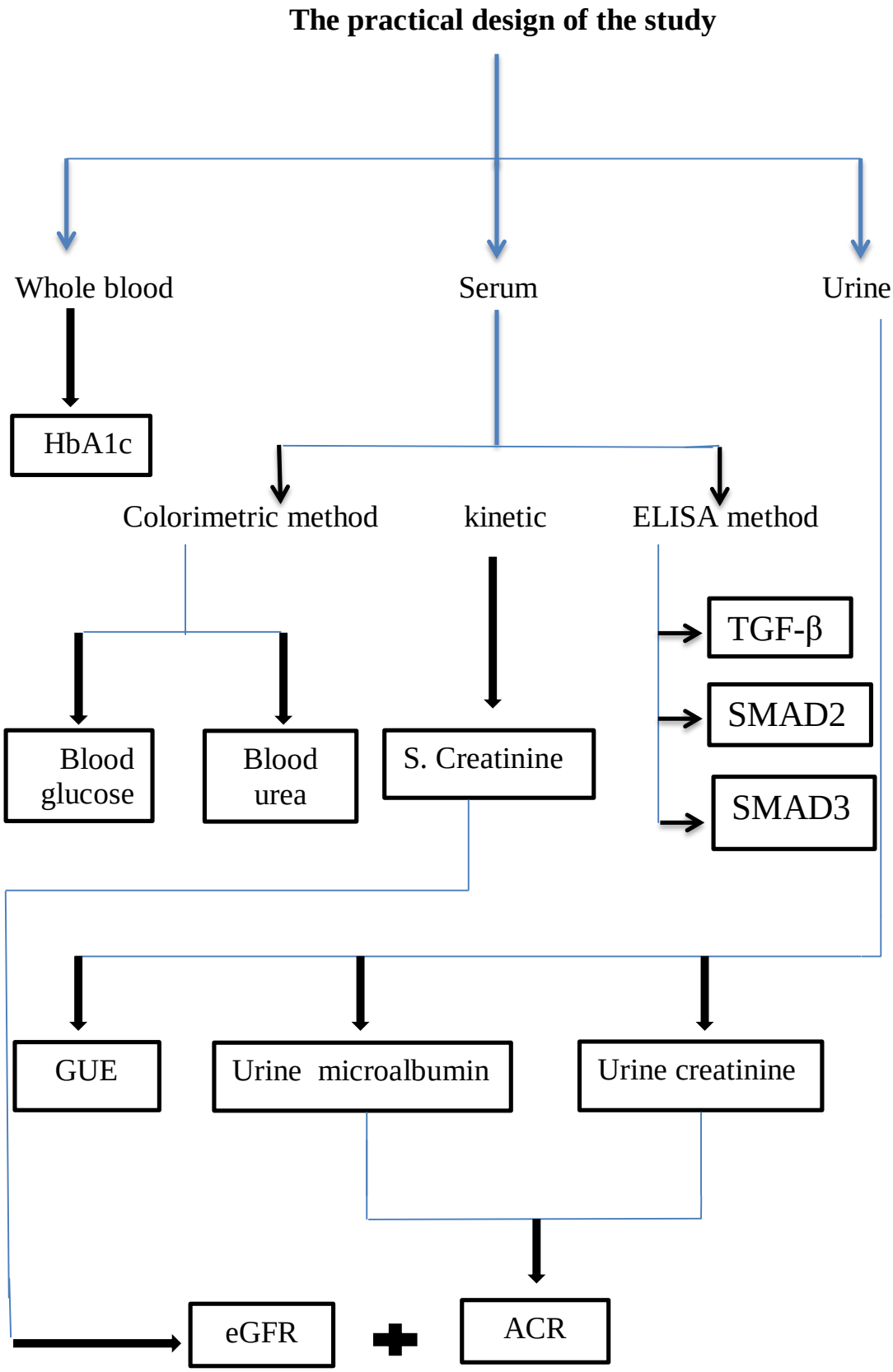
2.3.1.2 Urine Samples:

Ten milliliters of freshly morning urine samples were collected into a clean container and divided into two aliquots.

Aliquot 1: Used for general urine examination which includes macro-examination using urine strip kit supplied by Human diagnostics, and microscopic.

Aliquot 2: The urine sample was centrifuged to remove particulate matter, and used for the determination of urinary albumin and urinary creatinine.

The practical design of the study was illustrated in figure 2-2.



Figure(2-2): A scheme illustrated the practical design of the study. GUE: General Urine Examination; ELISA: Enzyme-Linked Immunosorbent Assay; ACR: Albumin /Creatinine Ratio; eGFR: Glomerular Filtration Rate.

2.3.2. Body Mass Index (BMI)

Body mass index was calculated for all subjects according to a ratio depending on weight and height obtained by applying a mathematical equation, in which the weight in kilogram was divided by the square height in meters (Nuttall, 2015), and the results were considered as follows :

- BMI is less than 18.5 and 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.

Obesity is frequently subdivided into categories:

- Class 1: BMI of 30 to <35
- Class 2: BMI of 35 to <40
- Class 3: BMI of 40 or higher (Nuttall, 2015).

2.3.3 Determination of Serum Small Mother Against Decapentaplegic Homolog 2 (SMAD2)

2.3.3.1 Principle:

Enzyme-Linked Immunosorbent Assay (ELISA) kit was used. The plate has been pre-coated with a human SMAD2 antibody. SMAD2 present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human SMAD2 antibody was added to wells and binds to SMAD2 in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated SMAD2 antibody. After incubation unbound Streptavidin-HRP has washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human SMAD2 bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-3:- Components of SMAD2 ELIZA kits.

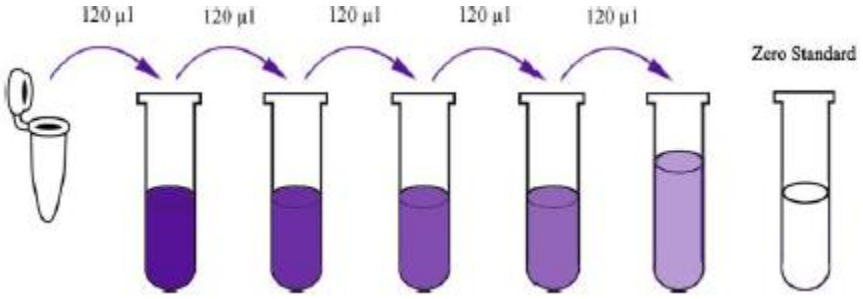
Components	Quantity (96T)
Standard solution (12.8ng/ml)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human SMAD2 antibody	1ml x1
User instruction	1
Plate sealer	2 pics

2.3.3.2 Reagent Preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

6 ng/ml	Standard No 5	120µl Original Standard + 120µl Standard Diluent
3 ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
1.5 ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
0.75ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
0.375ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
12.8 ng/ml	6 ng/ml	3 ng/ml	1.5 ng/ml	0.75ng/ml	0.375ng/ml

Figure(2-3): Standards concentration of SMAD2.

C- To yield 500 ml of 1x Wash Buffer dilute 20ml of Wash Buffer concentrate 25x into deionized or distilled water. When crystals have been made in the concentrates, mix gently until the crystals have fully dissolved.

2.3.3.3 Assay Procedure:

1. Before use, all reagents are put to room temperature. The assay is performed at room temperature.

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

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

4. A volume of 40µl from the sample was added and then added 10µl of Human SMAD2 antibody to the sample well, then 50µl of Streptavidin-HRP to both sample and standard wells. Well, mixed. The over a plate was covered with sealer and 60 min incubated at 37 °c.

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

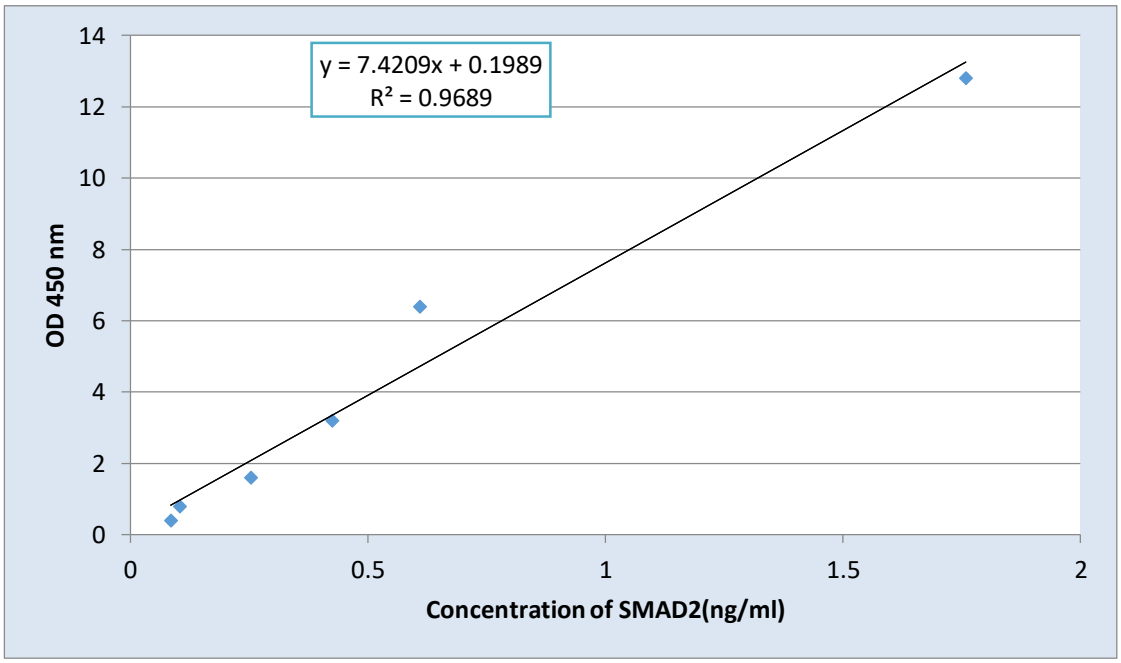
6. A volume of 50µl substrate solution A added and then 50µl substrate solution B for each well. The coated plate was incubated with a new sealer for 10 min. at 37°c in dark media.

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

8. The optical density (OD value) of each well-identified directly after applying the stop solution by utilizing a microplate reader set at 450 nm within 10 min.

Detection range:- 0.05 – 10 ng/ml

Sensitivity:- 0.019 ng/ml



Figure(2-4): Standard curve for Human SMAD2.

2.3.4 Determination of Serum Small Mother Against Decapentaplegic Homolog 3 (SMAD3)

2.3.4.1 Principle:

Enzyme-Linked Immunosorbent Assay (ELISA) kit was used. The plate has been pre-coated with a human SMAD3 antibody. SMAD3 present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human SMAD3 antibody was added to wells and binds to SMAD3 in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated SMAD3 antibody. After incubation unbound Streptavidin-HRP has washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human SMAD3 bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-4:- Components of SMAD3 ELIZA kits.

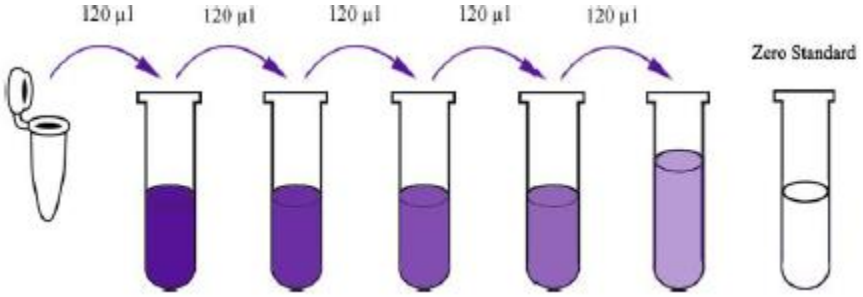
Components	Quantity (96T)
Standard Solution (40ng/ml)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human Smad3 Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

2.3.4.2 Reagent Preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

20 ng/ml	Standard No 5	120µl Original Standard + 120µl Standard Diluent
10 ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
5 ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
2.5 ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
1.25ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
40ng/ml	20ng/ml	10ng/ml	5ng/ml	2.5ng/ml	1.25ng/ml

Figure(2.5): Standards concentration of SMAD3.

C- To yield 500 ml of 1x Wash Buffer dilute 20ml of Wash Buffer concentrate 25x into deionized or distilled water. When crystals have been made in the concentrates, mix gently until the crystals have fully dissolved.

2.3.4.3 Assay Procedure:

1. Before use, all reagents are put to room temperature. The assay is performed at room temperature.

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

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

4. A volume of 40µl from the sample was added and then added 10µl of Human SMAD3 antibody to the sample well, then 50µl of Streptavidin-HRP to both sample and standard wells. Well, mixed. The over a plate was covered with sealer and 60 min incubated at 37 °c.

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

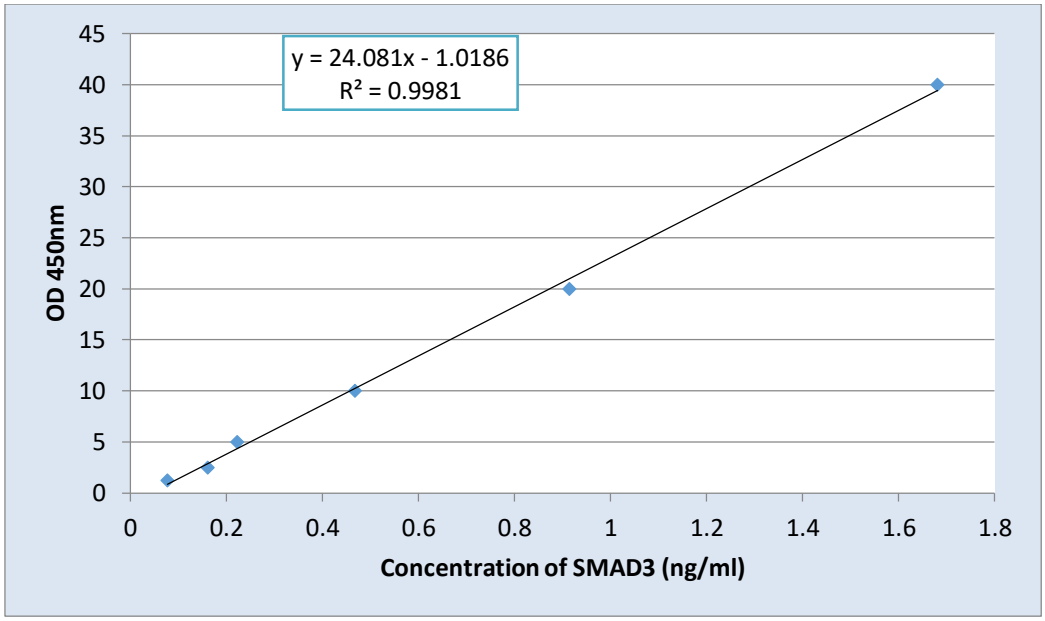
6. A volume of 50µl substrate solution A added and then 50µl substrate solution B for each well. The coated plate was incubated with a new sealer for 10 min. at 37°c in dark media.

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

8. The optical density (OD value) of each well-identified directly after applying the stop solution by utilizing a microplate reader set at 450 nm within 10 min.

Detection range:- 0.1 – 35 ng/ml

Sensitivity:- 0.047ng/ml



Figure(2-6): Standard curve for Human SMAD3.

2.3.5 Determination of Serum Transforming Growth Factor β (TGF-β):

2.3.5.1 Principle:

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

Table 2-5:- Components of TGF-β ELIZA kits.

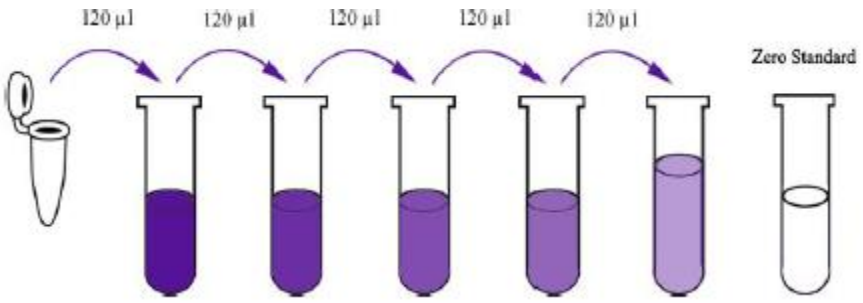
Components	Quantity
Standard Solution (4800ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated human TGF- β Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

2.3.5.2 Reagent Preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

2400ng/ml	Standard No 5	120µl Original Standard + 120µl Standard Diluent
1200ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
600ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
300ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
150ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
4800ng/ml	2400ng/ml	1200ng/ml	600ng/ml	300ng/ml	150ng/ml

Figure(2-7): Standards concentration TGF-β.

C- To yield 500 ml of 1x Wash Buffer dilute 20ml of Wash Buffer concentrate 25x into deionized or distilled water. When crystals have been made in the concentrates, mix gently until the crystals have fully dissolved.

2.3.5.3 Assay Procedure:

1. Before use, all reagents are put to room temperature. The assay is performed at room temperature.

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

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

4. A volume of 40µl from the sample was added and then added 10µl of human TGF-β antibody to the sample well, then 50µl of Streptavidin-HRP to both sample and standard wells. Well, mixed. The over a plate is covered with sealer. and 60 min incubated at 37 °c.

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

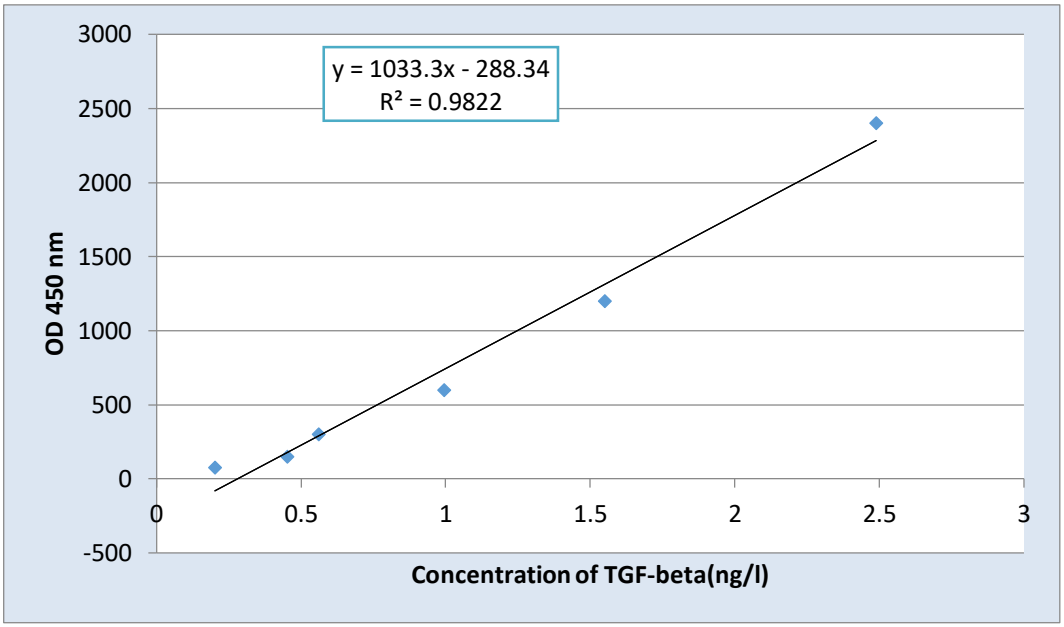
6. A volume of 50µl substrate solution A added and then 50µl substrate solution B for each well. The coated plate was incubated with a new sealer for 10 min. at 37°c in dark media.

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

8. The optical density (OD value) of each well-identified directly after applying the stop solution by utilizing a microplate reader set at 450 nm within 10 min.

Detection range:- 10 – 4000ng/ml

Sensitivity:- 5.11ng/ml



Figure(2-8): Standard curve for Human TGF-β.

2.3.6 Determination Fasting Blood Glucose Concentration

2.3.6.1 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 that glucose is oxidized to D-gluconate by the glucose oxidase (GOD) with the formation of hydrogen peroxide, in the presence of Peroxidase (POD) a mixture of phenol and 4-amino antipyrine (4-AA) is oxidized by hydrogen peroxide, to form a red quinone imine dye proportional to the concentration of glucose in the sample(Barham and Trinder, 1972). As the reactions below reveal:

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

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

2.3.6.2 Composition of the Reagents:

Table 2-6: 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).

2.3.6.3 Procedure of the kit:

1. Reagents and samples were brought to room temperature.

2. Into labeled tubes was Pipetted:

TUBES	Blank	Sample	CAL. 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.

2.3.6.4 Calculation of the result:

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

- Standard concentration = 5.56mmol/L.

2.3.7 Determination of Glycated Hemoglobin (HbA1c):

2.3.7.1 Principle:

The level of HbA1c among total hemoglobin was determined optically through a series of biochemical and enzymatic reactions(Weykamp, 2013). 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 → HbA0 + HbA2 + HbA1c

Step 2 : HbA1c + protease → glycated Val – His + other residues

Step 3 : Glycated Val – His + FPOX → H2O2

Step 4 : H2O2 + POD + dye → color change

As shown in Figure 2.9.

Figure (2.9): Assay principle for determination of HbA1c. FPOX: Fructosyl – peptide Oxidase, POD: Peroxidase.

2.3.7.2 Reagent Composition:

Table 2-7: 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.

2.3.7.3 Procedure:

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

2.3.7.4 Calculation:

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

2.3.8 Determination of Blood Urea:-

2.3.8.1 Principle:

By using an enzymatic colorimetric method with a commercially available kit, 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 salicylate in presence of sodium nitroprusside as a coupling agent to yield a green chromophore. the intensity of the color formed is proportional to the concentration of urea in the sample(Traynor *et al.*, 2006). As the following reactions:

$$\text{Urea} + H_2O \xrightarrow{\text{ureas}} 2NH3 + Co2$$

$$NH4+ + \text{Salycilate} + NaClO \xrightarrow{\text{NITROPRUSSIDE}} \text{Indophenol} + NaCl$$

OH-

2.3.8.2 Reagents Composition:

Table 2.8: 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).

2.3.8.3 Reagent Preparation:

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

2.3.8.4 Procedure:

1. Reagents and samples were brought to room temperature.

2. Into labeled tubes was pipetted:

TUBES	Blank	Sample	CAL. Standard
Working reagent	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. Pipetted:

R3	1.0 mL	1.0 mL	1.0 mL

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

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

2.3.8.5 Calculation:

$$\text{urea (mmol/L)} = \frac{Sample\ absorbance}{Standard\ absorbance} \times \text{Standard concentration}$$

- 8.33mmol/L= Standard concentration.

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).

2.3.9 Determination of Serum and urine creatinine:

2.3.9.1 Principle:

By using the kinetic colorimetric method with a commercially available kit, serum creatinine was determined. The principle that is based upon a modification of the original picrate reaction (Jaffe reaction) creatinine under alkaline conditions reacts with picrate ions forming a reddish complex. The formation rate of the complex measured through the increase of absorbance in a prefixed interval of time is proportional to the concentration of creatinine in the sample(Delanghe and Speeckaert, 2011).

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

2.3.9.2 Reagents Composition:

Table 2-9: Reagents for measuring serum and urine creatinine levels.

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

2.3.9.3 Reagent Preparation:

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

2.3.9.4 Procedure:

1. Working reagents, samples, and standards were brought to reaction temperature(37ºC).

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

3. Into cuvette was Pipetted:

Working reagent	1.0mL
Sample or standard	100µL

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).

- A urine sample was diluted at 1:50 with distilled water before the assay. The result was multiplied by 50.

2.3.9.5 Calculation:

The concentration of creatinine in serum or plasma:

$$\frac{(A2-A1)Sample}{(A2-A1)Standard} \times \text{C. standard (mg/dl creatinine)}$$

The concentration of creatinine in urine:

$$\frac{(A2-A1)Sample}{(A2-A1)Standard} \times \text{C. standard} \times 50$$

- **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).

Urine:

- male (63-166) mg/dl
- female (47-110) mg/dl

2.3.10 General Urine Examination:

This test was done by following the conventional common method. The aim of doing this simple test was to exclude all cases with abnormal urine findings (erythrocytes, leukocytes, and active sediments).

2.3.11 Determination of microalbuminuria and ACR

2.3.11.1 Semi-quantitative method:

Microalbuminuria was tested by dip strips(specific strips for testing microalbumin and other parameters in human urine) that are reacted with Sulfonephthalein 2.2% as a reagent to yield sky-like color ranging from mild, moderate to severe cases which are considered a semi-quantitative measurement 30-80 mg/L, 81-150 mg/L and >150 mg/L respectively. The procedure is used only when the sample was well mixed with uncentrifuged urine and which should not be older than two hours.

The test strip was immersed in the urine (approximately 2 seconds) so that all reagent areas are covered**.** The reagent areas were compared to the strip with the corresponding charts of color fields on the container 60 seconds after immersion(Naser *et al.*, 2015)**.**

2.3.11.2 Quantitative Method :

Turbidimetric test for the quantitative determination of microalbumin in human urine.

- **Principle of the Assay:**

Albumin in sample or standard reacts with anti-albumin antibodies in the reagent. The absorbance increase caused by the resulting aggregates is measured by a turbidimetric end-point method (Camblaao and Lievens, 1988).

- **Reagents Content:**

R1 **Buffer.** phosphate buffer, polyethylene glycol, sodium azide.
R2 **Antiserum.** Anti-human albumin.
STD **Microalbumin standard**.

- **Assay Procedure:**

Table 2.10: Pipetting method for measuring urinary albumin.

Reagents and samples were brought to room temperature. Zero the instrument with distilled water.	
Into cuvette was Pipetted:	
R1 **Buffer**	900 µL
STD Or sample	60 µL

The cuvette was mixed and inserted into the instrument, and the absorbance(A1) was read at 340nm.	
R2 **Antiserum**	150 Ml
The cuvette was mixed and incubated for 5 min at room temperature, and the absorbance(A2) was read at 340nm.	

❖ Calculation:

The difference between A2 and A1 was calculated which is represented by (ΔA = A2 – A1).

Normal reference values: (0-25) mg/l according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

❖ Urinary Albumin: Creatinine (A: C) Ratio:-

ACR is a ratio between two measured tests:

1- Measurement of creatinine in urine.
2- Measurement of microalbumin in urine.

The ratio was measured as follows:-

$$\textbf{ACR} = \frac{\text{Urine albumin (mg/dl)}}{\text{Urine creatinine (g/dl)}}$$

2.3.12 Measurement of Glomerular Filtration Rate (eGFR):

GFR is the best overall index of kidney function. Normal GFR varies according to age, sex, and body size, and declines with age. The National Kidney Disease Education Program (NKDEP) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Kidney Foundation (NKF), and the American Society of Nephrology (ASN) recommend estimating GFR from serum creatinine (Levey and Stevens, 2010)

eGFR was calculated by using two common equations: Modification of Diet in Renal Disease (MDRD) and Chronic Kidney Disease Epidemiology

Collaboration (CKD–EPI). The NKF recommends using the CKD – EPI creatinine equation to estimate GFR (Stevens and Levey, 2007)

- MDRD Equation: (Levey *et al.*, 2007)

GFR = 175 x (Standardized Scr)-1.154 x (age)-0.203 x (0.742 if female) x (1.210 if African American)

GFR is expressed in mL/min/1.73 m2.

SCR is standardized serum creatinine expressed in mg/dL.

Age is expressed in years.

- CKD – EPI Creatinine Equation(2009): (Levey *et al.*, 2009)

GFR = 141 x min(SCr /*k*, 1)*a* x max(SCr /*k*, 1)-1.209 x 0.993Age x 1.018 (if female) x 1.159 (if black)

GFR is expressed in mL/min/1.73 m^2.

SCR is standardized serum creatinine expressed in mg/dL.

K = 0.7 (female) or 0.9 (male)

α = -0.329 (females) or -0.411 (males)

Min = indicated the minimum of S_{Cr}/κ or 1

max = indicates the maximum of S_{Cr}/κ or 1

age = years

2.4 Statistical 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 chi-square and fisher-exact tests were used to find the association between categorical variables. Pearson correlation coefficient was used to assess the relationship between two continuous variables. The receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of TGF-Beta, SMAD2, and SMAD3 in 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. A *p*-value of ≤ 0.05 was considered significant.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Clinical and Predictors Characteristics of the Groups Investigated:

3.1.1 The study's demographic and clinical characteristics

3.1.1.1 Control group

The control group consisted of 60 healthy people of both sexes, with 33 (55%) males and 27 (45%) females as shown in Figure (3-2). Their ages ranged from 35 to more than 55 years, and biochemical variables such as Fasting Blood Glucose (FBG), glycated hemoglobin (HbA1c), blood urea, serum creatinine, albumin-creatinine ratio (ACR), and Microalbuminuria, The demographic features of the control group are shown in Table (3-1).

3.1.1.2 patient's group

The patient's group consisted of 60 patients of both sexes, with 36 (60%) males and 24 (40%) females as shown in Figure (3-2). Their ages ranged from 35 to more than 55 years, and biochemical variables such as fasting glucose (FBG), glycated hemoglobin (HbA1c), blood urea, serum creatinine, albumin-creatinine ratio (ACR), and Microalbuminuria, The demographic features of the patient's group are shown in Table (3-1).

The patients were separated into three age groups, with the first group (35-45) years having 14 (23.3%), the second group (45-55) years having 42 (70.0%), and the third group (>55) years having 4 (6.7%), as shown in Table (3-1) and Figure (3-1).

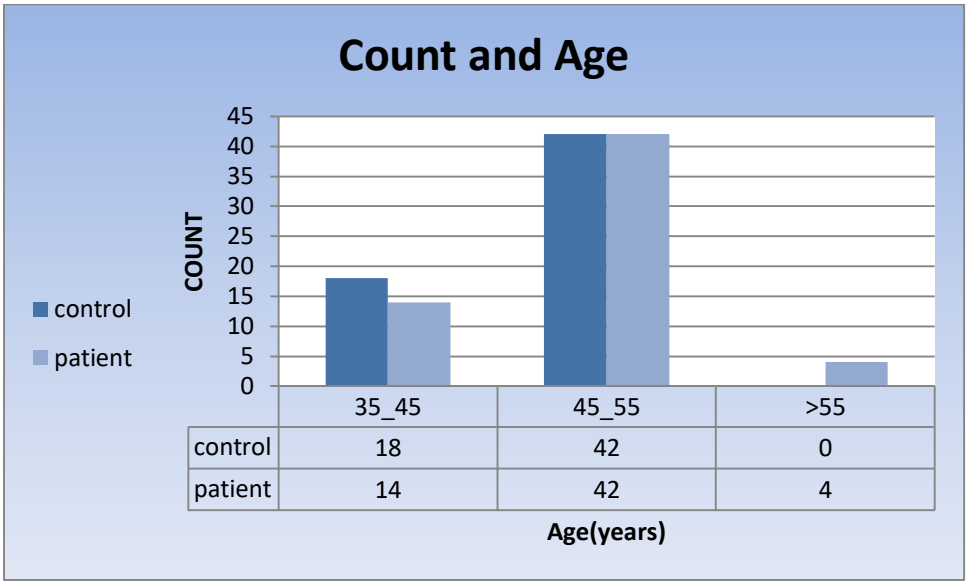
As well as separated into four groups based on BMI: the first group (<18.5) and its number 0, the second group (18.5-24.9) and its number 7 (11.7%), the third group (25-29.9) and its number 32 (53.3%), and the fourth group (>30) and its number 21 (15%), as shown in Table (3-1) and figure (3-3).

It was also separated into three groups based on the duration of the disease: the first group (5 years) had a number of 9 (15%), the second group (5-10 years) had a number of 29 (48.3%), and the third group(>10) had a number of 22 (36.7%), as shown in Table(3-1) and figure (3-4).

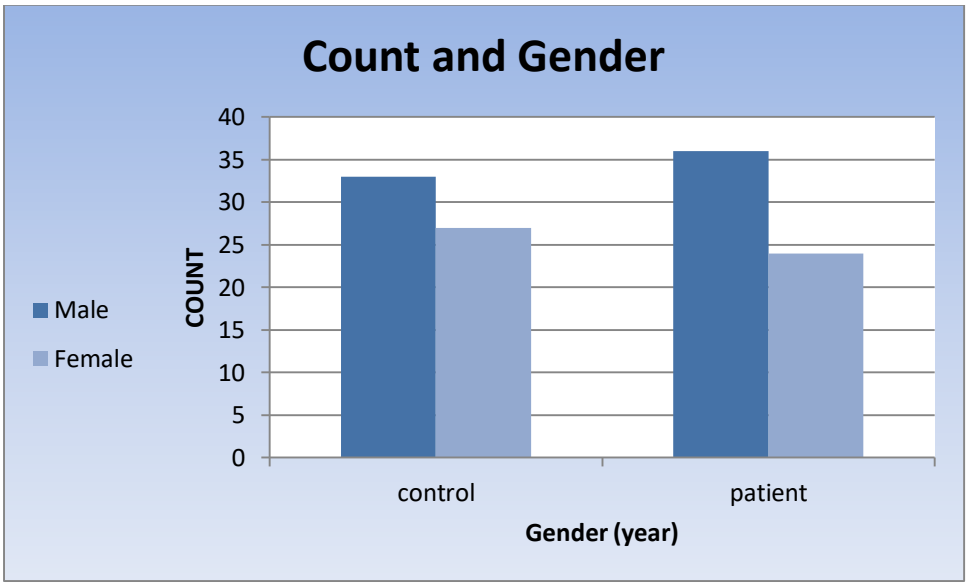
They were also separated into three groups based on the albumin-creatinine ratio (ACR): the first group (<30), its number is 20 (33.3%), the second group(30-299), its number is 20 (3.33%), and the third group(300), its number is 20 (33.3%), as shown in Table(3-1) and figure (3-5).

Table 3-1: Characteristics of diabetic nephropathy and control groups in terms of demographics:

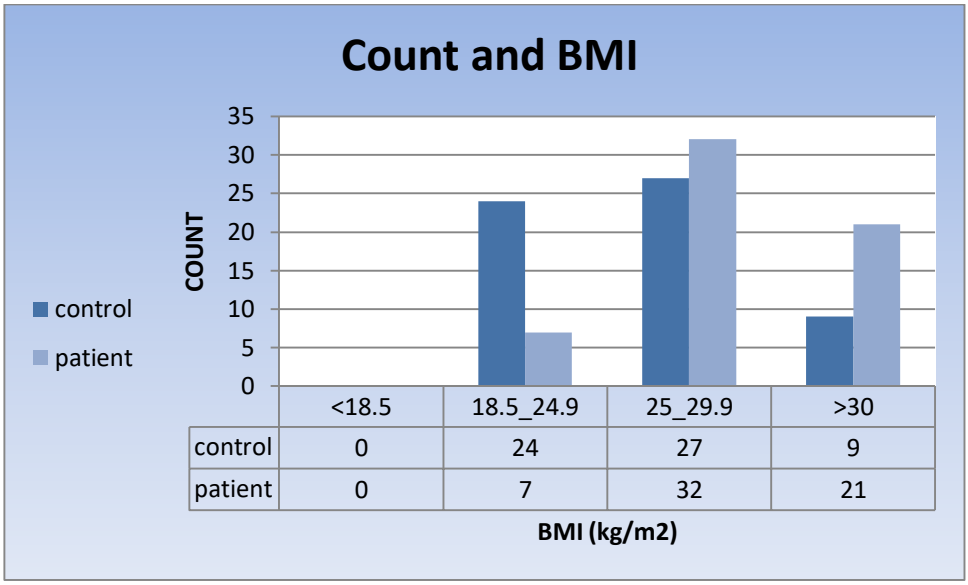
Variables	Control group	T2DM
Total number	60 (100%)	60 (100%)
Age(years)		
35-45	18 (30%)	14 (23.3%)
45-55	42 (70%)	42 (70.0%)
>55	0	4 (6.7%)
sex		
Male	33 (55%)	36 (60%)
Female	27 (45%)	24 (40%)
BMI(kg/m^2)		
<18.5	0	
18.5-24.9	24 (40%)	7 (11.7%)
25-29.9	27 (45%)	32 (53.3%)
>30	9 (15%)	21(35%)
Duration(years)		
<5	0	9 (15.0%)
5-10	0	29 (48.3%)
>10	0	22 (36.7%)
ACR(mg/g)		
<30	60 (100%)	20 (33.3%)
30-299	0	20 (33.3%)
>300	0	20 (33.3%)



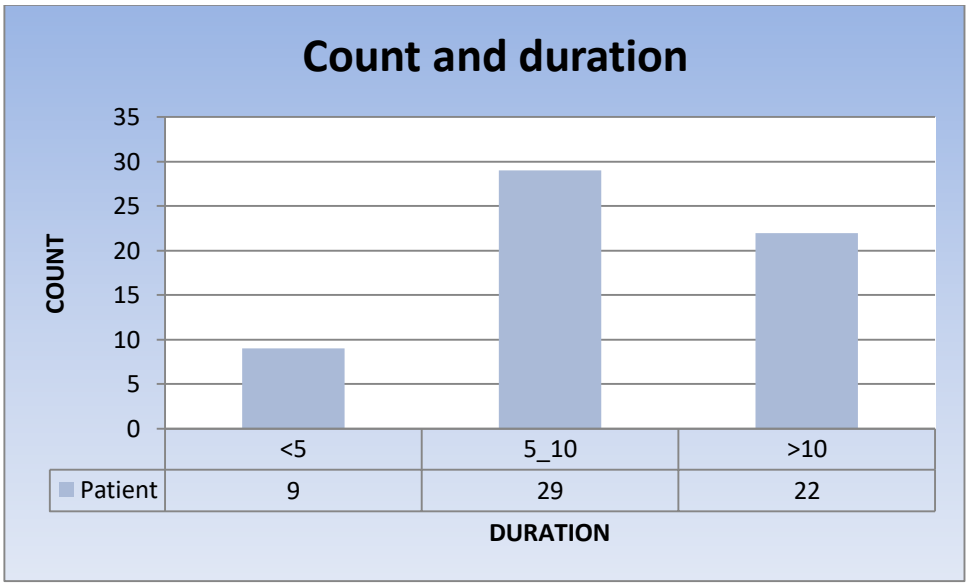
Figure(3-1): Distribution of control and patients among to the Age



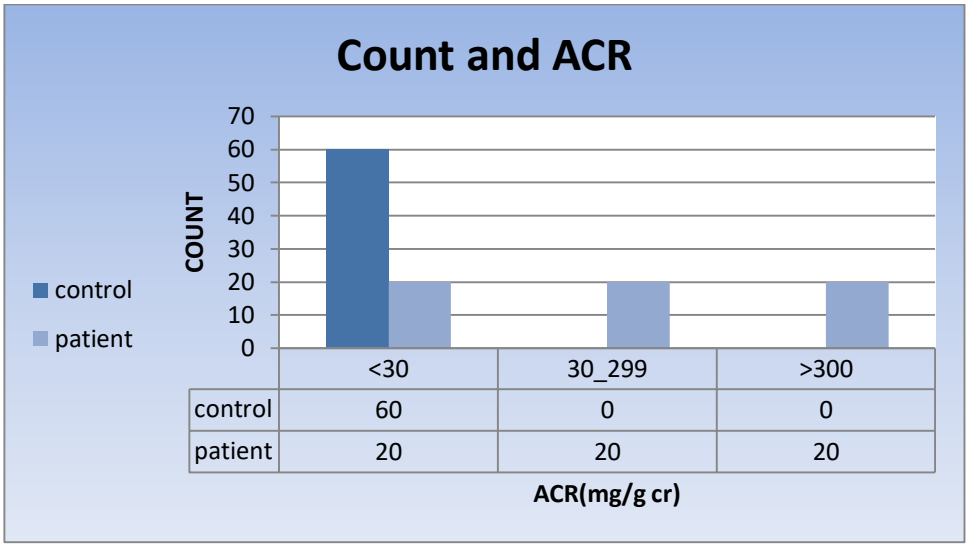
Figure(3-2): Distribution of control and patients among to the Gender



Figure(3-3): Distribution of control and patients among to the BMI



Figure(3-4): Distribution of patients among to the duration of DM



Figure(3-5): Distribution of control and patients among to the Albumin creatinine ratio

3.1.2 Age and BMI:

The results presented in Table (3-2) for age and BMI showed that there was no significant difference (P-value >0.05) between the studied groups (T2DM with normoalbuminuria, T2DM with microalbuminuria, T2DM with macroalbuminuria 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 age and BMI according to studied groups.

	T2DM			
Variables	Control group N=60 Mean ±SD	Normalbuminuria N=20 Mean ±SD	Microalbuminuria N=20 Mean ±SD	Macroalbuminuria N=20 Mean ±SD
AGE (year)	48±4.46[a]	50±6.6[a]	52±5.5[a]	53±9.4[a]
BMI(kg/m^2)	26.2±3.5[a]	28.6±3.2[a]	28.4±3.0[a]	28.1±3.5[a]

Data were expressed as mean ± SD .p-value > 0.05 considered not significant, by one-way ANOVA test. Similar letters (a) mean no significant difference in age and BMI between studied groups (Tukey's).

3.1.3 Gender

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

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

gender	Study groups				total	x^2	p-value
	Control	Normoalbuminuria	Microalbuminuria	Macroalbuminuria			
Male	33%	10%	12%	14%	69%	1.8	0.6
Female	27%	10%	8%	6%	51%		
total	60%	20%	20%	20%	120%		

The results presented in Table (3-3) for gender showed that there was no significant difference (P-value >0.05) between the studied groups (T2DM with normoalbuminuria, T2DM with microalbuminuria, T2DM with macroalbuminuria, and the control group).

3.1.4 Duration of type 2 Diabetes Mellitus:

DM duration was significantly (P-value < 0.001) longer in T2DM Patients with macroalbuminuria as compared T2DM Patients with microalbuminuria and T2DM Patients with normoalbuminuria. The mean duration and standard deviation(SD) were (6.6±2.88),(8.9±3.8),(13.7±5.4) respectively. As shown in Table (3-4).

Table 3-4: Duration of Diabetes in the T2DM patient's groups.

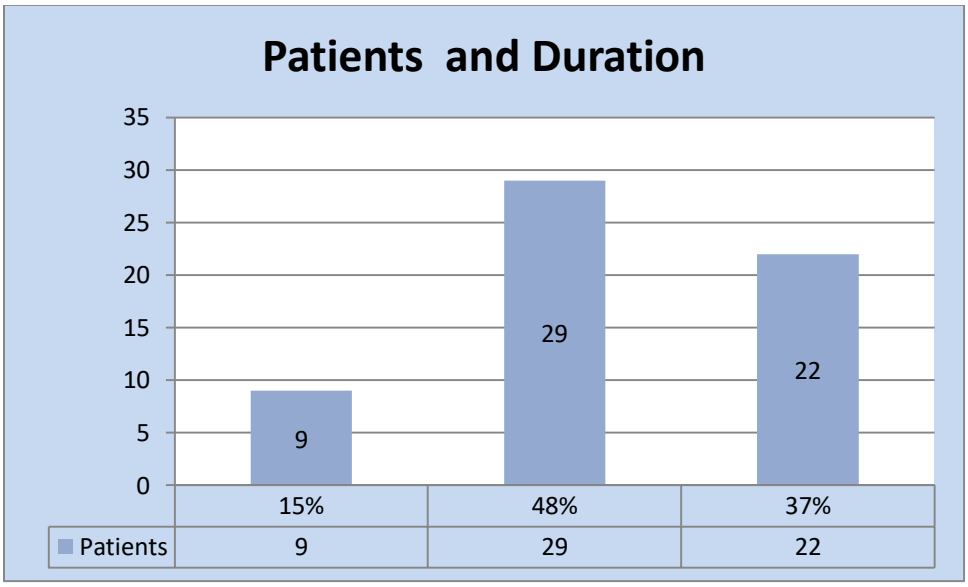
Duration (years)	**T2DM**		
	Normoalbuminuria N=20 Mean±SD	Microalbuminuria N=20 Mean±SD	Macroalbuminuria N=20 Mean±SD
	6.6 ±2.88[a]	8.9± 3.8[a]	13.7± 5.4[b]

Data were expressed as mean ± SD .p-value <0.05 is significant, by one-way ANOVA test. Different letters (a and b) mean a significant difference between studied groups, Similar letters mean no significant difference between studied groups (Tukey's).

The duration of DM has a significant impact on the development of diabetic complications. Long-term diabetes is one of the common manifestations of diabetic kidney disease, according to American Diabetic Association criteria for microvascular complications (Association, 2020).

The present study agreed with (Indriani, Lestari and Dewantari, 2020) who show the frequency of macroalbuminuria was strongly associated with diabetes duration (P < 0.001). Another study (El-Ashmawy *et al.*, 2015) showed that the duration of diabetes was a predictor of nephropathy in T2DM patients and the development of nephropathy in diabetic patients was linked to the duration of diabetes mellitus. This is because a long period of hyperglycemia-induced tissue damage leads to the progression of the disease. The duration of DM from history is not always the true duration of type II DM as many had a delay in diagnosis(Porta *et al.*, 2014).

Figure (3-6) shows the distribution of patients according to the duration of diabetes including (≤ 5 years, 5-10 years, and >15 years). A majority (No.= 29, 48%) of patients presented with a duration of DM between 5-10 years.



Figure(3-6): Distribution of patients among to the Duration of DM.

3.2 Parameters:

3.2.1- Fasting Blood Glucose and HbA1c:

The mean concentrations of FBG and HbA1c levels were significantly increased in T2DM patients with macroalbuminuria, T2DM patients with microalbuminuria, and T2DM patients with normoalbuminuria compared with the control group, as shown in Table (3-5).

Table (3-5): The mean differences and multiple comparisons of study variables according to studied groups

	T2DM			
Biochemical parameters	Control group N=60 Mean±SD	Normoalbuminuria N=20 Mean±SD	Microalbuminuria N=20 Mean±SD	Macroalbuminuria N=20 Mean±SD
FBG(mmol/l)	4.4±0.68 **a***	8.5±1.01 **b***	12.1±1.32 **c***	17.5±2.71 **d***
HbA1c(%)	4.3± 0.45**a***	7.7± 0.47**b***	8.9 ±0.51**c***	11.7 ±1.84**d***

Data were expressed as mean ± SD .p-value <0.05 is significant, by one-way ANOVA test. Different letters (a,b,c and d) mean a significant difference between studied groups, Similar letters mean no significant difference between studied groups (Tukey's).

According to the results of the present study that shown the levels of FBG and HbA1c were significantly elevated in T2DM patients with macroalbuminuria, microalbuminuria and normoalbuminuria compared with the healthy control. These results support previous research that suggests hyperglycemia is the driving force behind the development of DN (Sun *et al.*, 2013). 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 (Fakhruddin, Alanazi and Jackson, 2017). Furthermore, elevated HbA1c has been linked with the production of microangiopathy in diabetes due to the special affinity for oxygen, resulting in tissue anoxia (Kundu *et al.*, 2013).

The high concentration of HbA_{1C} 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 (Sherwani *et al.*, 2016).

In this study, poor glycemic control (elevated HbA1c) increased in T2DM with macroalbuminuria and microalbuminuria and this is in agreement with the study done by (Al-Eisa *et al.*, 2017) who reported a poor glycemic control is a risk factor of diabetic nephropathy, the finding of a higher mean HbA1c in patients with macroalbuminuria and microalbuminuria in this study supports the fact that there are most likely due to poor glycemic control.

3.2.2- Renal Function tests.

The finding demonstrated a significant difference in urea, creatinine, GFR, and ACR between T2DM patients with macroalbuminuria and microalbuminuria as compared with T2DM with normoalbuminuria and the control group, As shown in the Table (3-6).

Table (3-6): Renal Function Parameters in studied groups.

	T2DM			
Biochemical parameters	Control group N=60 Mean±SD	Normalbuminuria N=20 Mean±SD	Microalbuminuria N=20 Mean±SD	Macroalbuminuria N=20 Mean±SD
Blood urea(mmol/l)	4.7± 0.80^{a*}	5.4 ±1.16^{a*}	8.2± 1.38^{b*}	10.1± 2.90^{c*}
Serum creatinine(μmol/l)	63.0± 8.5^{a*}	76.0± 21.8^{ab*}	78.5 ±16.5^{b*}	120.5± 21.0^{c*}
eGFR (ml/min/1.73/m^2)	92.2 ±5.0^{a*}	88.9± 14.6^{a*}	86.9 ±17.1^{a*}	56.5 ±14.2^{b*}
ACR (mg/g)	10.5± 3.2^{a*}	15.6± 6.4^{a*}	132.4 ±63.6^{b*}	585.6± 167.2^{c*}

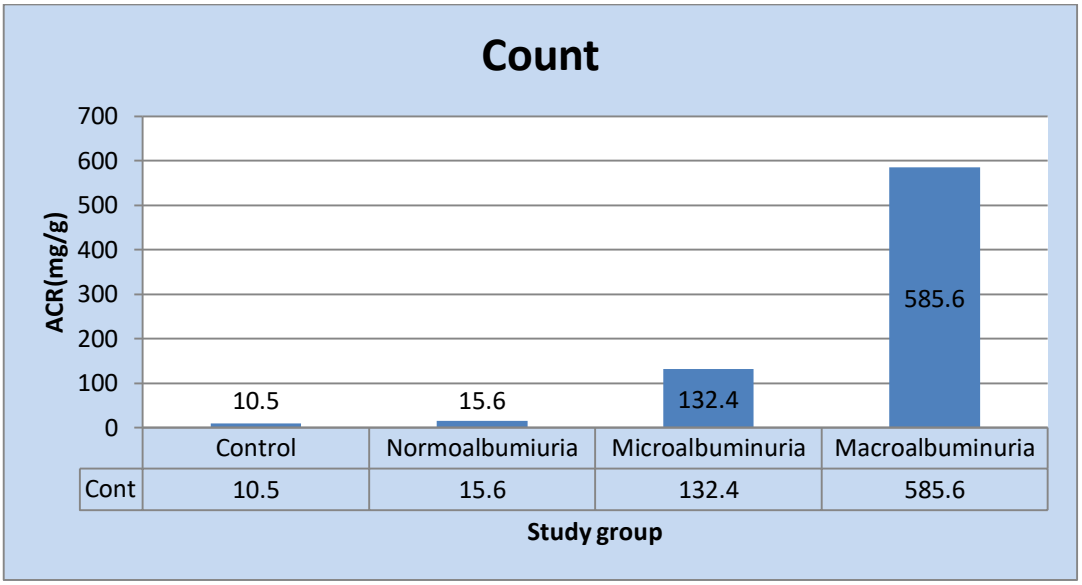
Data were expressed as mean ± SD .p-value <0.05 is significant, by one-way ANOVA test. Different letters (a,b, and c) mean a significant difference between

studied groups, Similar letters mean no significant difference between studied groups (Tukey's).

Blood tests for urea and creatinine are the simplest way to monitor renal function. This work shows a significant difference in blood urea and creatinine between the T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria compared with normoalbuminuria and control groups. It is important to note that urea and creatinine are not useful parameters in the early detection of DN. Urea and creatinine were elevated in patients as the disease progressed to late stages (Kiconco, Rugera, and Kiwanuka, 2019).

The present study showed a significant difference in eGFR between the T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria compared with normoalbuminuria and control groups. It is important to note that eGFR is not a useful parameter in the early detection of DN. It is decreased in CKD and declines in renal in the late stage of the disease. According to this study, the result was agreed with the study of (Pavkov *et al.*, 2013).

Albumin creatinine ratio (ACR) (Mean ± SD) in T2DM with macroalbuminuria, T2DM with microalbuminuria, T2DM with normoalbuminuria, and control groups were (585.6± 167.2), (132.4 ±63.6), (15.6± 6.4),(10.5± 3.2) respectively with highly significant difference P(<0.001). Urinary ACR was significantly elevated in T2DM with macroalbuminuria patients and T2DM with microalbuminuria as compared to those with normoalbuminuria and control group P(<0.001). whereas there was no significant difference between T2DM with normoalbuminuria and control group P (0.858).



Figure(3-7): The mean differences of ACR among to studied groups

Microalbuminuria was considered the conventional biomarker for detection and prediction of DN in T1DM, and T2DM and remains the standard marker for early diagnosis of DN and albuminuria is the strongest predictor of ESRD in DM patients (Uwaezuoke,2017).

The early microalbuminuria found in diabetes patients may be partly due to injury caused by hyperglycemia. However, renal damage in DN was described by alterations in glomerular permeability and structure. The glomerular wall consists of 3 layers: endothelial cells, epithelial cells, and basement membrane. The basement membrane, which removes proteins based on their size and charge, is responsible for a large portion of filtration selectivity(Maestroni and Zerbini, 2018). However, in DN, the permeability barrier is damaged, resulting in proteinuria of plasma proteins that are usually not freely filtered through the glomerulus, such as albumin. Structural modifications in diabetic kidney disease include the deposition of mesangial matrix and thickening of the basement membrane in the glomeruli, as well as renal tubular hypertrophy and related basement membrane alterations in the tubulointerstitial with tubolo-interstitial fibrosis(Moresco *et al.*, 2013).

The present study was in agreement with the study of (Taqwaa *et al)*. which demonstrated that urinary ACR levels were significantly increased in the macroalbuminuria and microalbuminuria groups as compared with other groups

and it has good diagnostic power in predicting the severity of nephropathy in patients with diabetes (Sueud *et al.*, 2019).

3.2.3 Novel Biochemical Parameters:

The finding demonstrated a significant difference in Serum TGF-beta, SMAD2, and SMAD3 between T2DM patients with macroalbuminuria and microalbuminuria as compared with T2DM with normoalbuminuria and the control group, As shown in the Table (3-7).

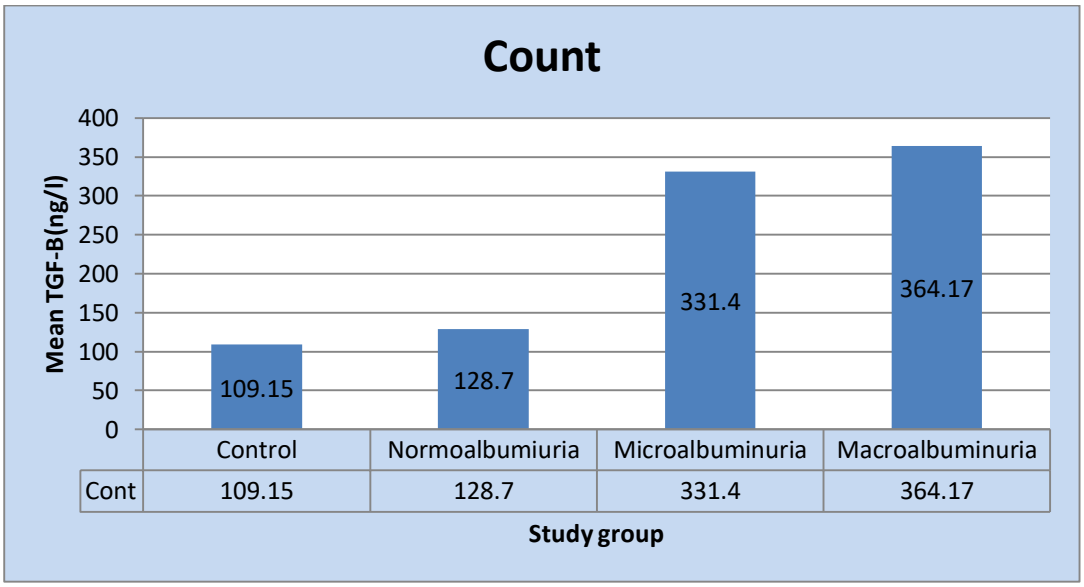
Table (3-7): Biochemical parameters according to studied groups

Biochemical parameter	**T2DM**			
Study groups	**Control group N=60** Mean±SD	**Normoalbuminuria N=20** Mean±SD	**Microalbuminuria N=20** Mean±SD	**Macroalbuminuria N=20** Mean±SD
TGF-B (ng/l)	109.1±14.8^{a*}	128.7±32.9^{a*}	331.4±101.0^{b*}	364.1±98.1^{b*}
SMAD2 (ng/ml)	2.20± 0.60^{a*}	2.60± 0.80^{a*}	3.60± 1.01^{b*}	4.11±1.06^{b*}
SMAD3 (ng/ml)	1.76± 0.42^{a*}	2.04 ±0.67^{a*}	3.98 ±1.27^{b*}	7.21±1.87^{c*}

Data were expressed as mean ± SD .p-value <0.05 is significant, by one-way ANOVA test. Different letters (a,b, and c) mean a significant difference between studied groups, Similar letters mean no significant difference between studied groups (Tukey's).

3.2.3.1 Transforming Growth Factor-Beta (TGF- β) :

Serum TGF-beta levels (Mean ±SD) in the T2DM with normoalbuminuria, T2DM with microalbuminuria, T2DM with macroalbuminuria, and control groups were (128.7±32.9), (331.4±101.0), (364.1±98.1),(109.1±14.8) respectively with highly significant difference P(<0.001), as shown in figure (3-8).



Figure(3-8): Serum TGF-beta level among to studied groups.

The most common microvascular consequence of diabetes is DN. Diabetes affects 20% to 40% of T2DM patients and might progress to ESRD, impacting T2DM patients' mortality and morbidity. Early identification, however, is vital in improving clinical care, and therapeutic intervention from the earliest stages of DN is critical to avoid progression to ESRD and dialysis (Balducci *et al.*, 2014).

Microalbuminuria, also known as ACR (30-299 mg/g creatinine), has been considered the gold standard diagnostic and prognostic biomarker for the past three decades. It was the first and most widely used clinical measure of DN and is linked to cardiovascular risk in T2DM patients (Association, 2020). Microalbuminuria, on the other hand, may not be a sensitive or specific predictor of DN due to several limitations. First, only approximately 30 % of patients with incipient nephropathy(microalbumin) progress to macroalbuminuria after ten years of follow-up (Rossing, Hougaard, and Parving, 2005). Second, some patients do not progress to overt nephropathy but stay at

microalbuminuria or even regress to normoalbuminuria (Satirapoj, 2018). Third, Some patients without microalbuminuria display advanced renal pathological changes, indicating that microalbuminuria may not be an optimal marker for "early" detection of DN. Furthermore, some patients may be presented with decreased glomerular filtration rate whereas normoalbuminuria, indicating that albuminuria is not a perfect marker for early detection (Roux *et al.*, 2018). For these reasons, it's crucial to look into new biomarkers that can help with the early detection of DN. At this time, a lot of attention was gained to inflammation biomarkers because they were considered sensitive and specific biomarkers for the early detection of kidney damage (Vijay *et al.*, 2018). One of these biomarkers is the transforming growth factor(TGF-beta) plays a critical role in DN.

The results of the present study demonstrated that serum TGF-β levels were significantly higher in T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria as compared to normoalbuminuria and normal control groups. These results suggest that TGF-β may be involved in the development of DN. This can be explained on the basis that TGF-β upregulation in diabetes could be attributed to elevated glucose, AGEs, PKC, and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. The high glucose induces serine/threonine-protein kinase/protein kinase B (Akt/PKB) phosphorylation in a protein kinase C-β (PKC-β)-dependent manner resulting in the upregulation of TGF-β1 transcription(Chuang *et al.*, 2015). The present work was in agreement with(the El Mesallamy *et al.*, 2012) study.

On the other hand, it was observed that there was a significant elevation in serum TGF-β1 values in diabetic microalbuminuric and macroalbuminuric groups in comparison with a diabetic normoalbuminuric group which comes in agreement with the significant positive correlation between TGF-β and ACR. The prolonged overexpression of TGF-β is associated with the development of severe glomerulonephritis and glomerulosclerosis (El Mesallamy *et al.*, 2012).

TGF-β is one of the most important Extra Cellular Matrix (ECM) regulators, both as a potent inducer of ECM synthesis and an inhibitor of the degradation of ECM components, by directly suppressing matrix metalloproteinases and inducing tissue inhibitors of metalloproteinases, resulting in a net accumulation of ECM, Studies have

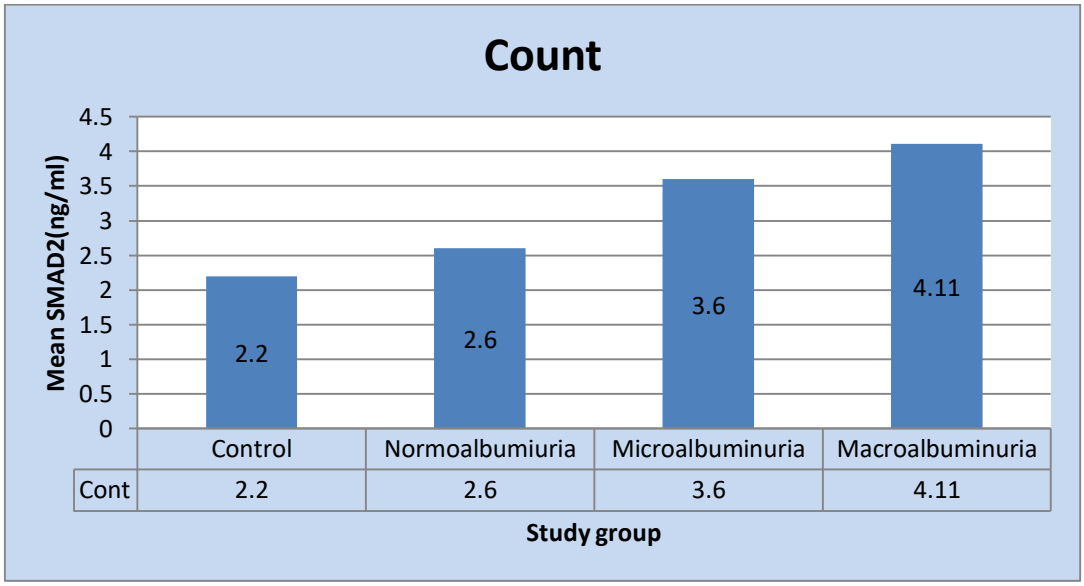
also shown that TGF-β is a key mediator in experimental models of diabetic kidney disease and patients with diabetic nephropathy (Lee, Kim and Choi, 2015). An overactive TGF-β signaling pathway has been implicated as a critical profibrotic factor in the progression of chronic kidney disease in human DN. In animal studies, TGF-β neutralizing antibodies and TGF-β signaling inhibitors were effective in ameliorating renal fibrosis in DN (Zhao, Zou and Liu, 2020).

The TGF-β signaling pathway is activated in DN, and the inhibition of TGF-β attenuates fibrosis in animal models of diabetes (Meng, 2019). Pathogenic stimuli in DKD activate TGF-β signaling. Angiotensin-II, which was elevated in mesangial cells and glomerular endothelial cells, has been implicated in activating TGF-β by the generation of ROS from nicotinamide adenine dinucleotide phosphate oxidases or by activating protein kinase C- and p38 MAPK-dependent pathways. Hyperglycemia, mechanical stretch, and advanced glycation end products were found to upregulate TGF-β1 in DKD (Zhao, Zou, and Liu, 2020). TGF-β is a critical factor in the pathophysiological progression of DKD, having both pro-and anti-inflammatory properties (Sureshbabu, Muhsin, and Choi, 2016). The results observed were in agreement with the previous study was done by (Mou *et al.*, 2016) who demonstrated that TFG-β was significantly higher in three groups of type 2 diabetic patients(Normoalbuminuria, Microalbuminuria, and Macroalbuminuria) compared to the control group and that TGF-β values were increased in diabetic patients without early signs of glomerular injury. On the other hand, a study (by Liu and Desai, 2015) reported that the pathophysiology of DN is very complex, involving many molecules and abnormal cellular activities. Given the respective pivotal roles of TGF-β in inflammation, oxidative stress, and fibrosis during DN, found that the level of serum TGF-β was significantly elevated in diabetic patients with macroalbuminuria and diabetic patients with microalbuminuria as compared with the control subject. Suggesting that S.TGF-β is a potential marker of tubular damage and precedes the appearance of microalbuminuria as a marker of glomerular damage. Moreover, the present results agreed with the study(Elwi *et al.*, 2022) which reported that the mean of serum TGF-β was significantly elevated in T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria compared to both T2DM with normoalbuminuria and control subjects. Indicating that serum TGF-β may be an independent and reliable marker for early detection of DN.

Our data strongly support the hypothesis that hyperglycemia may trigger the activation of transforming growth factor-beta which in turn mediates progressive renal damage in type 2 DM. Increased serum transforming growth factor-beta may be useful as a marker of diabetic renal disease as it shows a close association with the parameters of renal injury in type 2 Diabetes Mellitus.

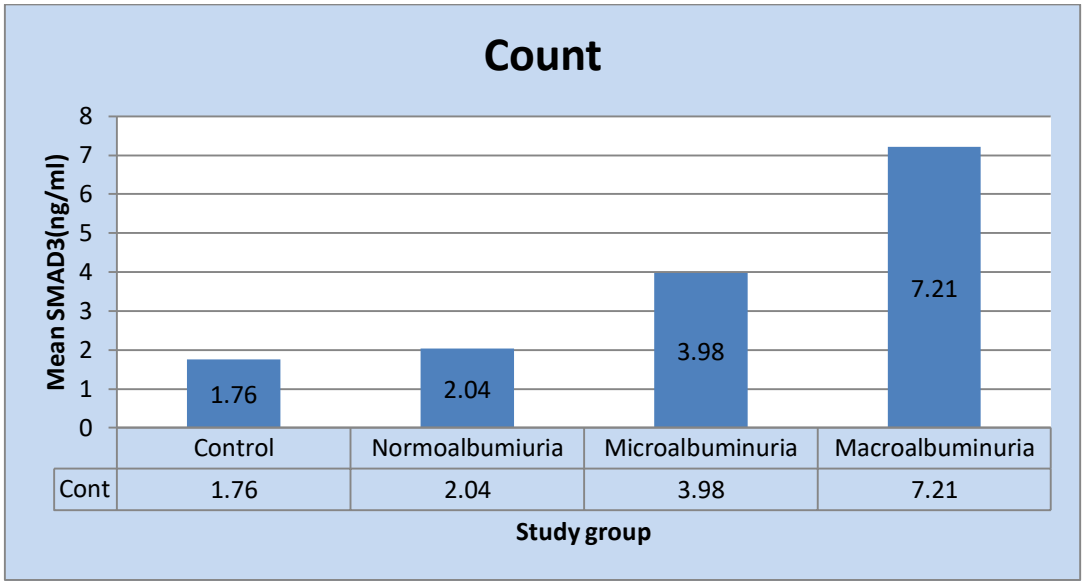
3.2.3.2 Serum Small Mother Against Decapentaplegic Homolog 2 (SMAD2) and Serum Small Mother Against Decapentaplegic Homolog 3 (SMAD3):-

Serum SMAD2 levels (Mean±SD) in T2DM with normoalbuminuria, T2DM with microalbuminuria, T2DM with macroalbuminuria, and control groups were (2.60±0.80),(3.60±1.01), (4.11±1.06), (2.20±0.60) respectively with highly significant difference P(<0.001), as shown in figure (3-9).



Figure(3-9) Serum SMAD2 level among to studied groups

On the other hand, serum SMAD3 levels (Mean ± SD) in T2DM with normoalbuminuria, T2DM with microalbuminuria, T2DM with macroalbuminuria, and control groups were (2.04 ±0.67), (3.98 ±1.27), (7.21 ±1.87), (1.76 ±0.42) respectively with highly significant difference P(<0.001), as shown in figure (3-10).



Figure(3-10): Serum SMAD3 level among to studied groups

The results of the present study pointed out a significant difference in the levels of SMAD2 in T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria as compared to those with normoalbuminuria and control groups. These results suggest that SMAD2 may be involved in the development of DN.

On other hand, SMAD3 levels were significantly increased in T2DM patients with macroalbuminuria as compared with T2DM patients with microalbuminuria, T2DM patients with normoalbuminuria, and the control group. Furthermore, there was a no significant difference between T2DM patients with normoalbuminuria compared with the control group in SMAD2. By comparison with SMAD2, there was a significant difference between T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria in SMAD3.

The TGF-β/SMAD pathway plays a critical role in renal fibrosis and also inflammation. Researchers have suggested the TGF-β/SMAD pathway as a potential therapeutic target for treating chronic kidney diseases(Lan and Chung, 2012). A study done by (Zuo *et al.*, 2019) showed that the TGF-β/SMAD pathway plays a critical role in the renal fibrosis process by promoting the expression of key components of the ECM by promoting the synthesis of adhesive proteins, collagen, and the proteoglycans found in extracellular matrix; it can also attenuate the decrease in protease synthesis, prevent decomposition of newly synthesized ECM, disrupt the balance between ECM synthesis and degradation, and accelerate the development of renal fibrosis. In rodents

with diabetic nephropathy, the intracellular SMAD pathway is significantly activated, transducing the TGF-β signal (Wang *et al.*, 2021).

The present study showed the levels of TGF-*β* and SMAD2/3 were higher in T2DM patients with macroalbuminuria and microalbuminuria than in the normal control group, indicating that the TGF-*β*/SMAD signaling pathway was activated during the development of diabetic kidney disease. In the TGF-β/SMAD signaling pathway, TGF-β binds to the TβRII receptor and phosphorylates it, and then forms a TGF-β complex by binding to the TβRI receptor. The complex further activates the downstream signaling proteins SMAD2 and SMAD3 and is finally transported to the nucleus and directly combines with DNA, regulating the regulation of transcription of target genes(Ren *et al.*, 2022). Also, the current results are in agreement with previous studies' pathway proteins (TGF-β and SMAD2/3) were significantly increased in the diabetic renal tissues (Bian *et al.*, 2022).

Importantly, the results of the present study revealed that the mean levels of TGF-β, SMAD2, and SMAD3 elevated with long diabetes duration as shown in table(3-8). That means as the duration is prolonged, the levels of these markers are important for detecting the prognosis of the disease.

Table (3-8): Levels of TGF-Beta, SMAD2, and SMAD3 with diabetic duration.

GROUP OF DURATION	**NO.**	**Biochemical parameter**		
	60	**TGF-B (ng/l)** Mean±SD	**SMAD2(ng/ml)** Mean±SD	**SMAD3(ng/ml)** Mean±SD
<5	9	269.9±168.3ab*	2.80±0.95ab*	2.94±1.70ab*
5-10	29	225.7±121.2a*	3.18±1.05b*	3.67±2.30b*
>10	22	341.3±107.4b*	4.03±1.09c*	5.98±2.36c*

3.3 Correlation Between Serum TGF-Beta, SMAD2, and SMAD3 with Other Variables in T2DM

3.3.1 Correlation Between Serum TGF-Beta, SMAD2, and SMAD3 with Diabetic Duration, Blood Urea, Serum Creatinine, eGFR, ACR, FBS, and HbA1c in T2DM Patients.

The data were evaluated using linear regression analysis to determine the correlation between the Duration of disease, Blood Urea, Serum Creatinine, eGFR, ACR, FBS, HbA1c, TGF-B, SMAD2, and SMAD3 in patients. As shown in Table (3-9).

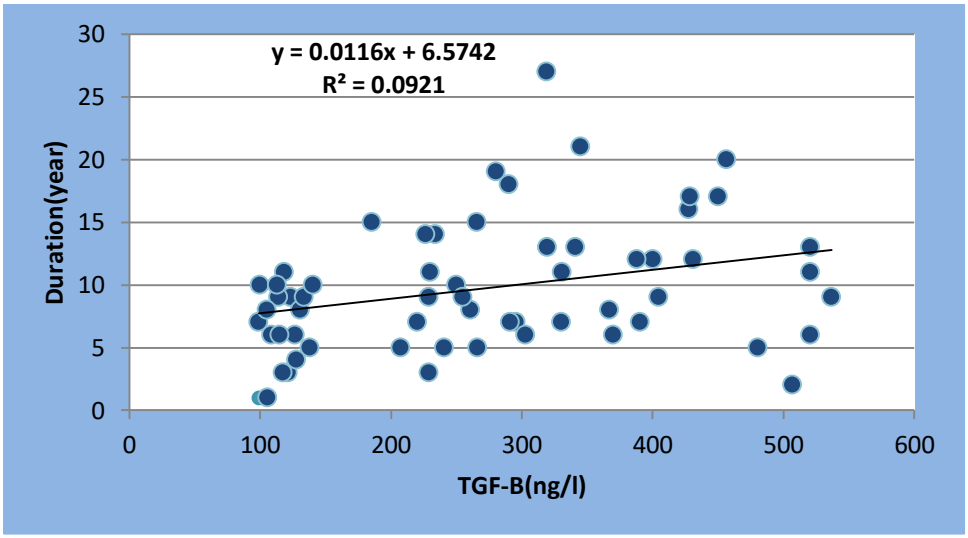
Table (3-9): Person correlation between TGF-Beta, SMAD2, and SMAD3 with other study variables in T2DM Patients.

Study variables	TGF-Beta (ng/l)		SMAD2 (ng/ml)		SMAD3(ng/ml)	
	r	P-value	R	P-value	r	P-value
TGF-B (ng/l)			**0.577****	**<0.001**	**0.640****	**<0.001**
SMAD2(ng/ml)	**0.577****	**<0.001**			**0.569****	**<0.001**
SMAD3(ng/ml)	**0.640****	**<0.001**	**0.569****	**<0.001**		
Diabetic Duration	**0.303***	**<0.01**	**0.330****	**<0.01**	**0.497****	**<0.001**
Blood Urea	**0.646****	**<0.001**	**0.500****	**<0.001**	**0.659****	**<0.001**
Creatinine	**0.442****	**<0.001**	**0.392****	**<0.001**	**0.641****	**<0.001**
eGFR	**-0.447****	**<0.001**	**-0.409**	**<0.001**	**-0.609**	**0.001**
ACR	**0.620****	**<0.001**	**0.548****	**<0.001**	**0.840****	**<0.001**
FBS	**0.593****	**<0.001**	**0.545****	**<0.001**	**0.772****	**<0.001**
HbA1c	**0.536****	**<0.001**	**0.478****	**<0.001**	**0.694****	**<0.001**

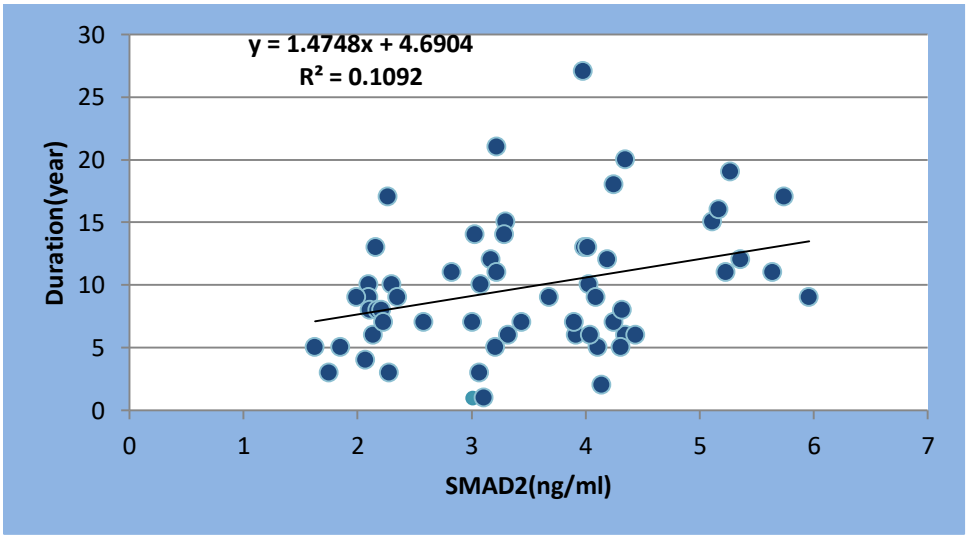
r: correlation coefficient

3.3.1.1 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite Diabetic Duration

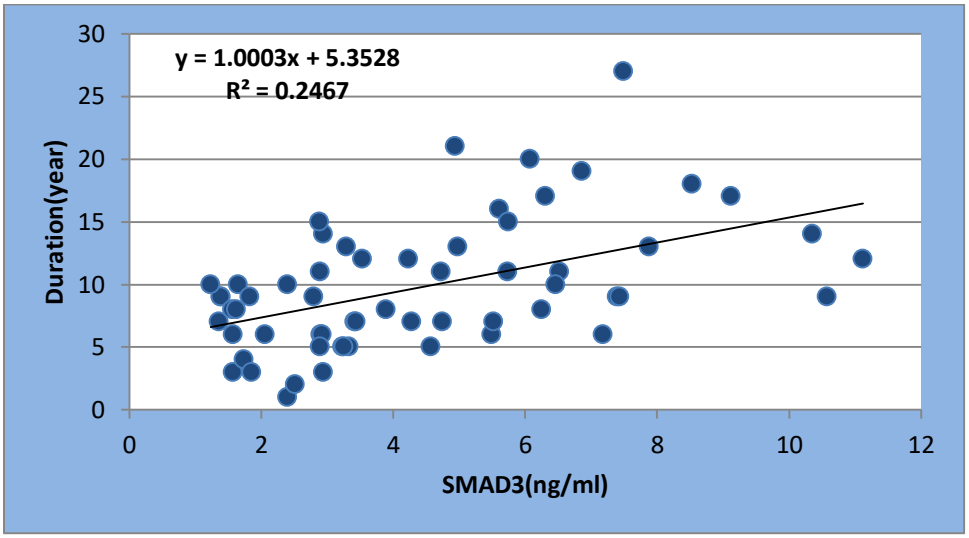
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with diabetic duration. The positive linear correlation in TGF-B (Figure 3-11) with a level of P<0.01 and a correlation coefficient of r=0.303. In contrast, SMAD2 positive linear correlation (Figure 3-12) at level P<0.01 and a correlation coefficient of r=0.330. while SMAD3 positive linear correlation (Figure 3-13) at level P<0.001 and a correlation coefficient of r= 0.497.



Figure(3-11): Correlation between TGF-BETA (ng/l) and Diabetic Duration(year) (r=0.303*, P<0.01)



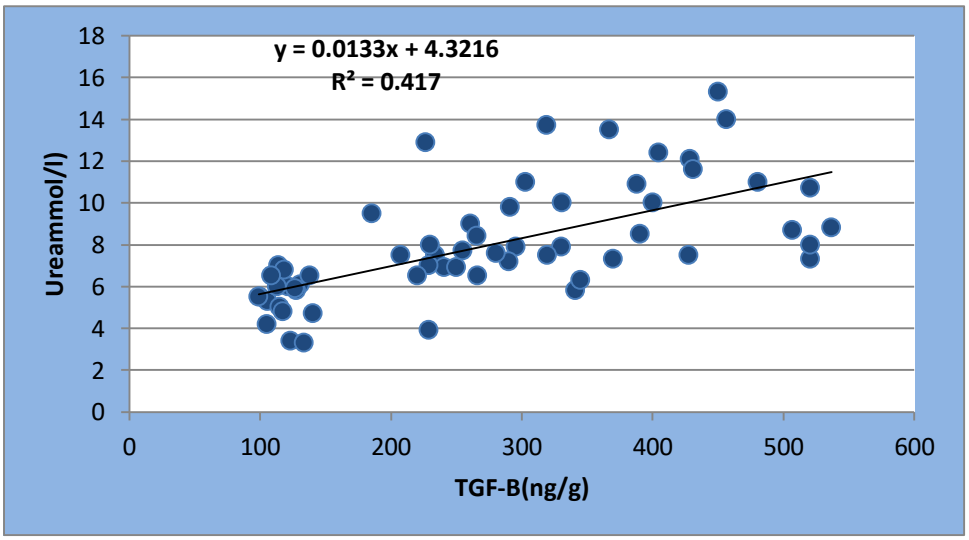
Figure(3-12): Correlation between SMAD2 (ng/ml) and Diabetic Duration(year) (r=0.330, P<0.01)**



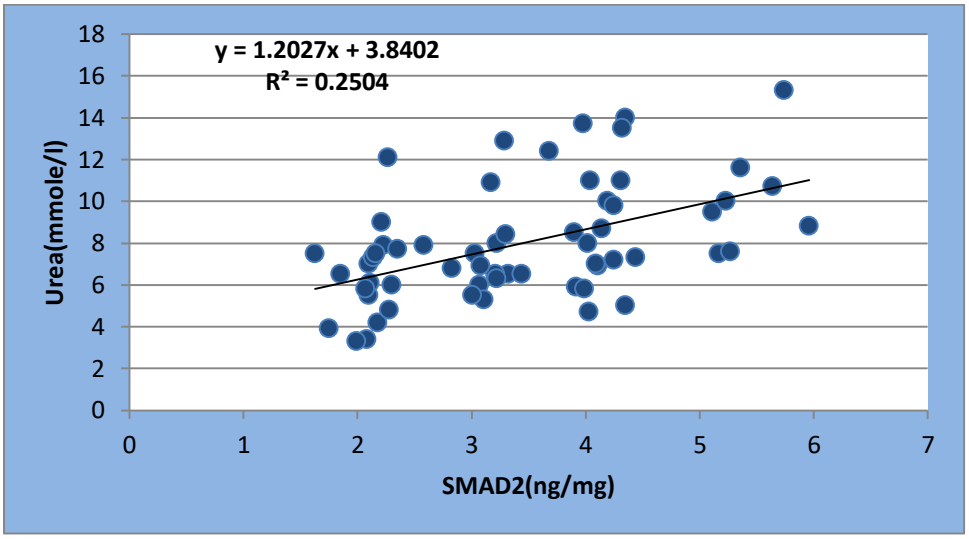
Figure(3.13): Correlation between SMAD3 (ng/ml) and Diabetic Duration(year) (r=0.497, P<0.001)**

3.3.1.2 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite Blood Urea

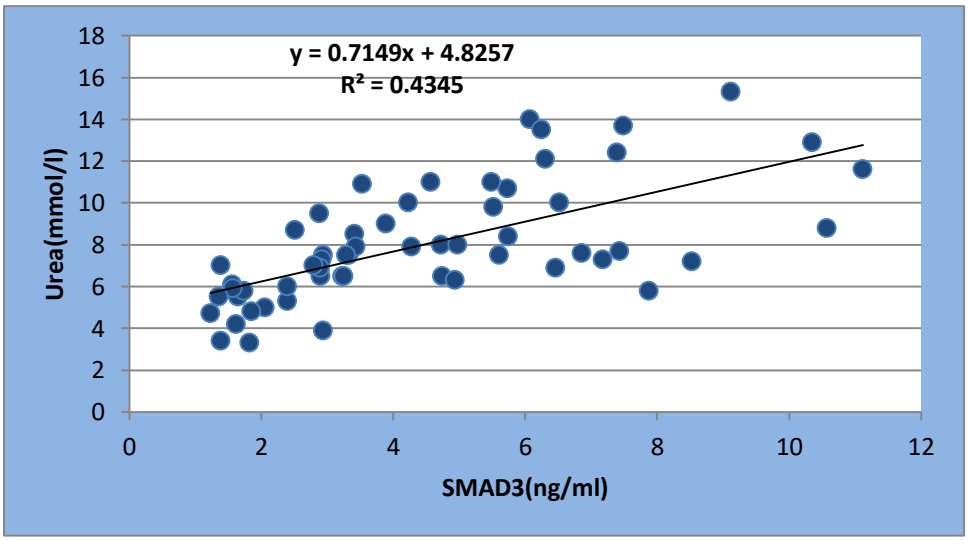
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with blood urea. The positive linear correlation in TGF-B (Figure 3-14) with a level of P<0.001 and a correlation coefficient of r=0.646. In contrast, SMAD2 positive linear correlation (Figure 3-15) at level P<0.001 and a correlation coefficient of r=0.500. while SMAD3 positive linear correlation (Figure 3-16) at level P<0.001 and a correlation coefficient of r= 0.659.



Figure(3.14): Correlation between TGF-B (ng/g) and Blood Urea(mmol/l) (r=0.646, P<0.001)**



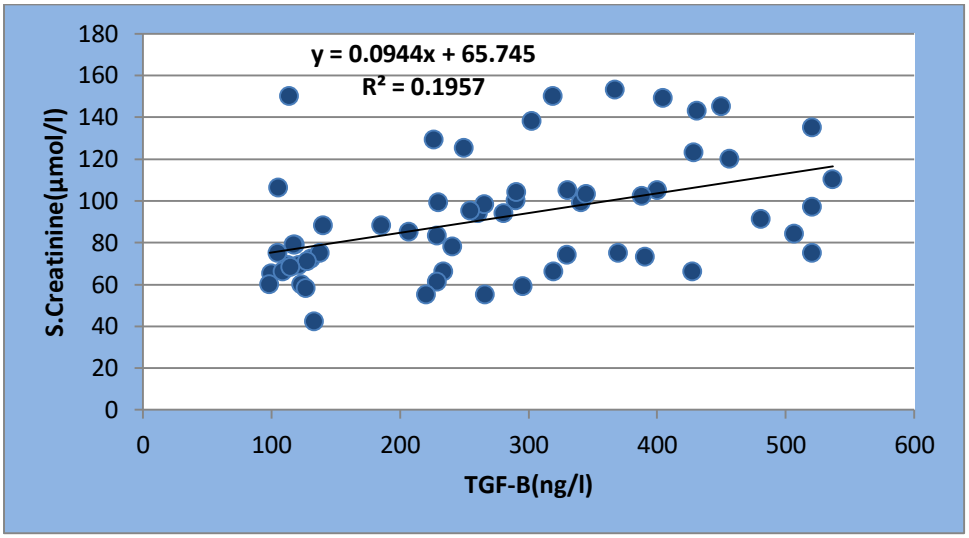
Figure(3.15): Correlation between SMAD2 (ng/ml) and Blood Urea(mmol/l) (r=0.500, P<0.001)**



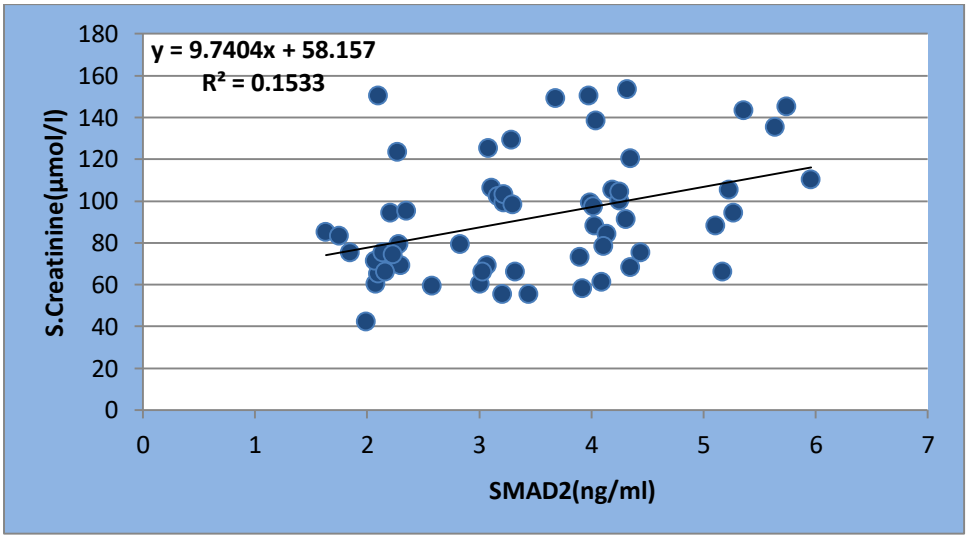
Figure(3.16): Correlation between SMAD3 (ng/ml) and Blood Urea(mmol/l) (r=0.659, P<0.001)**

3.3.1.3 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite Serum Creatinine

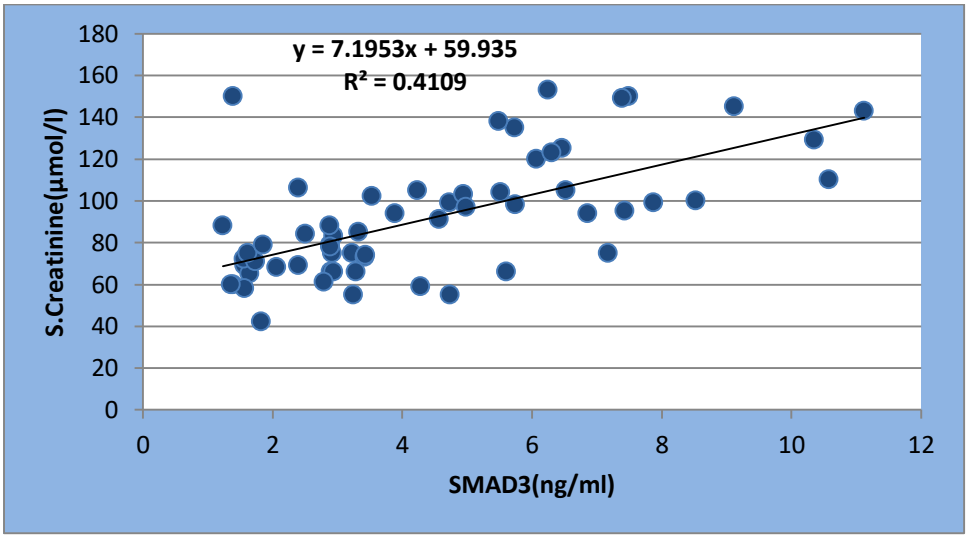
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with Serum Creatinine. The positive linear correlation in TGF-B (Figure 3.17) with a level of P<0.001 and a correlation coefficient of r=0.442. In contrast, SMAD2 positive linear correlation (Figure 3.18) at level P<0.001 and a correlation coefficient of r=0.392. while SMAD3 positive linear correlation (Figure 3.19) at level P<0.001 and a correlation coefficient of r= 0.641.



Figure(3.17): Correlation between TGF-B (ng/l) and S.Creatinine(mmol/l) (r=0.442, P<0.001).**



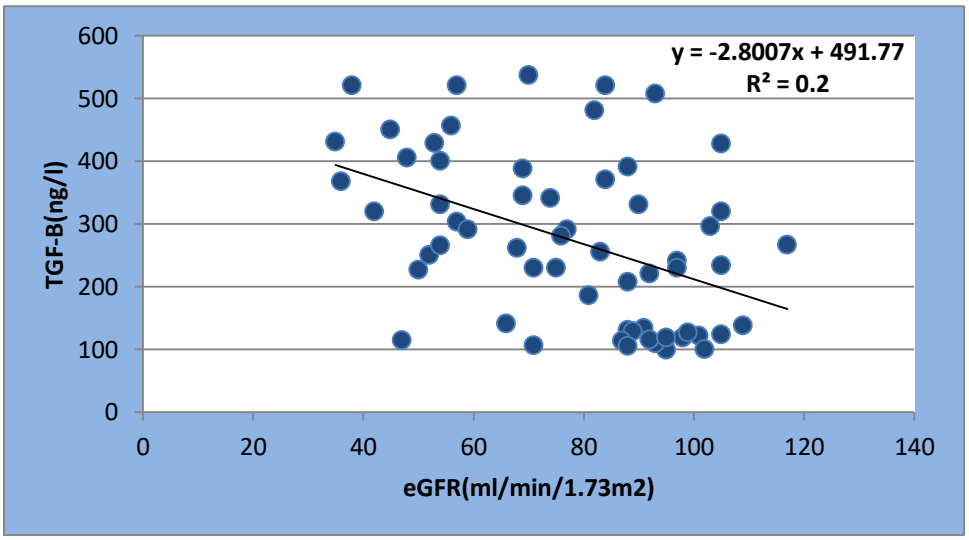
Figure(3.18): Correlation between SMAD2 (ng/ml) and S.Creatinine(mmol/l) (r=0.392, P<0.001)**



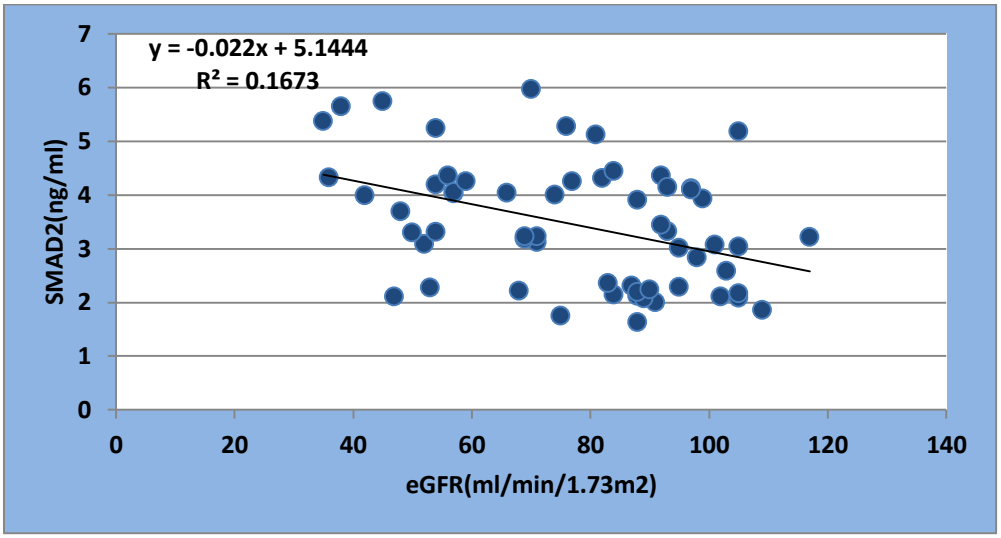
Figure(3.19): Correlation between SMAD3 (ng/ml) and S.Creatinine(mmol/l) (r=0.641, P<0.001)**

3.3.1.4 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite eGFR

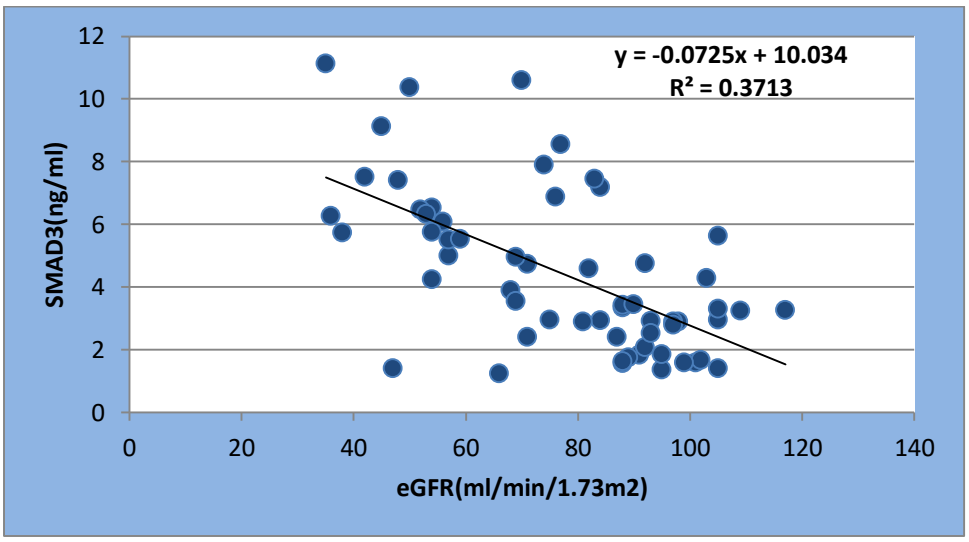
According to the study, their was a negative linear correlation of TGF-B, SMAD2, and SMAD3 with eGFR. The linear negative correlation in TGF-B (Figure 3-20) with a level of P<0.001 and a correlation coefficient of r=-0.447. In contrast, SMAD2 negative linear correlation (Figure 3-21) at level P<0.001 and a correlation coefficient of r=-0.409. while SMAD3 negative linear correlation (Figure 3-22) at level P<0.001 and a correlation coefficient of r=- 0.609.



Figure(3-20): Correlation between TGF-B (ng/l) and eGFR(ml/min/1.73m2) (r=-0.447, P<0.001)**



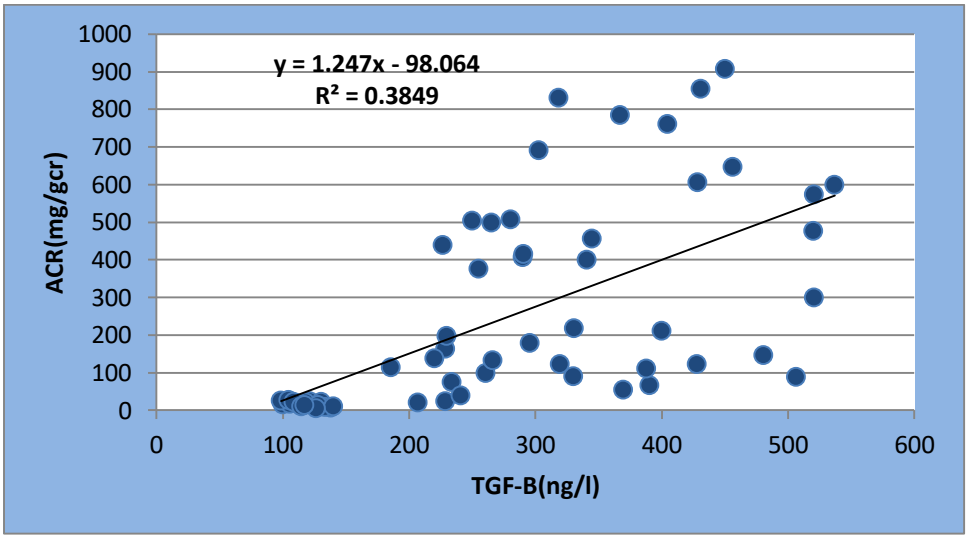
Figure(3-21): Correlation between SMAD2 (ng/ml) and eGFR(ml/min/1.73m2) (r=-0.409, P<0.001)**



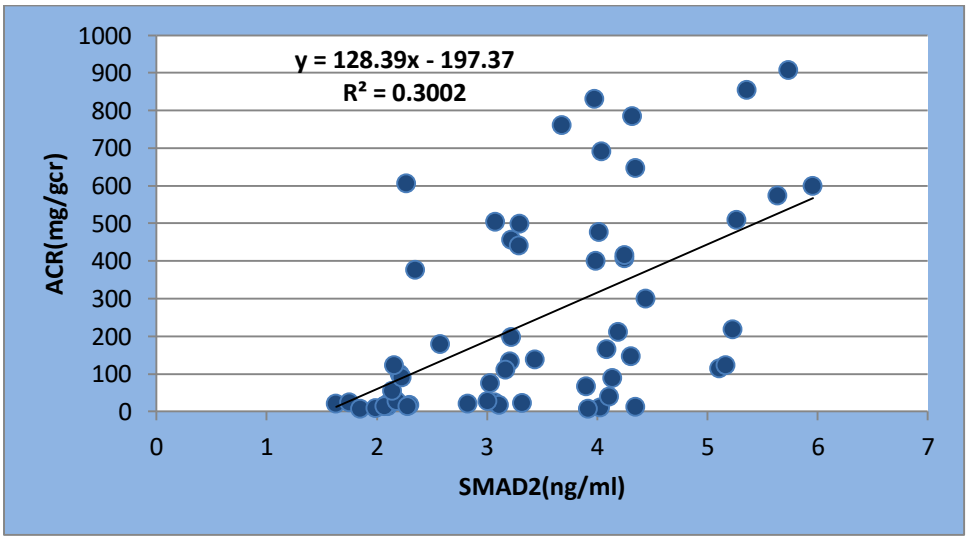
Figure(3-22): Correlation between SMAD3 (ng/ml) and eGFR(ml/min/1.73m2) (r=-0.609, P<0.001)**

3.3.1.5 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite Albumin-Creatinine Ratio(ACR)

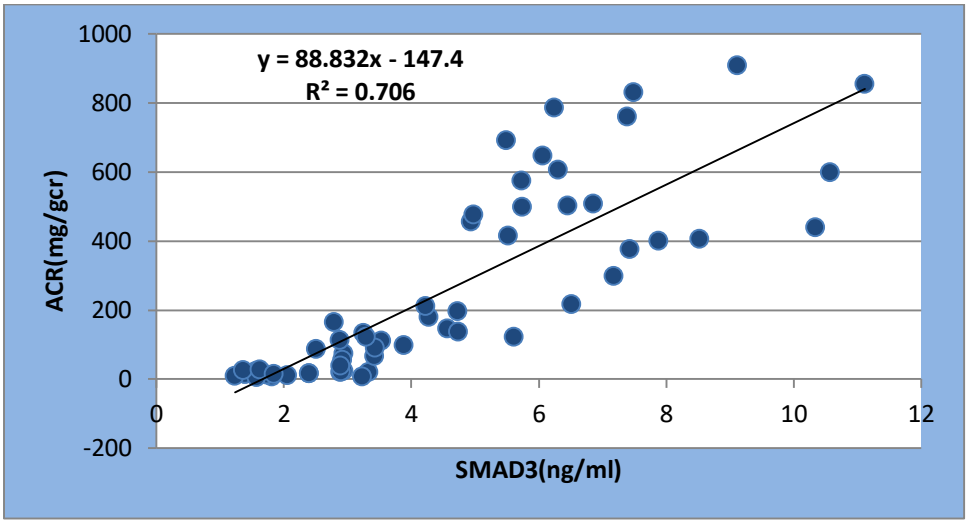
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with urinary albumin to creatinine ratio(ACR). The positive linear correlation in TGF-B (Figure 3-23) with a level of P<0.001 and a correlation coefficient of r=0.620. In contrast, SMAD2 positive linear correlation (Figure 3-24) at level P<0.001 and a correlation coefficient of r=0.548. while SMAD3 positive linear correlation (Figure 3-25) at level P<0.001 and a correlation coefficient of r= 0.840.



Figure(3-23): Correlation between TGF-B (ng/l) and ACR(mg/gcr) (r=0.620, P<0.001)**



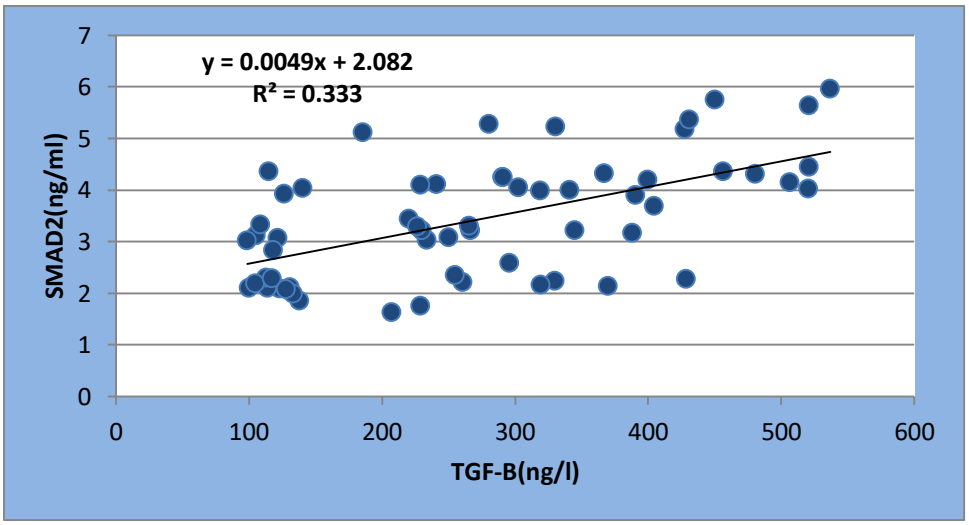
Figure(3.24): Correlation between SMAD2 (ng/ml) and ACR(mg/gcr) (r=0.548, P<0.001)**



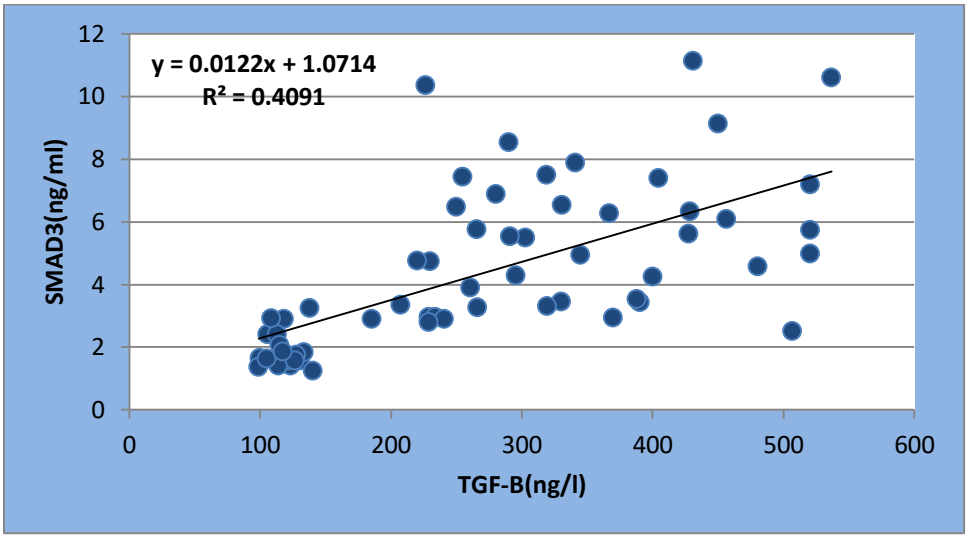
Figure(3-25): Correlation between SMAD3 (ng/ml) and ACR(mg/gcr) (r=0.840, P<0.001)**

3.3.1.6 Correlation Between TGF-Beta Opposite SMAD2, and SMAD3

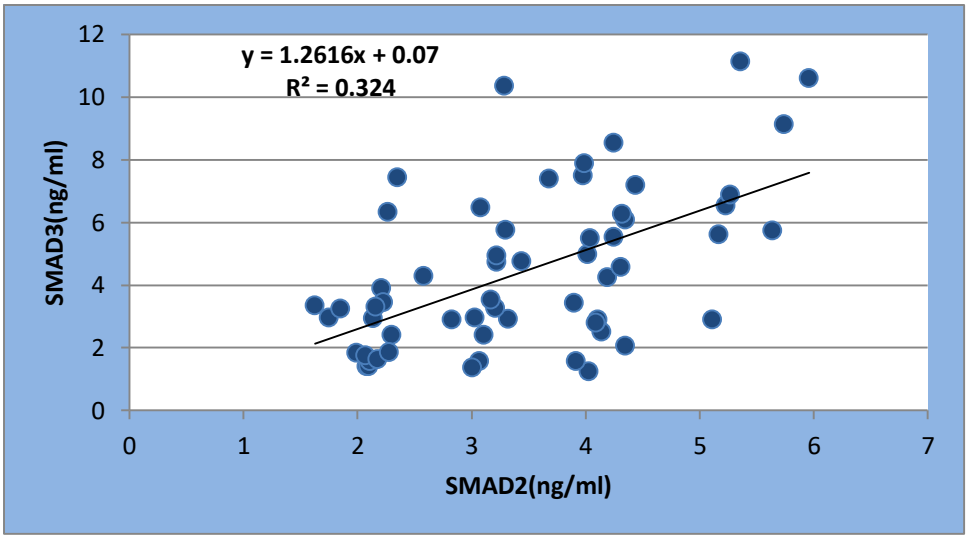
According to the study, TGF-B positively increased with SMAD2 and SMAD3. The positive linear correlation of TGF-B with SMAD2 (Figure 3-26) with a level of P<0.001 and a correlation coefficient of r=0.577. The positive linear correlation of TGF-B with SMAD3 (Figure 3-27) with a level of P<0.001 and a correlation coefficient of r=0.640. On other hand, the SMAD2 positive linear correlation with SMAD3 (Figure 3-28) with a level of P<0.001 and a correlation coefficient of r=0.569.



Figure(3-26): Correlation between TGF-B (ng/l) and SMAD2(ng/ml) (r=0.577, P<0.001)**



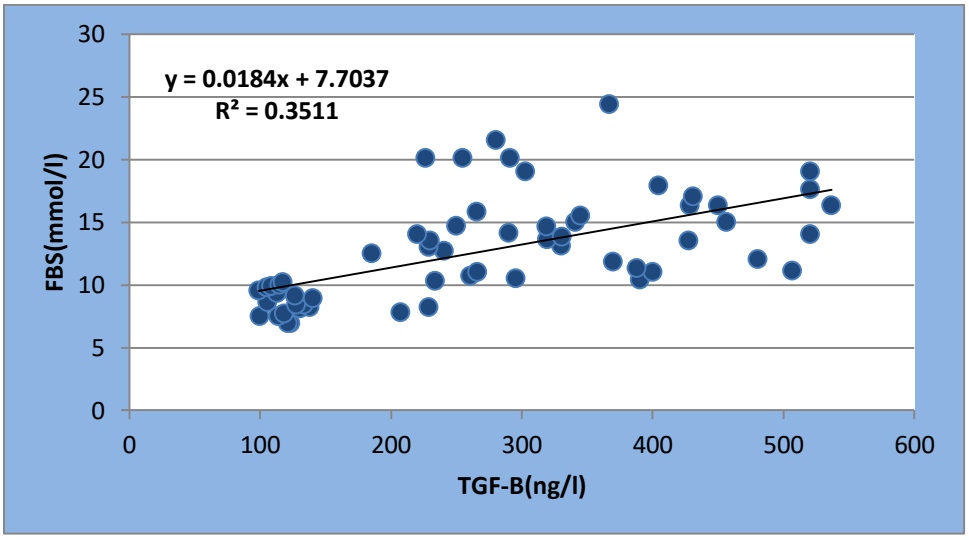
Figure(3-27): Correlation between TGF-B (ng/l) and SMAD3(ng/ml) (r=0.640, P<0.001)**



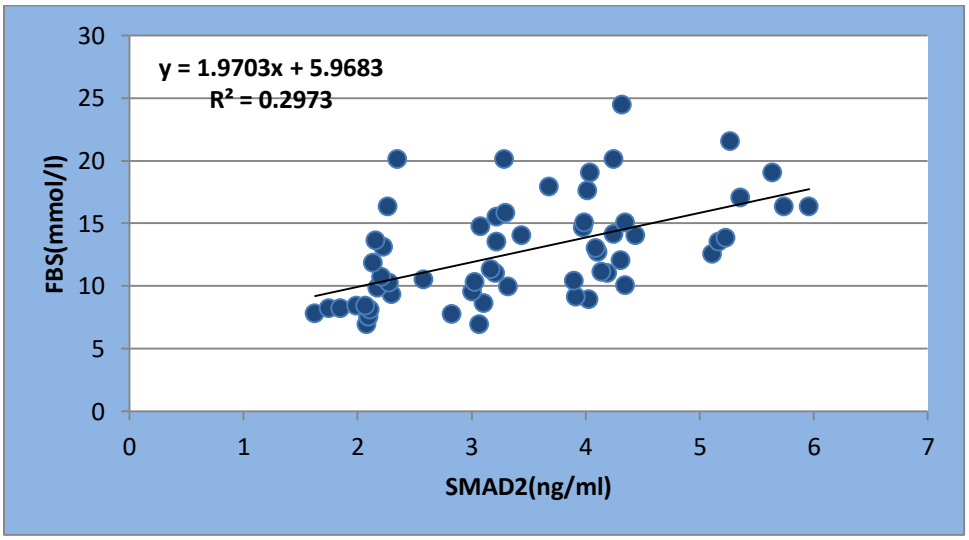
Figure(3-28): Correlation between SMAD2(ng/ml) and SMAD3(ng/ml) (r=0.569, P<0.001)**

3.3.1.7 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite Fasting Blood Sugar(FBS).

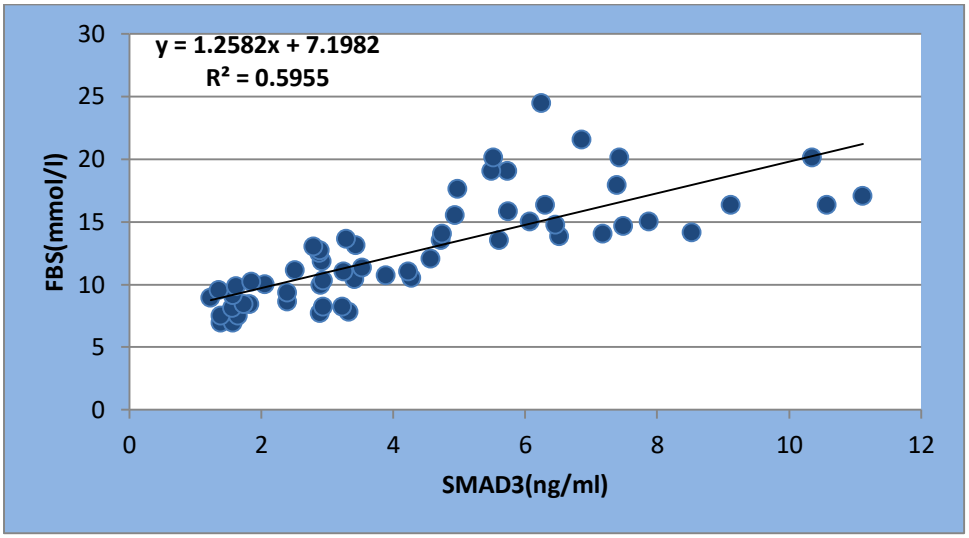
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with Fasting Blood Sugar(FBS) the positive linear correlation in TGF-B (Figure 3-29) with a level of P<0.001 and a correlation coefficient of r=0.593. In contrast, SMAD2 positive linear correlation (Figure 3-30) at level P<0.001 and a correlation coefficient of r=0.545. while SMAD3 positive linear correlation (Figure 3-31) at level P<0.001 and a correlation coefficient of r= 0.772.



Figure(3.29): Correlation between TGF-B(ng/l) and FBS(mmol/l) (r=0.593, P<0.001)**



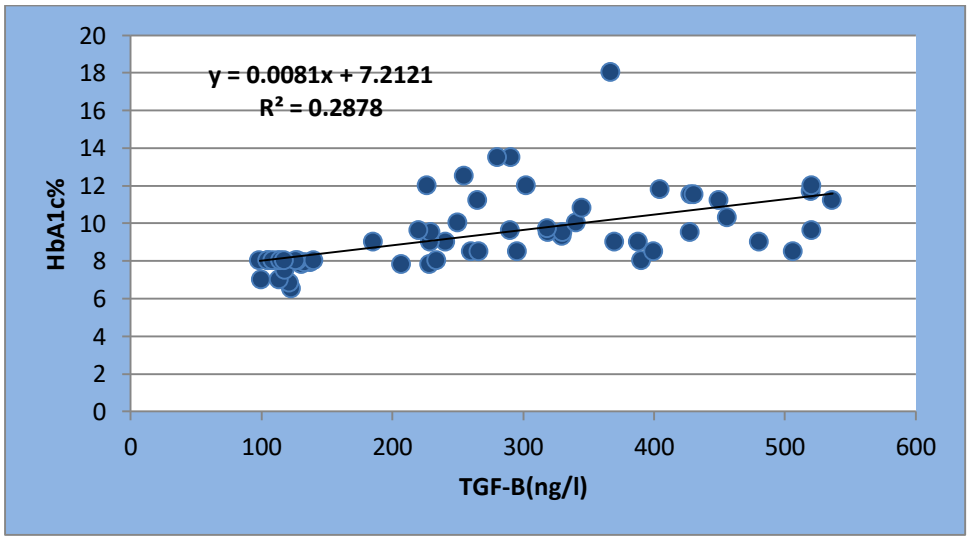
Figure(3-30): Correlation between SMAD2(ng/ml) and FBS(mmol/l) (r=0.545, P<0.001)**



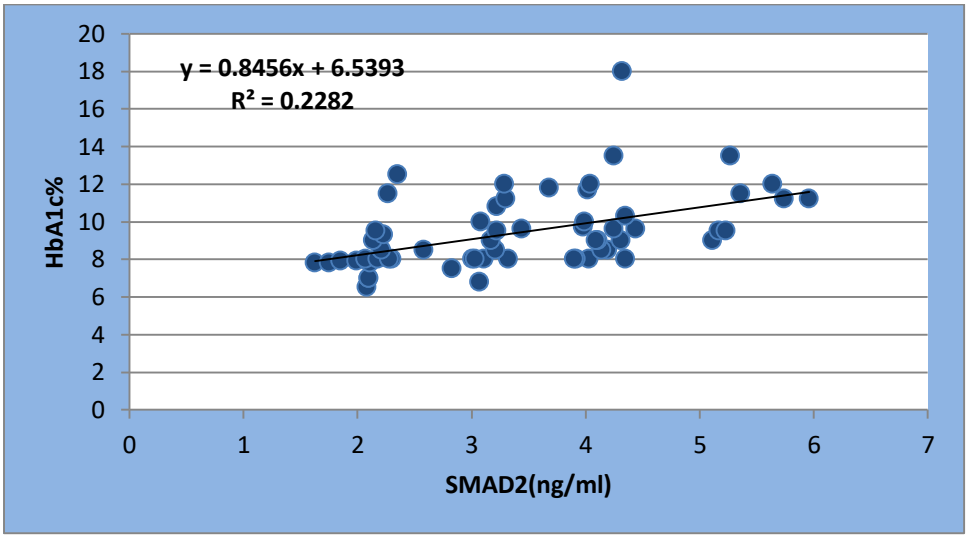
Figure(3-31): Correlation between SMAD3(ng/ml) and FBS(mmol/l) (r=0.772, P<0.001)**

3.3.1.8 Correlation Between TGF-Beta, SMAD2, and SMAD3 Opposite HbA1c%.

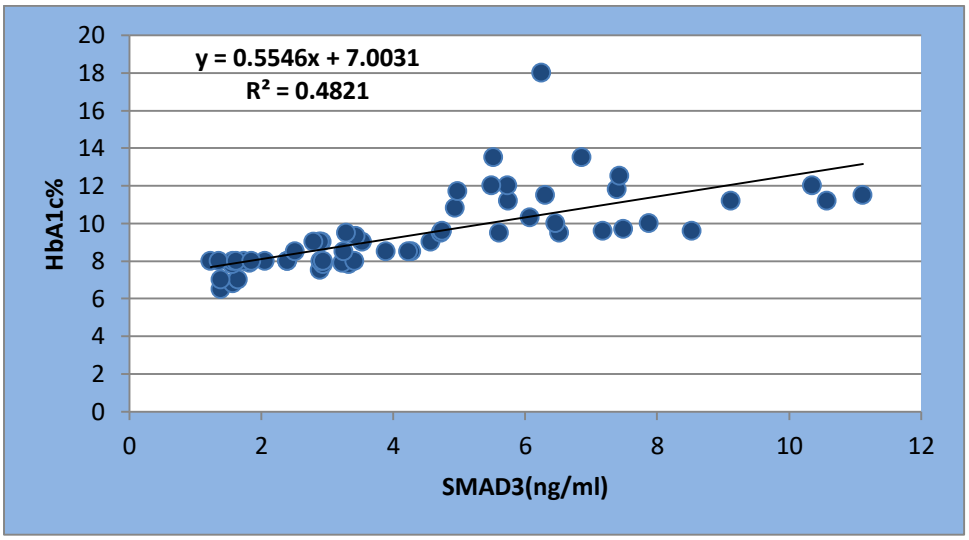
According to the study, TGF-B, SMAD2, and SMAD3 positively increased with HbA1c% the positive linear correlation in TGF-B (Figure 3.32) with a level of P<0.001 and a correlation coefficient of r=0.536. In contrast, SMAD2 positive linear correlation (Figure 3.33) at level P<0.001 and a correlation coefficient of r=0.478. while SMAD3 positive linear correlation (Figure 3.34) at level P<0.001 and a correlation coefficient of r= 0.694



Figure(3-32): Correlation between TGF-B(ng/l) and HbA1c% (r=0.536, P<0.001)**



Figure(3-33): Correlation between SMAD2(ng/ml) and HbA1c% (r=0.478, P<0.001)**



Figure(3-34): Correlation between SMAD3(ng/ml) and HbA1c% (r=0.694, P<0.001)**

The study showed a positive linear correlation between TGF-B, SMAD2, and SMAD3 with serum urea, as in Figure (3-14), Figure (3-15), and Figure (3-16), as well as a positive correlation between TGF-B, SMAD2 and SMAD3 with serum creatinine, as in Figure (3-17), Figure (3-18) and Figure (3-19). This is accordance with the study (Shukla *et al.*, 2018). The reason is due to TGF-β is an essential mediator that stimulates glomerular ECM formation in DN leads to TGF-β activation (Q. Zhang *et al.*, 2021).

Altogether, these factors lead to deteriorated kidney function as evidenced by increased serum urea and creatinine levels.

The present study describes a negative linear correlation between TGF-B, SMAD2, and SMAD3 with eGFR, as in Figure (3-20), Figure (3-21), and Figure (3-22). The main reason is due to the formation of fibrosis, mainly manifested in the deposition of matrix proteins. The deposition of extracellular matrix (ECM), is a major stage of structural and functional impairment of the kidney (significant reduction of effective nephrons and further decline of glomerular filtration rate)(Liu, 2006).

The present study describes a positive linear correlation between TGF-B, SMAD2, and SMAD3 with urinary ACR, as in Figure (3-23), Figure (3-24), and Figure (3-25). The results of the present study was agreed with previous study (Hu and Feng, 2011)

For serum TGF-Beta, the results demonstrated a positive correlation with UACR, BUN, and Creatinine and a negative correlation with eGFR in T2DM patients, which implies circulating TGF-B may participate in the pathogenesis of DN. The results of the present study were agreed with previous studies (Yang et al., 2021), (Qiao et al., 2017), (El Mesallamy et al., 2012). Additionally, for serum SMAD2 and SMAD3, the results demonstrated a positive correlation with both BUN and creatinine and a negative correlation with eGFR in T2DM patients (Thipsawat, 2021).

Also, we observed a positive and significant correlation of serum TGF-β level with HbA1c and fasting blood glucose. (Shaker *et al*., 2014) also reported a positive significant correlation between serum TGF-β1 and both glucose concentration and HbA1c. (Ibrahim and Rashed, 2007) have also found that serum TGF-β1 was significantly increased in patients with poor glycemic control with variable degree of renal dysfunction as compared to those with good glycemic control and comparable degree of renal dysfunction. These finding conform the direct link between hyperglycemia and activation of TGF-β1. It is known that glucose stimulates de novo synthesis of diacylglycerol (DAG) and activated DAG then leads to activation of protein kinase C (PKC) which increases TGF-β1 synthesis in mesangial cell and tubular cell (Ibrahim and Rashed, 2007).

Transforming growth factor-beta promotes the generation of reactive oxygen species; considerable variance was evident for FBG and HbA1c. The noteworthy finding revealed various factors, including lifestyle choices and environmental

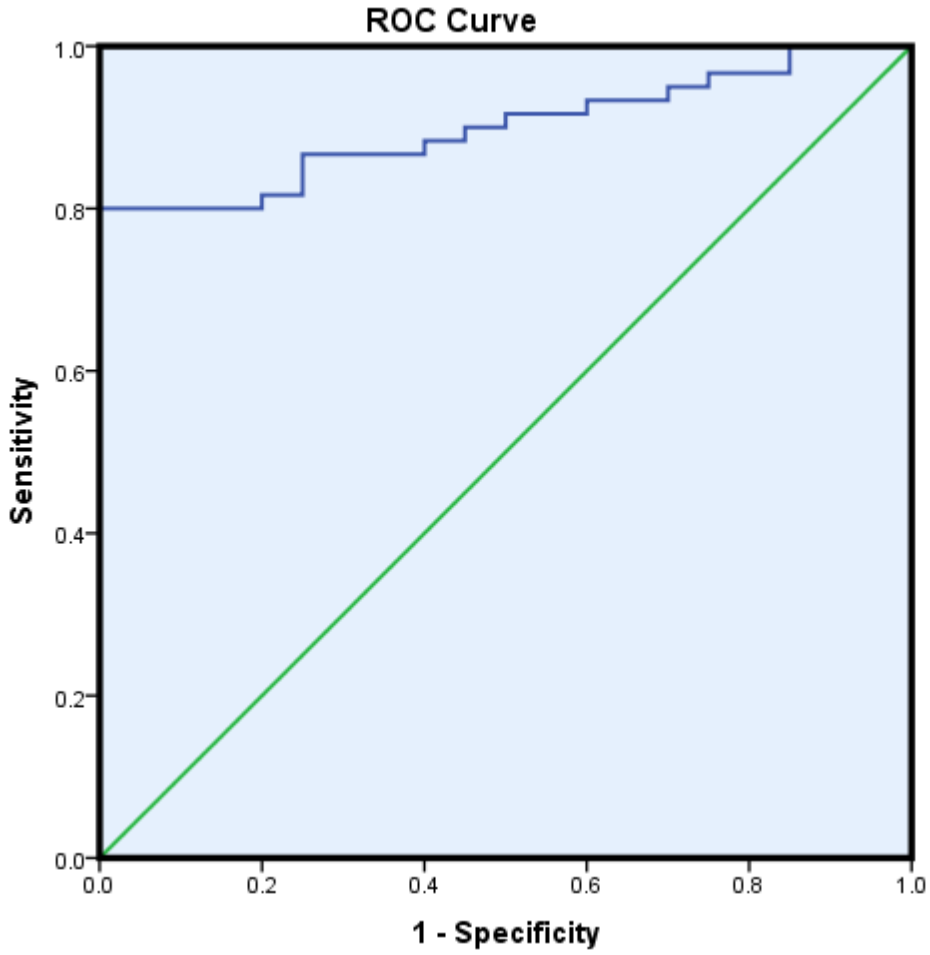
contamination, as causes of diabete have high levels of oxidative stress, which means high levels of free radicals, which are dangerous and speed up the diabetes complications are present(Tangvarasittichai, 2015). Evidences from in vivo and in vitro studies have indicated that increased concentration of glucose could stimulate TGFβ1 expression both in cultured renal cells and in the kidney which suggested that TGF-β1 might play an important role in the etiology of T2DN in T2DM (Tang *et al.*, 2018). This study showed that high glucose activated the fibrotic TGF-β/SMADs pathway, thereby activating the downstream factors and aggravating renal deterioration, which were agreed with previous studies (Isono *et al.*, 2002)(Zhang *et al.*, 2016)(Wang, Guo and Wang, 2021).

3.4 Diagnostic Values for Serum TGF-beta, SMAD2, and SMAD3 :

The receiver operating characteristic curve (ROC) was used to assess the diagnostic values of TGF-beta, SMAD2, and SMAD3 in identifying diabetic patients, and which of them is more specific or sensitive in the diagnosis of diabetic nephropathy.

3.4.1 Serum TGF-beta value:

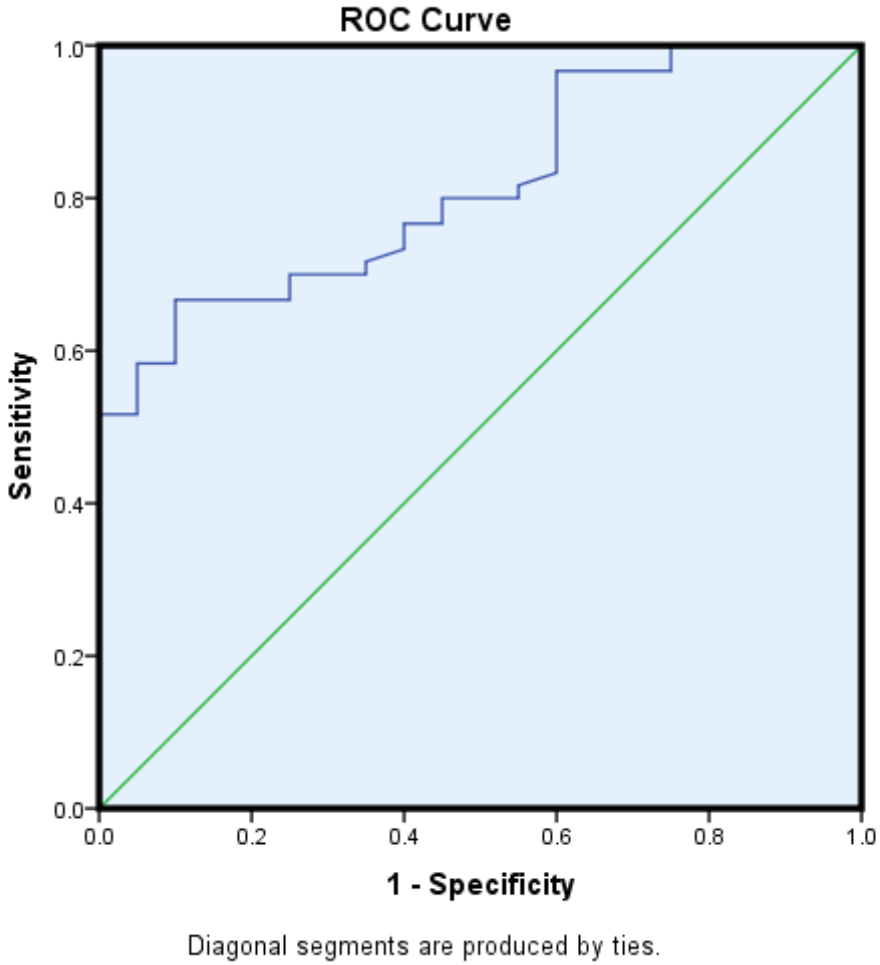
The area under the curve (AUC) was 0.899, p-value=0.000. The sensitivity and specificity of the test at the cut-off value of TGF -beta ng/ml ≥ 126.19 were 80 % and 95%, respectively. As shown in figure(3-35).



Figure(3-35): ROC Curve for Sensitivity and Specificity of TGF-beta to Predict Diabetic Nephropathy

3.4.2 Serum SMAD2 Value:

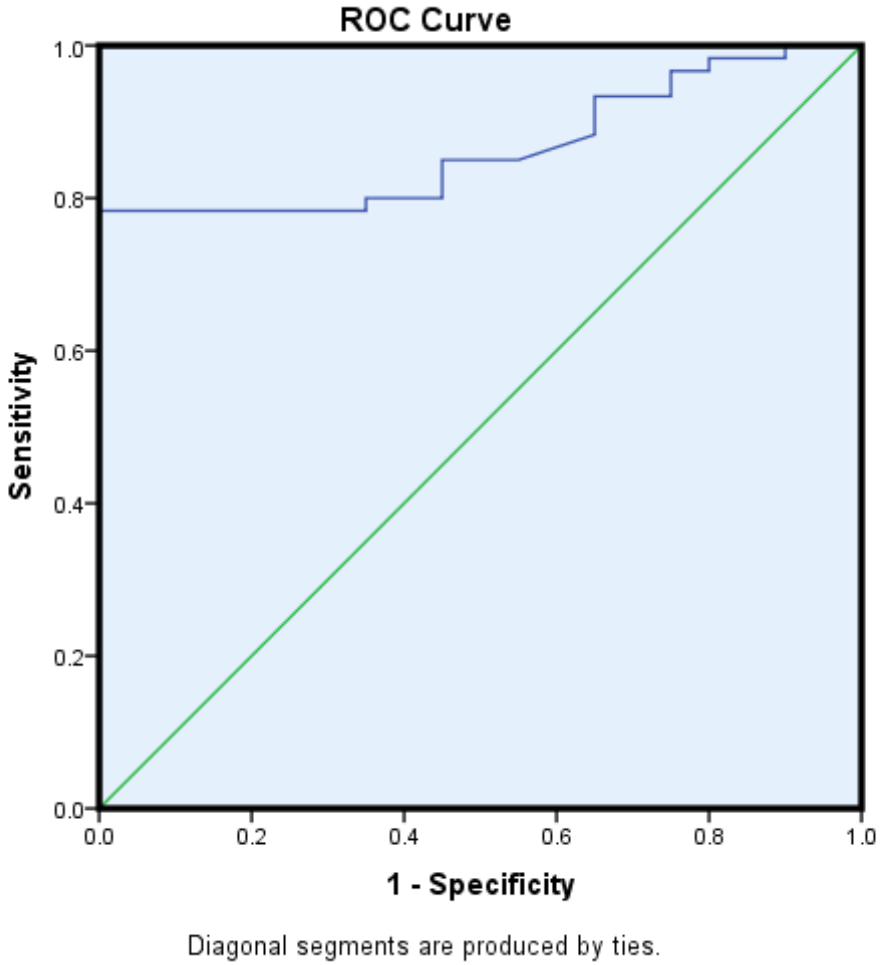
The area under the curve (AUC) was 0.66, p-value=0.033. The sensitivity and specificity of the test at the cut-off value of SMAD2 ng/ml ≥ 3.015 were 56.7 % and 95%, respectively. As shown in figure(3-36).



Figure(3-36): ROC Curve for Sensitivity and Specificity of SMAD2 to Predict Diabetic Nephropathy

3.4.3 Serum SMAD3 Value:

The area under the curve (AUC) was 0.866, p-value < 0.001. The sensitivity and specificity of the test at the cut-off value of SMAD3 ng/ml ≥ 2.33 were 78.30 % and 95%, respectively. As shown in figure(3-37).



Figure(3-37): ROC Curve for Sensitivity and Specificity of SMAD3 to Predict Diabetic Nephropathy

The current findings revealed that a good diagnostic profile of serum TGF-β at the optimal cut-off value for early detection of DN is 126.19 ng/l, with a sensitivity of 80%, specificity of 95%, positive predictive value (PPV) of 97%, negative predictive value (NPV) of 61%, and accuracy of 83%.

Also, the current findings revealed that a good diagnostic profile of serum SMAD3 at the optimal cut-off value for early detection of DN is 2.33 ng/ml, with a sensitivity of 78.30%, specificity of 95%, positive predictive value (PPV) of 95%, negative predictive value (NPV) of 58 %, and accuracy of 81%.

Our findings for SMAD2 revealed a good diagnostic profile with a sensitivity of 66.70 %, specificity of 90 %, positive predictive value (PPV) of 95%, negative predictive value (NPV) of 47%, and accuracy of 72% at a cutoff value of 2.995 ng/ml. As illustrated in Table (3-10)**.** These findings show that TGF-β, SMAD2,

and SMAD3 are more specific and sensitive in the early detection of nephropathy. As shown in Table(3-10).

Table 3-10 : Receiver Operating Characteristic Curve Analysis for TGF-Beta ,SMAD2 and SMAD3

Variable	TGF-Beta	SMAD2	SMAD3
AUC	0.899	0.816	0.866
Threshold	≥126.19	≥2.995	≥2.33
P-value	0.000	0.000	<0.001
SN	80%	66.70%	78.30%
SP	95%	90%	95%
PPV	97%	95%	95%
NPV	61%	47%	58%
Accuracy	83%	72%	81%

SN=sensitivity, SP=specificity, PPV= positive predictive value, NPV=negative predictive value, AUC= area under the curve. (P-value <0.05* was considered to be statically significant).

CHAPTER FOUR

CONCLUSION AND RECOMMENDATIO

Conclusions:-

1- Serum TGF-β, SMAD2, and SMAD3 are associated with macroalbuminuria and microalbuminuria as compared with normoalbuminuria and the control group.

2- Serum TGF-β, SMAD2, and SMAD3 are used as a predictor of renal disease in a patient with type 2 diabetes mellitus because of their high sensitivity and high specificity, This indicates that serum TGF-β, SMAD2, and SMAD3 are critical in the early prediction of diabetic nephropathy.

3- Serum TGF-β, SMAD2, and SMAD3 levels are positively correlated with albuminuria, and negatively correlated with eGFR in T2DM patients. This suggests that circulating TGF-β, SMAD2, and SMAD3 may have a role in the etiology of DN by influencing blood glucose levels, and inflammatory responses in the kidney.

4- Serum TGF-β, SMAD2, and SMAD3 levels were increased as the duration of diabetes is prolonged.

5- Serum TGF-β, SMAD2, and SMAD3 levels were significantly elevated in T2DM patients with macroalbuminuria and T2DM patients with microalbuminuria Results showed that TGF-β, SMAD2, and SMAD3 was an independent and excessively activated pathway that contributed to renal profibrotic and collagen deposition in kidney cells, indicating an earlier measurable marker of renal injury compared with the others traditional glomerular marker.

Recommendations:-

1-Large, multicenter studies are recommended for detecting the normal values of serum TGF-β, SMAD2, and SMAD3 to imply the early role of diagnosis of Diabetic Nephropathy.

2-Adipocyte differentiation, white to brown fat phenotypic transition, glucose and lipid metabolism, pancreatic function, insulin signaling, adipocytokine secretion, inflammation, and reactive oxygen species production is just a few of the pleiotropic effects of TGF-β/SMAD2/3 signaling on metabolism and energy homeostasis that must be discussed.

3- Introduce TGF-Beta as a common test in clinical practice, in addition to albuminuria, for early diagnosis of renal damage in diabetes patients.

4- A follow-up research is needed to see how patients with TGF-β, SMAD2, and SMAD3 elevations with normoalbuminuria fare in the future.

5-To prevent Diabetic Nephropathy, more attention should be paid to good Blood Sugar and HbA1c control in patients.

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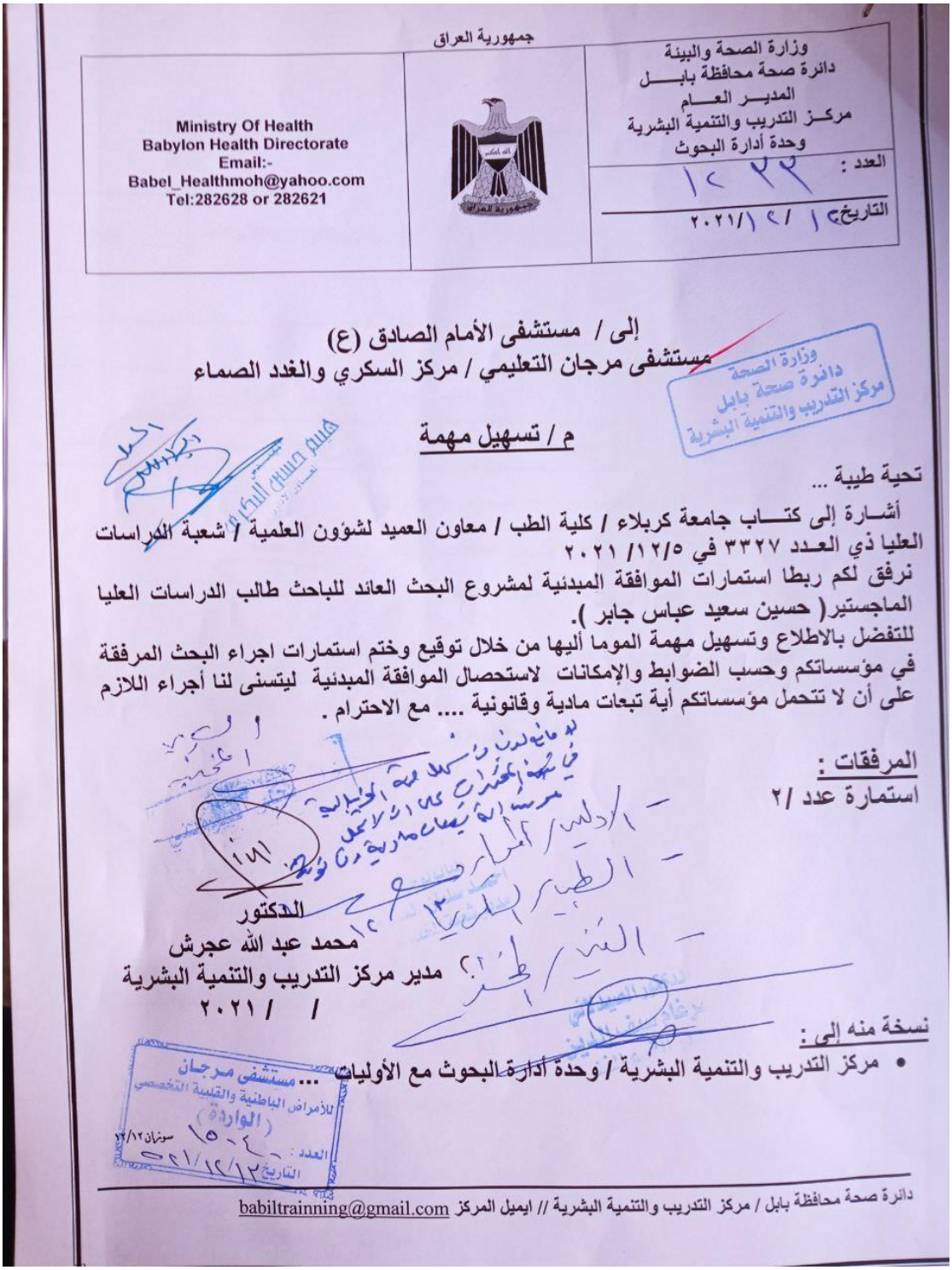
Appendix A: Questionnaire

Case number		**Date**	
Name			**Tel:**
Age			**Note**
Sex	**Male**	**female**	
Body max index (BMI)	**Height:**	**Weight:**	

Duration of diabetes(years)			
History of cardiovascular diseases (coronary artery disease, myocardial infraction, cerebrovascular or peripheral artery diseases			
Chronic Hypertension			
Renal disease rather than diabetic nephropathy			
Smoking Current			
Hepatic diseases			
Endocrine diseases			
Any type of tumors			
Acute and chronic inflammatory diseases			
Acute infections			
Type 1 diabetes mellitus			
Pregnancy			
Surgery or trauma within 6 months			
Retinopathy			
Medications: Dietary restriction: Hypoglycemic tablets: Insulin therapy:			

Appendix B



جمهورية العراق

وزارة الصحة والبيئة
دائرة صحة محافظة بابـــل
المديـــر العـــام
مركـــز التدريب والتنمية البشرية
وحدة أدارة البحوث

العدد : ١٢٢٢
التاريخ : ١ / ١٢ / ٢٠٢١

Ministry Of Health
Babylon Health Directorate
Email:-
Babel_Healthmoh@yahoo.com
Tel:282628 or 282621

إلى / مستشفى الأمام الصادق (ع)
مستشفى مرجان التعليمي / مركز السكري والغدد الصماء

وزارة الصحة
دائرة صحة بابل
مركز التدريب والتنمية البشرية

م / تسهيل مهمة

تحية طيبة ...

أشـارة إلى كتـــاب جامعة كربلاء / كلية الطب / معاون العميد لشؤون العلمية / شعبة الدراسات العليا ذي العـدد ٣٣٢٧ في ١٢/٥/ ٢٠٢١

نرفق لكم ربطا استمارات الموافقة المبدئية لمشروع البحث العائد للباحث طالب الدراسات العليا الماجستير(حسين سعيد عباس جابر).

للتفضل بالاطلاع وتسهيل مهمة الموما أليها من خلال توقيع وختم استمارات اجراء البحث المرفقة في مؤسساتكم وحسب الضوابط والإمكانات لاستحصال الموافقة المبدئية ليتسنى لنا أجراء اللازم على أن لا تتحمل مؤسساتكم أية تبعات مادية وقانونية .... مع الاحترام .

المرفقات :
استمارة عدد /٢

الدكتور
محمد عبد الله عجرش
مدير مركز التدريب والتنمية البشرية
/ / ٢٠٢١

نسخة منه إلى :
- مركز التدريب والتنمية البشرية / وحدة أدارة البحوث مع الأوليات

مستشفى مرجان ... للأمراض الباطنية والقلبية التخصصي
الواردة
العدد : ١٥٠٤
التاريخ ٢١/١٢/١٢

دائرة صحة محافظة بابل / مركز التدريب والتنمية البشرية // ايميل المركز babiltrainning@gmail.com

الخلاصة

داء السكري هو مرض استقلابي يتميز بفرط سكر الدم الناتج عن عيوب في إفراز الأنسولين أو عمل الأنسولين أو كليهما. يرتبط ارتفاع السكر المزمن في الدم بمرض السكري بالضرر طويل الأمد والخلل الوظيفي وفشل الأعضاء المختلفة ، وخاصة العين والكلى والأعصاب والقلب والأوعية الدموية. اعتلال الكلية السكري أحد أكثر المضاعفات شيوعًا وشدة لمرض السكري ويرتبط بزيادة معدلات الاعتلال والوفيات لدى مرضى السكري وأمراض الكلى. ويمثل السبب الرئيسي لأمراض الكلى المزمن (CKD) في المراحل النهائية (الداء الكلوي بمراحله الأخيرة). يعد الكشف المبكر عن اعتلال الكلية السكري (DN) أمرًا بالغ الأهمية في تحسين الإدارة السريرية وتطور التداخل العلاجي لداء الكلى بمراحله الأخيرة وغسيل الكلى. يتميز مرض الكلى السكري بضرر كلوي تدريجي ينعكس عن طريق زيادة البول الزلالي (نسبة الألبومين في البول إلى الكرياتينين (ACR) التي تتجاوز 30 ملجم / جم كرياتينين) مع ضعف في وظائف الكلى وانخفاض في معدل الترشيح الكبيبي (GFR) ، ارتفاع ضغط الدم ، والاعتلال والوفيات الزائدة بسبب مضاعفات القلب والأوعية الدموية.

تم التعرف على عامل النمو المحول (TGF-β) على أنه بروتين التهابي تليفي رئيسي يشارك في امراض التليف ، لا سيما في الأوعية الدموية والكلى. يتم تنشيط مسار إشارات TGF-β في مرضى اعتلال الكلية السكري (DN) من خلال زيادة مستويات الجلوكوز خارج الخلية ، وتمدد الخلية ميسانجيل ، وتنشيط نظام الرينين-أنجيوتنسين ، وأنواع الأكسدة التفاعلية (ROS) ، والمنتجات النهائية للكلايكيشن المتقدمة (AGEs) التي تعمل على تنشيط تخليق TGF-β عبر بروتين كاينيز C. ينشط مسار(TGF-β) ذاتيا او عن طريق الصماء . عامل النسخ للأمهات الصغيرات ضد عائلة منزوعة الرأس (SMAD) لها أدوار رئيسية في قرار مصير الخلية في نقل الإشارات من خارج الخلية إلى النواة من خلال تحويل مستقبلات عامل النمو بيتا (TGF-β) لتنشيط النسخ الجيني المستهدف. بالنسبة لإشارات (TGF-β) ، فإن الفسفرة والتفعيل بواسطة النوع النشط من النوع الثاني والنوع الأول من معقد المستقبل يتسبب في تكوين SMAD2 و SMAD3 لتكوين مركب غير متجانس مع SMAD4 وينقل المركب بأكمله إلى النواة. هناك يتفاعلون مع خلايا مختلفة ، ويرتبطون بالحمض النووي ، وينظمون الاستجابة المختلفة لنسخ الجينات.

تهدف الدراسة الحالية إلى تحديد دور SMAD2 و SMAD3 وعلاقتهما مع TGF-β في مرضى السكري من النوع الثاني المصابين باعتلال الكلية السكري المبكر والمزمن في عينة من السكان العراقيين ، وكذلك دراسة العلاقة بين هذه المؤشرات الحيوية مع المعلمات البايوكيميائية التقليدية لوظائف الكلى.

أجريت الدراسة على مرضى واصحاء في مدينة الحلة من أكتوبر 2021 إلى مايو 2022. وتم جمع العينات من مركز السكري والغدد الصماء في مستشفى المرجان التعليمي في بابل / مدينة الحلة.

تضمنت الدراسة الحالية 120 فردًا، منهم 60 مريضًا يعانون من داء السكري من النوع الثاني (T2DM) و 60 مشاركًا يتمتعون بصحة جيدة وتم استخدامهم كمجموعة اصحاء. تم تقسيم المرضى إلى ثلاث مجموعات متساوية وفقًا لنسبة الألبومين البولي لديهم إلى نسبة الكرياتينين (ACR) ، بما في ذلك المرضى الذين يعانون من البيلة الألبومينية الطبيعية (العدد = 20 30> ACR) (مجم / جم كرياتينين) وأولئك الذين يعانون من بيلة الالبومينية

الدقيقة (العدد = 20 ACR = 30-299) (مجم / جم كرياتينين) وبيلة الالبومينية الكبيرة (العدد = 20 ACR) (=أكثر من 300 مجم / جم كرياتينين). تم تقدير مستويات الجلوكوز، HbA1c ،اليوريا في الدم، مصل الكرياتينين، TGF-Beta ، SMAD2 و SMAD3 لجميع المشاركين ، بالإضافة إلى البيلة الالبومينية الدقيقة في البول.

وجدت نتائج الدراسة الحالية أن متوسط تركيز مستويات FBG و HbA1c قد زاد بشكل ملحوظ في مرضى T2DM الذين يعانون من البيلة الألبومينية الكبيرة ، البيلة الألبومينية الدقيقة ، والبيلة الألبومينية الطبيعية مقارنة بمجموعة الاصحاء (P <0.001).

كما كانت مستويات TGF-β، SMAD2 و SMAD3 في المصل في T2DM مع بيلة الألبومين الكبيرة وفي T2DM مع بيلة الألبومين الدقيقة أعلى بكثير مما كانت عليه في T2DM مع بيلة الألبومين الطبيعية ومجموعة الاصحاء مع فروقات ذات دلالة إحصائية كبيرة (P<0.001).من ناحية أخرى، هنالك زيادة في مستوى TGF-β، SMAD2وSMAD3 بشكل ملحوظ في البيلة الألبومينية الكبيرة مقارنة بالبيلة الألبومينية الدقيقة مع وجود فروقات ذات دلالة إحصائية عالية (P<0.001) بالإضافة إلى ذلك ، أظهر مصل TGF-β ، SMAD2 و SMAD3 ارتباطًا إيجابيًا مع ACR ، اليوريا في الدم ، مصل الكرياتينين ، FBG، HbA1c، ومدة مرض السكري ، وارتباطا سلبيا مع eGFR.

كشفت منحنيات خصائص تشغيل جهاز الاستقبال (ROC) المستخدمة من أجل الكشف المبكر عن اعتلال الكلية السكري (DN)، هناك صورة تشخيصية جيدة لمصل TGF-β للكشف المبكر عن DN ، مع حساسية 80٪ ، وخصوصية 95٪. وأيضًا كان المظهر التشخيصي جيدا لمصل SMAD3 للكشف المبكر عن DN ، بحساسية 78.30٪ ، خصوصية 95٪. كذلك اظهر SMAD2 تشخيصا جيدا للكشف المبكر عن DN ، مع حساسية 66.70٪ ، وخصوصية 90٪. وتعتبر جميعها مؤشرات حيوية مستقلة وموثوقة للكشف المبكر عن اعتلال الكلية السكري (DN).

في الختام ، كشفت هذه الدراسة عن أهمية هذه العلامات في التسبب في اعتلال الكلية السكري (DN) والتي يتم دعمها من خلال ارتباطها بالبيلة الزلالية وكذلك النوعية العالية والحساسية العالية من خلال تحليل ROC ، وبالتالي تم اقتراح إمكانية استخدامها كعلامات كيميائية حيوية في اعتلال الكلية السكري (DN).

جمهورية العراق

وزارة التعليم العالي

والبحث العلمي

جامعة كربلاء/كلية الطب

فرع الكيمياء الحياتية السريرية

تقييم SMAD2 و SMAD3 و علاقتهما بتحويل عامل النمو TGF-BETA في مرضى السكري من النوع الثاني المصابين باعتلال الكلية السكري المبكر والمزمن

رسالة

مقدمة الى مجلس كلية الطب/ جامعة كربلاء

كجزء من متطلبات نيل شهادة الماجستير

في الكيمياء الحياتية السريرية

من قبل

حسين سعيد عباس جابر

بكالوريوس علوم كيمياء- كلية العلوم/ جامعة بابل، (2006)

إشراف

الأستاذ المساعد

د. شيماء زهراو ندى

إشراف

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د. علاء عباس فاضل

1443 هـ

2022 م