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CORRELATION OF OXIDATIVE STRESS AND SERUM INFLAMMATORY STATUS IN DIABETIC NEPHROPATHY

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AHMED SAPOORI SACHIT SACHIT

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CORRELATION OF OXIDATIVE STRESS AND SERUM INFLAMMATORY STATUS IN DIABETIC NEPHROPATHY

By Ahmed Sapoori Sachit SACHIT

July 2021

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

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ABSTRACT

CORRELATION OF OXIDATIVE STRESS AND SERUM INFLAMMATORY STATUS IN DIABETIC NEPHROPATHY

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Master of Science in Biochemistry

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July 2021

This study investigates the interaction between DN, inflammatory status, and oxidative stress. As well as the effect of oxidative stress biomarkers on patients with DN. The study population was 87 participants (Patients = 57, HCs = 30), the patients were divided into three stages (3, 4, and 5) based on the GFR values; according to (MDRD) equation. The current study showed that Adiponectin (ADP), Malondialdehyde (MDA), and Tumor necrosis factor-alpha (TNF-α) levels were elevated in all stages of disease progression as compared with healthy controls. When compared the increased levels of ADP, MDA, and TNF-α, we observed that the levels of ADP and MDA was higher increased compared to the slight increase in TNF-α. Where TNF-α considered an inflammatory state that leads to increased levels of ADP. Through the study of receiver operating characteristics curve analysis (ROC curve) the results have been showed that ADP and MDA have a high AUC in all stages (3, 4, and 5). While TNF-α was in lower AUC in all three stages. The results of the current study indicate that ADP can be considered as a good indicator to diagnosis in early stage of disease development, since it is an anti-inflammatory who's increased due to the presence of inflammatory cells represented by TNF-α. MDA is also considered as a good indicator of the progression of diabetic nephropathy, which explains the association of oxidative stress with DN.

2021, 100 pages

Keywords: Adiponectin, Malondialdehyed, Oxidative stress, Inflammatory status

ÖZET

DİYABETİK NEFROPATİDE OKSİDATİF STRES İLE SERUM İNFLAMATUAR DURUMUNUN KORELASYONU

Ahmed Sapoori Sachit SACHIT

Biyokimya, Yüksek Lisans

Tez Danışmanı: Prof. Dr. Ayşe ŞAHİN YAĞLIOĞLU

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Temmuz 2021

Bu çalışma DN, inflamatuar durum ve oksidatif stres arasındaki etkileşimi araştırmaktadır. Oksidatif stres biyobelirteçlerinin DN'li hastalar üzerindeki etkisinin yanı sıra. Çalışma popülasyonu 87 katılımcıydı (Hastalar = 57, SK = 30), hastalar GFR değerlerine göre üç aşamaya (3, 4 ve 5) ayrıldı; (MDRD) denklemine göre. Mevcut çalışma, Adiponektin (ADP), Malondialdehit (MDA) ve Tümör nekroz faktör-alfa (TNF-α) düzeylerinin, sağlıklı kontrollere kıyasla hastalık ilerlemesinin tüm aşamalarında yükseldiğini göstermiştir. Artan ADP, MDA ve TNF-α seviyeleri karşılaştırıldığında, TNF-α'daki hafif artışa kıyasla ADP ve MDA seviyelerinin daha yüksek arttığını gözlemledik. TNF-α'nın artmış ADP seviyelerine yol açan bir inflamatuar durum olarak kabul edildiği durumlarda. Alıcı çalışma karakteristikleri eğri analizi (ROC eğrisi) çalışması yoluyla, sonuçlar ADP ve MDA'nın tüm aşamalarda (3, 4 ve 5) yüksek bir AUC'ye sahip olduğunu göstermiştir. TNF-α, her üç aşamada da daha düşük AUC'de iken. Mevcut çalışmanın sonuçları, ADP'nin, TNF-α tarafından temsil edilen inflamatuar hücrelerin varlığı nedeniyle artan bir anti-inflamatuar olduğu için, hastalık gelişiminin erken evresinde tanı için iyi bir gösterge olarak kabul edilebileceğini göstermektedir. MDA, oksidatif stresin diyabetik nefropati ile ilişkisini açıklayan DNnin ilerlemesinin iyi bir göstergesi olarak da kabul edilir.

2021, 100 sayfa

Anahtar Kelimeler: Adiponektin, Malondialdehit, Oksidatif stres, İnflamatuvar durum

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Ahmed Sapoori Sachit SACHIT

Çankırı-2021

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LIST OF ABBREVIATIONS

8oHdG	8-oxo-7,8-dihydro-2-deoxyguanosine
AGEs	Advanced Glycated End-products
AII	Angiotensin II
AKI	Acute kidney Injury
BP	Blood pressure
CKD	Chronic kidney Disease
CKF	Chronic kidney failure
CPN	Chronic progressive nephropathy
CT	Computed tomography
CVD	Cardiovascular disease
DCKD	Diabetic chronic kidney disease
DKD	Diabetic Kidney Disease
DM	Diabetes mellitus
DM	Diabetes mellitus
DN	Diabetic Nephropathy
DPP-4	Dipeptidyl peptidase-4
DR	Diabetic retinopathy
E GFR	Estimated glomerular filtration rate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF	Epidermal growth factor
ELIZA	Enzyme linked immunosorbent assay
ESAs	Erythropoiesis-stimulating agents
ESRD	End-stage kidney disease
ET-1	Endothelin-1
EURODIAB	European diabetes patients
FGF	Fibroblast growth factor
FPF	False positive fraction
GAGs	Glycosaminoglycan's
GBM	Glomerular basement membrane
GBM	Glomerular basement membrane
G-CSF	Granulocyte colony-stimulating factor
GDF-15	Growth differentiation factor-15
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GLP-1	Glucagon-like peptide 1
HAC	Histone acetylation
HbA1C	Hemoglobin A1C
HDL	High-density lipoprotein

H-FABP	Heart fatty acid-binding protein
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IL	Interleukin
IP-10	Interferon gamma-induced protein
KIM-1	Kidney injury molecule 1
LDL	Low-density lipoproteins
L-FABP	Liver-type fatty acid-binding protein
L-PGDS	Lipocalin-type prostaglandin D synthase
MCP-1	Monocyte chemoattractant protein
MDA	Malondialdehyde
NAG	N-acetyl-β-D- glucosaminidase
NDRD	Non-diabetic kidney disease
NF-kB	Nuclear factor kappa B
NGAL	Neutrophil gelatinase-associated lipocalin
NGAL	Neutrophil gelatinase-associated lipocalin
NLRP3	Pyrin-like receptor family pyrin domain-containing 3
OD	Optical density
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PDR	Proliferative Diabetic Retinopathy
PKC	Protein kinase C
RAAS	Renin-angiotensin-aldosterone system
RANTES	Regulated on activation, normal T cell expressed and secreted
ROC	Receiver Operating Characteristic Curve
ROS	Reactive oxygen species
SNA	Sympathetic nervous activity
SNA	Sympathetic nervous activity
T1DM	Type1 Diabetes mellitus
T2DM	Type2 Diabetes mellitus
TG	Triglycerides
TKF	Terminal kidney failure
TNF-a	Tumor necrosis factor-alfa
TPF	True positive fraction
UAE	Urinary albumin excretion
UKPDS	United Kingdom Prospective Diabetes Study
VC	Vascular calcification
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

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1. INTRODUCTION

Metabolic diseases lead to diabetes, which in turn Sugar levels in the blood rise as a result of this, and usually this increase in sugar is caused by a defect in the work or secretion of insulin. High blood sugar in the long term, this results in a malfunction of the organs' functioning and eventual failure, the most important of which is the eyes, heart, kidneys, blood vessels and nerves (Association 2014). Diabetes is one of the leading causes of chronic kidney disease (CKD) all over the world. Diabetes is a condition that is closely linked to renal disease, and among these diseases is Diabetic Nephropathy (DN) which is known as Kimmelsteil-Wilson Syndrome and can also be known as Inter-capillary or Nodular Glomerulosclerosis (Vujicic *et al.* 2012). Kimmelstiel and Wilson, British and American physicians, originally defined DN Syndrome in 1936 (Kimmelstiel and Wilson 1936). In DN syndrome, proteinuria occurs, the rate of glomerular filtration decreases, and blood pressure increases (Muhammad and Nazar 2014).The prevalence or progression of DN may be caused by an increase in the microalbuminuria of diabetic patients, according to studies of European diabetes patients (EURODIAB), (Orchard *et al.* 1990), and a Danish study over 18 years (Chaturvedi *et al.* 2001). Microalbuminuria may occur after 7.3 years in patients with diabetes in type I by 12.6% and in By 33%, T2DM according to the findings of a research conducted in the yearly incidence of microalbuminuria in persons with T2DM in the United Kingdom is 2%, according to the United Kingdom Prospective Diabetes Study (UKPDS), and after 10 years the prevalence can progress to 25% (Adler *et al.* 2003). In the period 15-20 years Microalbuminuria can occur in 15-40% of T1DM patients (Hovind *et al.* 2004), while in T2DM the prevalence rate is between 5-20% (Adler *et al.* 2003). As for Native Americans and Americans of African and Asian descent, the prevalence of DN is very common (Young *et al.* 2003). In T1DM patients, kidney disease is the highest percentage of T2DM complication in Caucasian (Vujicic *et al.* 2012). In India, DN is interesting, in a study published where 15% of people have DN after 20 years, out of 50% have T2DM (Craig *et al.* 2003). To detect the Kidney disease, including DN, can occur in newly diagnosed diabetic patients, the sugar level in the blood must be managed. as well as testing the level of albumin, and controlling the diastolic and systolic blood pressure (NICE 2011). In terms

of renal and cardiovascular disease (CVD) prevention and postponement in individuals with DN, controlling blood sugar levels and managing comorbidities including anemia, dyslipidemia, hypertension, and hyperphosphatemia are critical. Thus, to optimize treatment for these complex patients, a multidisciplinary approach is necessary. In addition, focus is now being given to innovative therapies directed at the complicated pathophysiology of DN (Kim *et al.* 2016). In the pathophysiology of DN, the same sequence found is: hyperglycemia, GBM thickening, hyper-filtration of glomerular, deterioration of endothelial integrity, microalbuminuria onset, impaired transport of nitric oxide, loss of auto-regulatory power of afferent/efferent and continuing loss of potential for glomerular filtration (Muhammad and Nazar 2014). Early and timely diagnosis for the diagnosis and treatment of DN with specific diagnostic modalities is advantageous. In order to detect kidney injury beyond albuminuria, several Researchers are increasingly concentrating on early biomarkers. In addition, the use of a combination of cutting-edge treatments and tried-and-true traditional methods might help to alleviate the enormous burden of DN (Kim *et al.* 2016). Three major mechanisms have been established for the acquisition of possible markers: kidney injury, Low-grade inflammation and oxidative stress, as well as atherosclerosis and vascular damage (Agnes *et al.* 2010). Since Tubulointerstitial injury may potentially play a role in the development of DN (Kim *et al.* 2016).

Importance of this study Diabetes mellitus (DM) has long been recognized as one of the four main non communicable illnesses, it needs immediate attention from all key stakeholders throughout the world in order to address its prevalence and consequences. It is seen as the world's third biggest risk factor for early death owing to hyperglycemia and hyperglycemic-induced oxidative stress and inflammation (Oguntibeju 2019). Diabetic kidney disease (DKD) is a serious diabetic consequence. causes significant morbidity and death. It is defined as chronic renal failure over a duration of three months or more (Amorim *et al.* 2019). In the development of diabetes problems, oxidative stress has been identified as a recognized route (Rehman and Akash 2017). Hyperglycemic-induced oxidative stress is thought to raise pro-inflammatory protein levels, with infiltrating inflammatory cytokines are secreted by macrophages, As a result, local and systemic inflammation develops (Yaribeygi *et al.* 2020). Biomarkers

are becoming more significant for predicting illness prognosis, personalizing therapy (precision medicine), and identifying early therapeutic and adverse drug reactions. Biomarker discovery, validation, and application, on the other hand, are difficult due to a variety of factors, including as an understanding of the biology of the biomarker and its relevance to illness, as well as the technological features of the assay used to assess biomarkers (Ziegler *et al.* 2019). Therefore, it is important to find new biomarkers for predicting initiation and progression of the disease.

This study examines the relationship between diabetes, inflammation, and oxidative stress, as well as the implications of possible diagnostic oxidative stress biomarkers for patients with DN.

2. LITERATURE REVIEW

2.1 Kidney

One of the most relevant tissues studied is the kidneys. It is also because of its function in filtering, it is the location of test-article-induced lesions, digestion, as well as compounds excretion. Moreover, it is possible to find a wide variety of random renal lesions. Chemical administration can worsen chronic progressive nephropathy (CPN) is a rat illness that develops spontaneously as they become older. And in the analysis of renal toxicologic and carcinogenic results, it is a troubling element (Seely and Brix 2005), as shown in (Figure 2.1).

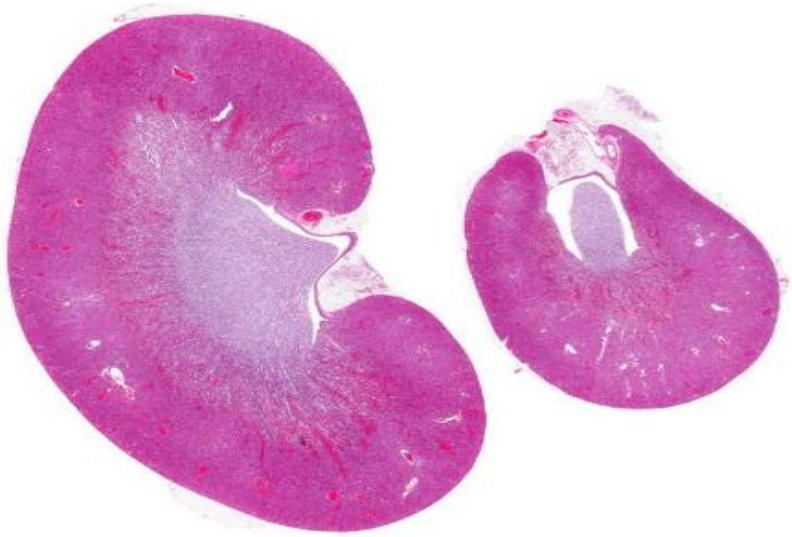

Figure 2.1 Cross-sectioned right kidneys Generally and Longitudinally sectioned left The two kidneys are at the rear of the body, behind the waist, behind the lower ribs (Seely and Brix 2005)

2.2 Kidney Anatomy

Each adult kidney weights about 150 g and is about the size of a hand. Two areas are seen as the kidney is sectioned (Figure 2.2). The cortex is the outermost section, and the medulla is the innermost part. Normally, the cortex is a dark reddish brown color, with a granulated texture. The cortex is found in both the glomeruli, tangled tubules and

cortical collecting ducts. The medulla has a striated appearance and is lighter in color. look, arising from the concurrent structure of Henle chains, the medulla blood vessels and the medullary collecting ducts the medulla, on the other hand, can be split into an outer and an inner medulla. That is similar the cortex has an outer medulla that is closer to the cortex and an inner medulla that is farther away from the cortex. The human kidney is divided into a series of lobes, usually eight to ten in number. A medullary tissue pyramid makes up each lobe, plus the cortical tissue that covers its base (Delanaye 2019).

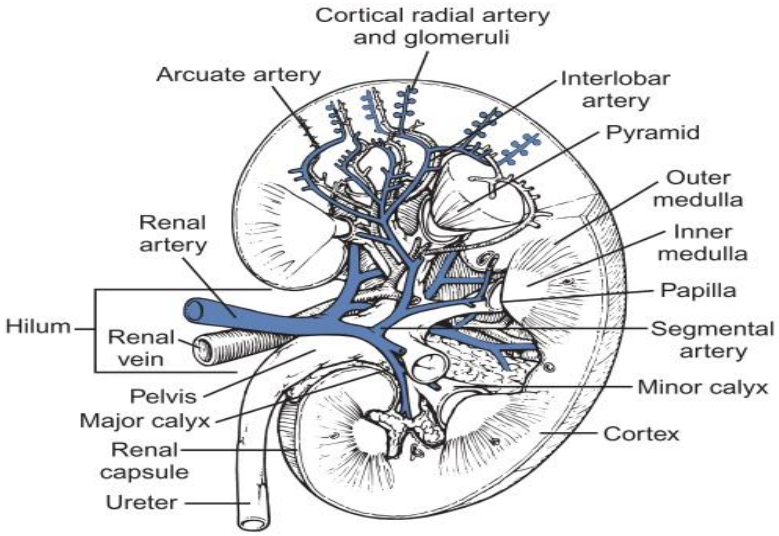

Figure 2.2 The human kidney, sectioned vertically

2.3 Kidney Functions

1. By excreting osmotically filtered or condensed urine, they regulate bodily fluids' osmotic pressure (osmolality).
2. The levels Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and bicarbonate are some of the ions found in blood plasma (HCO_3^-), phosphate, and sulfate, are controlled.
3. They contribute to acid-base balance by excreting H^+ when there is an excess of acid. When there is an overabundance of HCO_3^- excreted.
4. By regulating Na^+ and water excretion, they regulate the ECF's amount.

5. By changing Na^+, they aid in the monitoring of arterial blood pressure through excretion. and the development of different substances that can influence blood pressure (e.g., renin).

6. Metabolic waste products, includes urea (human protein metabolism's major nitrogen-containing end product), uric acid (purine metabolism's end product), and creatinine (muscle metabolism's end product) are removed.

7. Many medications (e.g., penicillin) and foreign or harmful substances are excluded.

8. They are the main development sites for some hormones, including erythropoietin and vitamin D_3 (1,25-dihydroxy).

9. Many insulin, glucagon, and parathyroid hormones are polypeptide hormones, are degraded.

10. They produce ammonia, which contributes to the acid-base balance.

11. They synthesize substances influencing the supply of renal blood and Na^+ excretions, including derivatives of arachidonic acid and kallikrein (Delanaye 2019).

2.4 Kidney Diseases

For having a healthy body, kidneys are necessary. They are largely responsible for washing the blood out of waste materials, excess water, and other impurities are all examples of impurities. In the bladder, these toxins are retained and later expelled during urination. In the body, the kidneys still regulate pH, sodium, and potassium levels. They manufacture hormones that stabilize blood pressure and monitor red blood cell development. A source of vitamin D that helps the body absorb calcium is also activated by the kidneys. Roughly 26 million American adults are affected by kidney failure. It happens when the kidneys get weakened and are unable to perform their work. Diabetes, high blood pressure, and a number of other chronic (long-term) illnesses can all result in damage. Other medical problems, kidney disease can be caused by a variety of factors, inclusive weak bones, nerve harm, and starvation. The kidneys can stop functioning entirely if the condition worsens over time. This suggests that the work of the kidneys would require dialysis. Dialysis is a method of filtering and purifying blood by the use of a machine. It won't help you recover from renal failure. but it can save or extend the lives of the patients (Hiß and Kielstein 2014)**.**

2.4.1 Diagnosis

For the purpose of diagnosing kidney disease, some representative tests can be performed

- Glomerular filtration rate (GFR)
- computed tomography or Ultrasound (CT) Scan
- Biopsy of the kidneys
- Albumin Levels in Urine
- Creatinine level in the blood

2.4.2 Etiology

Diabetes, hypertension, older age groups, and African American ethnicities are significant risk factors for growth and development of CKD. Almost 45% of kidney failure cases are due to diabetes, and chronic hypertension accounts for another 20%. Primary glomerulonephritis, lupus, and other less frequent conditions include polycystic kidney disease but relevant causes. More than 10 million Americans have diabetes, while 40-50 million individuals in the United States suffer from hypertension, making kidney disease a major at-risk population. Diabetes and hypertension are also significant CVD peril factors and the high incidence of the CVD in the CKD Patients is also influential to some degree (Weiner 2007).

2.5 Complications of CKD

A. Rectification of anemia. Anemia is normal in CKD patients, as Kidney disease is progressing, increasing the frequency and prevalence of anemia. CKD anemia is multifactorial in terms of etiology. The most frequent reasons are erythropoietin insufficiency, inflammation and iron deficiency

B. Iron deficiency correction. Iron deficiency is found in about 40% of non-dialysis CKD patients and is the most common source of apparent resistance to erythropoiesis-

stimulating agents (ESAs). There are several reasons for iron deficiency, including gastrointestinal injuries, causing unknown bleeding, nutritional deficiency and blood loss due to blood withdraw, the last of which is a defect and a decrease in iron absorption.

C. CKD-Mineral and Bone Dysfunction (CKD-MBD). CKD-MBD pathogenesis as seen in the (Figure 2.3) for serum phosphate, vitamin D, and parathyroid treatment (Daugirdas *et al.* 2014).

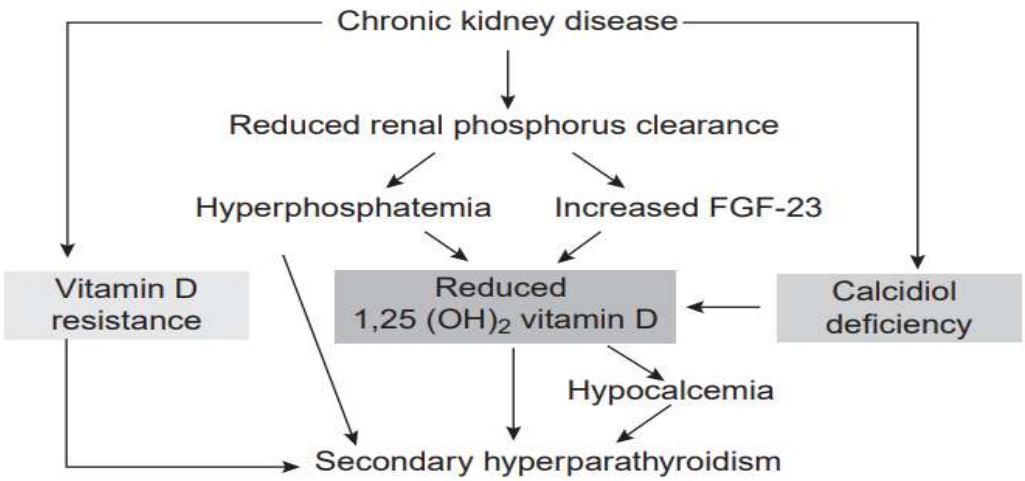

Figure 2.3 CKD-MBD pathogenesis

2.6 Dialysis and Types of Dialysis

For patients with metabolic aberrations of end stage renal diseases (ESRD) and uremia, renal dialysis is required. Two dialysis procedures are available hemodialysis and peritoneal dialysis are two treatments for uremia.

2.6.1 Hemodialysis

For people suffering from End-Stage Renal Failure, this is currently the primary care modality. In this process, the blood of the patient flows into an extracorporeal dialyzer, a system that reveals the highest possible region of a semipermeable membrane to a

minimal amount of blood. When the blood flows from one of the membrane's arms. A current of dialysate, a neutral electrolyte solution, running over the other, continues to come into diffusion equilibrium. Fluid elimination is achieved by differing blood pressure dialyzers

2.6.2 Peritoneal dialysis

It is used for acute renal dysfunction or hyperkalemia. The 1 or 2 liter dialysate bottles are hung so that the solvent flows through the peritoneal cavity through gravity, where diffusion and osmosis take place. Thus, fluids withdrawn flow into the drainage container or bag through gravity. This is called exchange, and it is generally considered one dialysis per 40 liters of exchange. Since this approach uses an open procedure, also called the manual method, the patient is also vulnerable to infection. This technique, however, is incredibly simple, requires little or no demand, and can be performed on an emergency basis in even the most remote or small hospitals (Tabish 1968).

2.7 Components of Glomerular Filtration Barrier

The extracellular matrix component of the selectively permeable glomerular filtration barrier (GFB) that separates the vasculature from the urine area is the glomerular basement membrane (GBM). The glomerular endothelial cells that line the glomerular capillaries and the podocytes (also known as visceral epithelial cells) that lie on the opposite side of the GBM inside the urine space are the first to produce it. Endothelium, GBM, and podocytes make up this three-layered structure the flow of plasma water and tiny solutes is aided, whereas the flow of big plasma proteins like albumin is restricted. The presence of excessive amounts of albumin in the urine is thought to be a sign of a malfunction in at least one of the GFB layers (Miner 2012).

The GBM is made of mostly of laminin, type IV collagen, nidogen, and heparin sulfate proteoglycan, as do all basement membranes. The GBM, however, is very dense and contains specific members of these broad protein families, such as laminin-521, collagen α3α4α5(IV), and, once again. The laminin and type IV collagen components are particularly important for GBM structure and function, according to knockout

studies in mice and genetic findings in humans, as laminin or collagen IV gene mutations cause filtration defects and renal disease of varying severity, depending on the nature of the mutations. According to these findings, the GBM plays a critical role in creating and sustaining the GFB (Miner 2012). In this systematic analysis of GBM-DP in DN, we found that the involvement of GBM-DP in patients with type 2 DN was correlated with an increased risk of ESRD, independent of clinical features such as eGFR, UP, and BP are all measures of kidney function. To our knowledge, this is the first study to establish a relationship between GBM-DP in DN and clinical outcomes. In 62.5 percent of our patients, GBM-DP was found in our However, the cohort has not yet been thoroughly tested for GBM-DP in DN,GBM-DP may be one of the most pathological results of diabetic patients (Yamakawa *et al.* 2019).

2.8 Basic Aspects of DN

2.8.1 Definition of DN

DN is a kidney illness that is complex in the final stages that occurs due to environmental and genetic factors, its many cellular mechanisms and molecular changes that cause kidney damage for diabetic patients. It is difficult in that there are no indications showing the pathological changes of the kidneys that allows this disease to be developed (Ortega et al., 2020).

According to Bright's description at Guy's Hospital in 1836, the existence of albumin in the urine, was a serious sign of kidney disease, and this description was a marker of the emergence of clinical nephrology. These notes were also made by Cotunnius Diabetic urine was discovered in 1770 and 1798 by Rollo. Patients contains proteins. In 1840 these observations led Rayer to suggest that diabetes causes a form of Bright's disease. Epidemiological studies have shown that 20-40% of diabetic patients will develop proteinuria, and on average diabetes usually develops 15-20 years after the beginning of the disease. they develop kidney failure. The average survival after the onset of proteinuria and not receiving kidney support treatment is only 5 years (Parchwani and Upadhyah 2012).

DN, also known as Kimmilsteel-Wilson syndrome, because this illness is defined by the existence of albumin in urine (albuminuria) as well as decreased glomerular filtration and is produced in the long run by TIDM and T2DM. This syndrome was first described by Paul Kimmelstiel and Clifford Wilson in 1936 (Kimmelstiel and Wilson 1936).

2.8.2 Epidemiology of diabetic nephropathy

Diabetes is a worldwide epidemic, along with the cost of health care and deaths. Diabetes can be defined as a burden, as the prevalence of diabetes in the year 2000 was about 171 million, and this number increased by 2035, the population is anticipated to grow to 592 million, up from 382 million in 2013. That's 8-10% of the world's population, which results in about $ 548 billion being spent on diabetes care. Type 2 diabetes makes up 85- 90% of people with diabetes in general. In the United States alone, there were 25.8 million people with diabetes in 2011.Adults and children suffer from diabetes, as do 79 million people with pre-diabetes. In 2009-2011 in most countries diabetes was caused in people with ESRD, as the long-term development of TIDM and T2DM leads to diabetic nephropathy. About 60% of people with ESRD in Singapore, Mexico and Malaysia were caused by long-term diabetes. Likewise, 40-50% of the countries in Israel, Korea, China, Taiwan, Japan, the USA, and New Zealand, ESRD can occur, due to high diabetes. In 2011 the accident rates for high-blood sugar-related kidney disease patients in the USA were 44,266 and 584 per million for the (20-44, 45-64) and (65-74) years age groups, respectively (Lim 2014).

Diabetes, which is the most joint cause of ESRD. It also leads to blindness in adults, amputation after trauma as well as a risk factor for vascular atherosclerosis. Due to the impact of diabetes on more than 285 million around the world, the disease is expected to increase to nearly 50% in the year 2030 in developing countries Africa, South America and Asia. India currently leads the world with the largest number of diabetics, 50 million, and it is expected to increase by 2030 to 87 million Indians, which puts a burden on the health budget in the country (Parchwani and Upadhyah 2012).

2.8.3 Stages and clinical course diabetic nephropathy

Protein is not found in the urine of a substantial percentage of diabetes individuals with impaired renal glomerular filtration. However, many TIDM patients show protein in the urine without any changes in the renal GFR. Thus, DN is classified as Diabetic Kidney Disease (DKD) and it is interesting in that these stages in TIDM diabetes do not occur with T2DM. As the late diagnosis is often made by the emergence of co-occurring disorders such as high blood pressure and protein in the urine and kidney failure, as a result, a new name for diabetic chronic kidney disease has emerged (DCKD) has been suggested instead of DN to know the extent of kidney damage. It is also recommended to have a periodic annual examination for patients with T2DM (Sulaiman 2019). DN is associated with TIDM and T2DM, and there are five stages of its development.

Stage I: In this stage, the GFR either increases or is within the normal level and this stage lasts 5 years, the kidney size increases by 20% and the rate of renal plasma flow increases 10-15%, while protein in the urine and blood pressure stays at acceptable limits (Vujicic *et al.* 2012).

Stage II: This stage, known as the calm phase, occurs two years after the commencement of the illness and is marked by mesangial proliferation, kidney injury, and basement membrane thickening. And usually no clinical signs appear at this stage and the renal glomerular filtration can return to normal values and it is normal for many patients are expected to stay in this stage till the end of their lives (Vujicic *et al.* 2012, Sulaiman 2019).

Stage III: At this stage, albumin appears in the urine, but in small quantities of 30-300 mg /day. This may be the first sign of its clinical detection, This stage may occur from 5-10 years from the onset of the disease and at this stage blood pressure either rises or remainsnormal, and the patients who reach this stage is 40% (Sulaiman 2019).

IIIA: The GFR may be slightly decreased and no evidence of kidney damage appears (Muhammad and Nazar 2014).

IIIB: Irreversible kidney damage may occur (Muhammad and Nazar 2014).

Stage IV: This stage leads to chronic kidney failure (CKF) which is an advanced stage of the disease, and the albumin increases in the urine greater than 300 mg /day. In addition, the rate of renal glomerular filtration decrease a flow rate of lower than 60 ml / min (Sulaiman 2019).

Stage V: This stage is called terminal kidney failure (TKF) where the rate of glomerular renal filtration decrease to lower than 15 ml / min. Patients who reach this stage are treated either by kidney replacement, peritoneal dialysis, or hemodialysis (Vujicic *et al.* 2012).

In early stage of DN, changes only appear in the size of the kidneys and Doppler indicators, while changes that occur in the rate of renal the amount of protein in the urine and glomerular filtration are two of the strongest markers of the degree of injury (Vujicic *et al.* 2012).

Usually, DN is linked to three factors, including high blood pressure, albumin in urine, and renal impairment. For an accurate diagnosis, there are 5stages of DN for the purpose of assessing kidney function including (Papadopoulou *et al.* 2017).

1- Hyper filtration.
2- The Silent Stage.
3- Microalbuminuria.
4- Macro albuminuria.
5- Renal impairment.

2.8.4 Differential diagnosis of diabetic nephropathy

One of the most important problems that led to the boost in the number of people with DN is the lack of clarity of the vital signs of people with this disease, and this justifies the need for vital indicators for early diagnosis of the disease, which identifies rapidly advanced patients in ERSD. De Bruyne method, which is discovered by conventional pathology, is new and one of the most important diagnostic methods that shows biochemical changes in the renal cortical tissues before the renal tissue damage. This method consists of applying near infrared spectroscopy to kidney biopsies which has no

tissue destructive effect and can be used on routine stained tissues. Where spectral changes appear related to the reactions of carbamoyl and glycation, which is a biochemical indicator of DN. This method can have an important role in analyzing pathological tissues and there is also an important point in the management of DN which is that it must calculate the ability or functions of the kidneys using mathematical algorithms, including levels of creatinine in serum or cystatin-C, gender, weight and height to obtain the estimated GFR.

According to the study by Luis-Lima, which showed an error in the estimated GFR of 30% in patients with diabetes compared to the GFR that was measured Patients with a GFR of lower than 60 ml/min have a higher danger of this mistake. The use of creatinine levels in serum and cysteine-C for type 2 diabetics showed a failure to reverse renal function in GFR. The use of microalbuminuria is an important urinary biomarker in diagnosing DN and knowing the extent of kidney damage.

However, better urinary biomarkers are required. A urinary proteomics method was used in Koziolek's manuscript to classify in a large cohort research (563 diabetic patients). tiny proteins and peptides were linked to pathological alterations in the early stages of diabetes illness. Between DN and healthy controls, they found many differentially expressed proteins, Apolipoprotein A-I, beta-2 microglobulin, cadherin in epithelial cells, Lithostathine-1alpha, and Lithostathine-1alpha (E-cadherin). They identified one of them as a critical protein in diabetic kidney damage. E-cadherin which showed that its levels of urinary excretion were sufficiently sensitive to differentiate between the various stages of CKD, indicating that E-cadherin is a possible diabetic patients have a urine biomarker. Knowing the vital signs of cardiovascular risk is critical since cardiovascular events are a leading cause of mortality in DN. Vascular calcification (VC) can be considered an important predictor of the risk of developing CVD in patients with CKD.VC can be caused by calcium phosphate mineral deposits in the middle and inner layers of blood vessels that upset internal calcification inhibitors abnormal mineral metabolism and inflammation (Ortega *et al.* 2020).

There are several early detection signs of DN, NGAL (neutrophil gelatinase-associated lipocalin), chitinase-3-like protein 1 (YKL-40), cystatin C, and plasma growth

differentiation factor-15 are all found in serum and urine (GDF-15) (Papadopoulou *et al.* 2017).

2.8.5 Pathogenesis of diabetic nephropathy

- Caveolae are 50 to 100 nm flask-shaped plaques of cholesterol-rich plasma membrane and glycosphingolipids in which functional changes occur when the membranes are irritated and known as the presence of cavolin necessary for their biosynthesis, acting as a center for intracellular signaling. Many signaling proteins are found in caveolae, primarily due to their interaction with caveolin. They are thought to function as a central hub for intracellular signaling, allowing for the compartmentalization of signaling proteins and therefore effective signal transduction, or sequestering and restricting the activation of signaling cascades in some circumstances. It works on arranging and dividing the signals that transmit them with high efficiency. The caveolin genes consist of cav1, cav2, and cav3 The cav1, 2 is found in most organs and the cav3 is found in the skeletal muscles, the heart, and the diaphragm.Whereascav1 was found essential as a signaling including the kidney.Cav1 stimulation is expressed in both human and experimental glomerular diseases, including DN, so the importance of cav1 and caveolae lie in their pathogenesis (Krieken and Krepinsky 2017). (Table 2.1) shows the effect of histone acetylation (HAc) on, oxidative stress, inflammation and fibrosis in progression and development of DN (Li *et al.* 2016).

Table 2.1 Showing the effect of histone acetylation (HAc) on, oxidative stress, inflammation and fibrosis in progression and development of DN (Li *et al.* 2016)

Ac proteins[1]	Acetylation site	Effects in DN
Histone lysine	H_3K_9	Diabetic glomerulosclerosis with advanced inflammation
	$H_3K_{9/14}$	Hypertrophy, fibrosis; increased miR-192 expression; inflammation
	H_3K_{18}	Glomerulosclerosis in advanced diabetics; inflammation
	H_3K_{23}	Glomerulosclerosis in advanced diabetics
	H_3K_{27}	Increased miR-192 expression
	H_4	Apoptosis of cells, proteinuria, and a rise in Scr
	$H_4K_{5/8/12}$	Inflammation

- Hyperglycemia may not be a factor in increased diabetic filtration, as glomerular filtration may persist in TIDM diabetics even after it is controlled and regulated. There are several potential pathways to the pathogenesis of DN (Papadopoulou *et al*. 2017).

1- mitochondrial reactive oxygen species (ROS).

2- cumulation of Advanced Glycated End-products (AGEs).

3- Protein kinase C (PKC).

4- Prorenin activity is higher in children and teenagers

5- Nephrin.

- The immune response is considered mainly in DN. The inflammatory immune response relies heavily on the inflammasome family, which includes receptors and regulators. Other inflammasome components, such as the nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3), can recognize innate danger signals, resulting. The result of which is the activation of caspase-1, interleukin (IL) -1β, IL-18 and other cytokines. These changes stimulate the inflammatory cascade reaction that leads to DN. Other factors that activate NLRP3 are high lipids, high sugar, and high uric acid in the blood (Qiu and Tang 2016).
- The Autophagic Route Upstream- in these intrinsic renal cells, for example, autophagy induction, a number of autophagic vacuoles, and so on, the direction, but not the entire course, was predominantly studied. In most cases, there was

[1] Acetylation proteins

autophagic inactivation, an underlying important mechanism progression of DN. Targeting the autophagic pathway to activate autophagy activity may have a Reno protective-effect. However, autophagic activation was also found in a few studies in which the role of activated autophagy was de-bate: mounting an adaptive response or leading to autophagic apoptosis (Figure 2.4) (Liu *et al.* 2018).

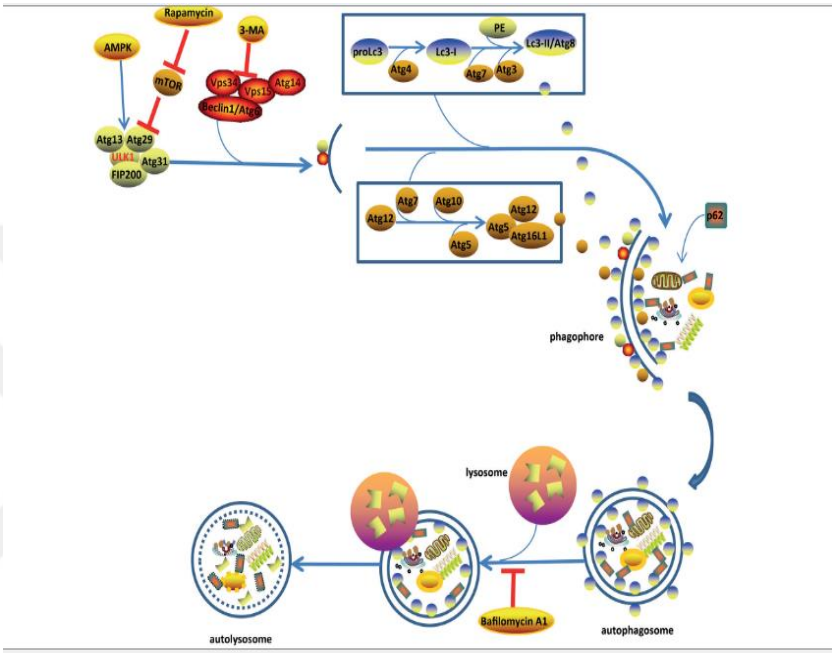

Figure 2.4 The diagram illustrates the pathway of autophagy (Liu *et al*. 2018)

2.9 Pathophysiology of DN

2.9.1 Renal structural damage

DKD is related to a series of changes in the pathological anatomy of the kidneys, and these changes reflect the functions of the kidneys, including the thickness of the GBM. These changes are quantifiable in DKD, this thickening is caused by an accumulation of extracellular matrix (ECM) components of these ingredients

- Collagen type IV
- Laminins
- Nidogen / entactin in the lamina rarainterna of the GBM.

The changes that take place in the GBM are a transition in the collagen chains between a1 and a2 to the a3 and a4 chains. This transfer affects the structural quality of GBM, which can be an indication of a change in the thickness (Khoury *et al.* 2020).

2.9.2 Risk factors of DKD

The risk factors for DKD are classified into several factors, including sensitivity factors (gender, age, family history, ethnicity) starting factors (hyperglycemia and acute kidney injury (AKI)) and factors of progress (obesity, nutritional factors, high blood pressure). Among the most important of these factors are high blood pressure and high blood sugar (Alicic *et al.* 2017).

2.9.2.1 Hyperglycemia factors

In TIDM with normal albumin in the urine, blood control is the indicator that there is no development in the appearance of albumin in the urine. There are two experiments conducted on patients with TIDM and T2DM. It is the management of blood glucose levels. and this control has shown a positive result in preventing the development of DKD. The control of the hemoglobin A1C (HbA1C) level was <7%, and this control the chance of acquiring microalbuminuria was decreased by 9 years. by 34% and macro albuminuria by 56%.Likewise, for people with T2DM , after controlling their blood glucose levels there was a decrease in the rate of development of microvascular complications, including DKD to 24%,as well as a decrease in the rate of development of proteinuria to 33% (Alicic *et al.* 2017).

2.9.2.2 Hypertension factors

Studies have shown that controlling blood pressure in TIDM and T2DM diabetics reduces the risk of developing microalbuminuria (Lin *et al.* 2018). In DN patients there is a risk of developing the disease with continuous high blood pressure, so the lifestyle must be modified, including microalbuminuria detection, blood glucose management, and blood pressure normalization (Wagnew *et al.* 2018).

2.9.2.3 Obesity, metabolic factors

Clinically, population and basic research indicated that visceral obesity is the main cause of metabolic syndrome and T2DM as a cause of high blood pressure. Population studies have indicated that 78% of men and 65% of women have high blood pressure due to weight gain. Clinical studies have indicated that weight loss reduces blood pressure in hypertensive patients (Maric and Hall 2011). Nuclear factor kappa B(NF-kB) may be activated via hyperglycemia, and obesity may activate ROS and PKC work together to boost cytokine expression quickly. When NF-kB trans is activated, it travels to the nucleus. Rapidly increasing the transcription of genes such as endothelin-1, intercellular adhesion molecule-1, VCAM-1, TNF-α, and IL-6, which promote DN development (Mima 2013).

2.9.2.4 Hemodynamic factors

The excess of amino acids in the blood (aminoacidemia) leads to the stimulation of several factors, including high blood sugar, increased glomerular filtration, increased perfusion. These factors lead to damage and inflammation in early diabetes as they have a hemodynamic effect in the kidneys. At the same time, high local development of angiotensin II (AII) in the efferent artery causes vasoconstriction. High intraglomerular pressure and glomerular pressure are the overall influences (Figure 2.5) (Alicic *et al.* 2017).

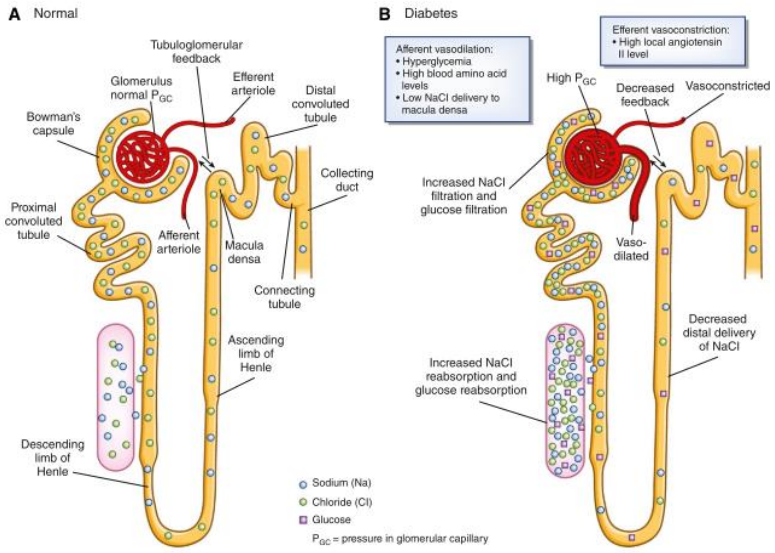

Figure 2.5 A drawing showing the normal and DN with renal hemodynamics (Alicic *et al.* 2017)

2.9.2.5 Cardiovascular complications

Diabetes is associated significantly with microvascular and macrovascular, the risk of developing cardiovascular atherosclerosis increases in patients with TIDM and T2DM diabetes. In long-term diabetics, the risk of CVD increases to 67% for men and 57% for women over the age of 50. Fatal Coronary Heart Disease Incidence in patients with diabetes was around 3.5-fold greater than in those without diabetes. The relative risk for diabetes-related fatal women have a 50% greater risk of coronary heart disease than males (Figure 2.6) (Lau and Shen 2013).

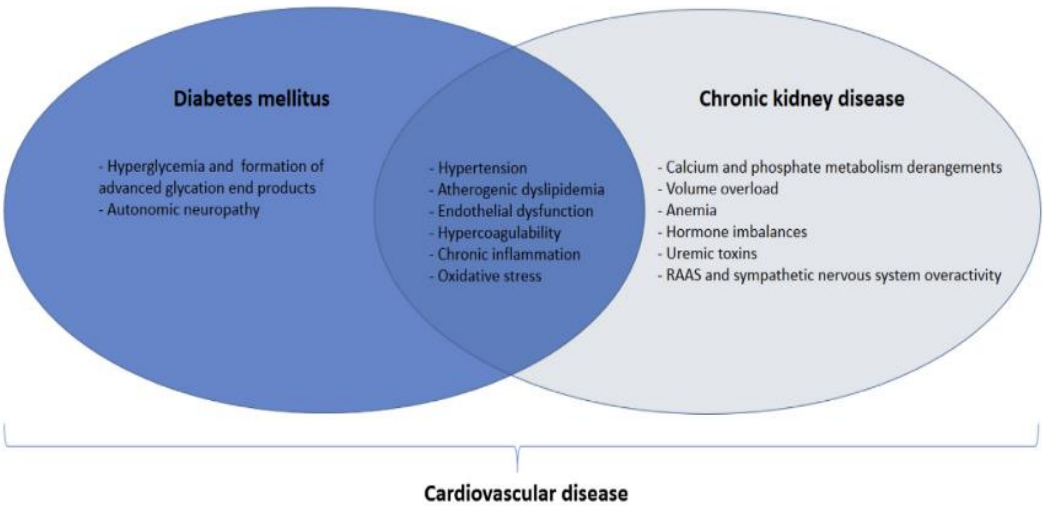

Figure 2.6 Patients with DM and CKD, an overview of the main known and proposed mechanisms of cardiovascular disease (Pálsson and Patel 2014)

2.9.2.6 Lipid disorder

Many lipoprotein abnormalities have been linked to DN, including increased plasma levels of VLDL, LDL, and triglycerides (TG), as well as decreased levels of HDL, are considered to be correlated with DN. Many studies have documented that through mediators such as chemokines, ROS, cytokines, and through hemodynamic changes, lipids can stimulate both glomerular and tubulointerstitial injury. Clinical trials in patients with DN have shown that an additional benefit of proteinuria reduction can be associated with lipid regulation. Experimental studies have shown that lipid-lowering agents exert a certain degree of Reno protection, both through indirect lipid-lowering effects and through a direct cell defense effect. In the prevention and treatment of DN, lipid regulation appears to be significant (Chen *et al.* 2005).

2.9.2.7 Adiponectin with DN

Adiponectin which is consequent from adipose tissue, is excreted only in the kidneys and a little in the liver, it is considered to serve a secondary role. clearance physiology.

Only monomers and dimers will cross the GFB and be contained in the urine because to the adiponectin monomer's large molecular weight (28 kDa). In the kidneys, adiponectin is primarily found in the arterial endothelium and smooth muscle cells. It is also found in the capillary endothelium and, to a lesser extent, in the epithelial cells of the proximal and distal tubules. Adiponectin is secreted in inflammatory state , oxidative stress and sympathetic nervous activity, which are joint in (CKD) (Przybyciński *et al.* 2020). It TNF receptor activation for fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), has anti-inflammatory properties (FGF). Overexpression of adiponectin in diabetic rats may maintain nephrin, reduce TGF expression, and lessen albuminuria. It is yet unclear if adiponectin will have a substantial impact on human DN (Mima 2013).

2.9.2.8 Tumor necrosis factor-α of DN

Increasing pro-inflammatory cytokine levels in diabetic patients indicates that diabetic complications may be associated with inflammation. Diabetic rats' peritoneal macrophages were cultured with GBM were documented to produce higher TNF-α and interleukin (IL)-1 levels than macrophages incubated with normal rat membranes. These authors indicated that the DN may include pro-inflammatory cytokines production. TNF-mRNA levels in the glomeruli of diabetic rats have been found to rise for a long time in previous investigations (24 and 52 weeks) after diabetes induction (Navarro *et al.* 2005).Inflammatory cytokines are TNF- α, IL-1, IL-6, and IL-18 are all implicated in the development and progression of DN, since inflammation plays a key part in the process. Remarkably, high levels of systemic TNF receptors were correlated with progression to ESKD in patients with T2DM. In patients with T2DM, urinary TNF-alpha excretion was correlated with the incidence of glomerular and tubulointerstitial injuries (Samuel 2017).

2.10 Genetics of Diabetic Nephropathy

DN's mode of genetic transmission is still controversial. Theoretically, it may occur in three distinct types, as in other diseases, which would contribute to DN growth. **Monogenic** mutations: are those that occur in a gene that has a dominant function.

Oligogenic form: mutations/polymorphisms in a few genes would enhance vulnerability in an independent and cumulative manner. **Polygenic** form: changes in several DNA loci, each with a little but cumulative influence on DN development.

Given that DN is a complex illness, the method of transmission is likely polygenic, as evidenced by genetic correlations with other environmental variables and clinical data. including DM length, arterial hypertension, dyslipidemia, and smoking are likely to contribute to DN development. Since multiple genes may be involved in DN development and several of them are not yet identified, in most cases it is impossible to know the exact pattern of heredity with certainty (Rippin *et al.* 2001).

2.11 Prevention and Treatment of DN

The treatment of the risk factors that have been identified, is the basis for preventing DN: These are also, CVD risk factors that should be addressed aggressively: hyperglycemia, hypertension, dyslipidemia, and smoking (Gross *et al.* 2005).There are several methods for preventing or treating DN, but there is no question that glycemic regulation is of vital importance. In a clinical study entitled 'Renal Protective Effect in T2DM Patients with Dipeptidyl peptidase-4 DPP-4 Inhibitors': Kim *et al.* investigated the effectiveness of dipeptidyl-peptidase IV (DPP-4i) inhibitors in the prevention of DN in 414 T2DM patients and discovered that long-term DPP-4i therapy might delay DN progression by reducing urine albumin excretion and reducing eGFR decline. Animal models, which include DN, are professional instruments for the study of novel treatment methods (Shen *et al.* 2017).

2.12 Biomarkers for Prediction of DN

In the tables below, it is possible to clarify the biomarkers by which the occurrence of diabetic nephropathy can be expected, the (Table 2.2) shows The Novel Biomarkers Classification. Also known as glomerular biomarkers. As for the (Table 2.3) shows the new diabetic nephropathy biomarkers Occurs prior to microalbuminuria in urine

Table 2.2 The Novel Biomarkers Classification (Samuel 2017)

Glomerular biomarkers	Tubular biomarkers	Biomarkers of oxidative stress	Biomarkers of inflammation	Miscellaneous biomarkers
Transferrin	NGAL	8oHdG	TNF-α ‡[1]	veGF ‡
Albumin	KIM-1		IL-1β ‡	Podocalyxin ‡
IgG	NAG		IL-8 ‡	Nephrin ‡
Ceruloplasmin	Cystatin C		IL-18 ‡	H-FABP #[2]
Type Iv collagen	L-FABP		IP-10 ‡	Retinol-binding protein #
Laminin	α-1-microglobulin		MCP-1 ‡	AGe
GAGs			G-CSF ‡	
Fibronectin			Eotaxin ‡	
L-PGDS			Orosomucoid	
			RANTES ‡	

Table 2.3 The new diabetic nephropathy biomarkers (Samuel 2017)

Biomarker	Type of renal injury	T1DM[3]	T2DM[4]	Pre-microalbuminuria[5]	Predicts microalbuminuria
Urinary transferrin	Glomerular injury	+	+	+	+
Urinary TNF-α	Glomerular injury	+	+	+	+
Urinary type Iv collagen	Glomerular/tubular injury	+	+	+	+
Urinary fibronectin	Glomerular injury	+	+		
Urinary GAGs	Glomerular injury	+	+	+	
Urinary NAG	Tubular injury	+	+	+	+
Urinary L-PGDS	Glomerular injury		+	+	+

[1] Glomerular biomarkers
[2] Tubular biomarkers
[3] Type1 diabetes mellitus
[4] Type2 diabetes mellitus
[5] Occurs prior to microalbuminuria in urine

2.13 The Dilemma of The Remnant Kidney Hypothesis of Clinical DN

When a disease or kidney defect occurs, it leads to the loss of the nephron, and when the loss occurs, it is compensated for by the remaining nephron. When the delamination occurs, this leads to the development of glomerular pathophysiology. Inflammation and cellular differentiation is created by excessive amounts of physiologically active molecules are delivered to the distal nephron and tubulointerstitium (Schnaper 2014).

- As with kidney activity, the fundamental unit of kidney failure is the nephron.
- Endocrine behavior and physiological responses guide the compensation for the remaining nephrons to preserve total body homeostasis as nephrons are lost.
- The increased demand for compensation with the remaining nephron leads to a loss of the ability to respond to compensation
- Subsequent structural and metabolic degradation results in additional injury and destruction of the nephron (Schnaper 2014).

2.14 Association DN and Diabetic Retinopathy (DR)

The existence and the severity of DR was shown to be a significant predictor. of nephropathy in a cross-sectional sample of T1DM and T2DM patients. Logistic regression with many variables found that nephropathy was independently correlated with DR as the only complication of diabetes, and that retinopathy raised 4.37 times the chance of getting nephropathy the DR predictive value for T1DM, however, was highest. These results have been validated in other studies that established the presence of DR as an independent predictor of micro- or macro albuminuria is noteworthy. progression in patients with T1DM or T2DM. Nephropathy it's been proven that danger rises with the severity of DR and In a 25-year follow-up analysis, proliferative diabetic retinopathy (PDR) was characterized as a significant independent incident nephropathy marker in T1DM (Pearce *et al.* 2019).

2.15 Non-Diabetic Kidney Disease

This study enrolled a total of 101 patients. The majority of common a reason for a kidney biopsy was the latest emergence of nephrotic syndrome 41%, accompanied with fast increasing RF 29% and active urine incontinence sedimentation 21%, 51% of patients had isolated DN for renal biopsy, NDRD was isolated in 20% of the cases, while DN plus NDRD was found in 29% of the cases. Isolated NDRD is caused by a variety of factors. was IgA nephropathy, while the major causes of NDRD superimposed on Acute tubular necrosis accounted for 39% of DN, while acute interstitial nephritis accounted for 33%, male gender, diabetes of limited term (<8 years). According to multiple logistic regression study, Lower active urinary sediment and glycated hemoglobin were NDRD predictors that were unrelated (Kritmetapak *et al.* 2018).

2.16 Oxidative Stress in DN

Numerous research have demonstrated show that they are both diabetic status Insulin resistance is a factor an important role in oxidative stress generation (Mima 2013). Production of oxygen-free radicals and antioxidant defense systems for reducing free radical toxicity, under physiological conditions, is balanced. Increased cytokine production is also involved in oxidative stress, Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is a commonly used marker of oxidative stress. Urinary 8-oxodG is specifically connected to the DNA oxidation ratio and DNA repair effectiveness. A major Japanese study included normalbuminuric or microalbuminuria T2DM patients. Different findings were found in a further T2DM patients with or without nephropathy, as well as healthy control participants, were included in the study. The contradictory findings of these clinical studies haven't shown any results. that markers of oxidative stress provide in individuals with extra prognostic information with diabetes, combined with risk markers already identified. In short, while oxidative stress markers were elevated relative to healthy controls in diabetic patients, these indicators have not been demonstrated to give new information regarding the development and progression of renal disease in diabetes individuals in clinical studies

(Hojs *et al.* 2016). Potential new therapies it has the potential to combine PKC-beta inhibitors with greater concentrations of Glucagon-like peptide 1agonists are available. Glucagon-like peptide 1(GLP-1) is one possible medication that could minimize angiotensin II to minimize the risk of DN by reducing toxic effects, inflammation and oxidative stress (Mima 2013).

2.17 Inflammatory Status in DN

Low-grade chronic inflammation and activation of the innate immune system are main factors in DM' pathogenesis. In diabetic patients, diverse inflammatory parameters are elevated and constitute good predictors of the progression of this disease, Chronic low-grade inflammation and innate immune activation surprisingly, inflammation, together with oxidative stress and fibrosis, is also indicated by a growing number of studies. Aside from hemodynamic abnormalities, metabolic disturbances and increased production of neurohumoral factors like angiotensin II are also common., the main links in the development of DN diverse components of the immune system, including adhesion molecules, chemokines, and pro-inflammatory cytokines, participate in the initiation and progression of DN. Increased inflammatory molecule levels, as well as the aggregation of large numbers of inflammatory cells were observed in the renal tissue of patients with DN.As nephropathy progresses, the concentration of these components increases, both of which are linked increased urine albumin excretion (UAE) and glomerular and tubulointerstitial clinical indicators harm (Javier *et al*. 2020).

3. MATERIALS AND METHODS

3.1 Design of Study

This research was carried out at the Specialized Dialysis Center and Al-Hussein Teaching Hospital in Al-Muthanna Governorate / Iraq, between (January to April 2021), included (87) cases, (57) male and female patients with T1DM and T2DM. Their average age was (25-80) years, with disease duration (25-45) years.

Third stage: GFR = 30-44 mL/min

Fourth stage: GFR = 15-29 mL/min

Fifth stag: GFR <15 mL/min

Three groups of patients were formed: -

Control: - Included (30) healthy control individuals, ages (25-45).

Patients: Patients are classified into three groups based on the stage of nephropathy. As shown in the (Table 3.1).

Table 3.1 Shows the division of diabetic nephropathy stages based on GFR

GFR[7] groups	GFR range	Number of patients
healthy controls	> 90 mL/min	30
Stage three	30-44 mL/min	9
Stage four	15-29 mL/min	19
Stage five	< 15 mL/min	29

3.2 Collection of Blood Sample

Each patient had (5 mL) of venous blood drawn and divided into an EDTA tube for the purpose of immediate HbA1C analysis and a gel tube and left for 2 hours at room temperature, after which it was separated in a centrifuge for 15 min. at 3000 rpm. The separated serum was taken, split, and pooled in an Eppendorf tube and frozen at -20 C$^{\circ}$ The medical history and information of the participants were taken and arranged as shown in the (Table 3.2).

[7] Glomerular filtration rate

Table 3.2 Shows the information of the participants and the analyzes that were conducted

Patient Profile
Name :-
Age :-
SEX :-
Weight :-
History :-
Duration of Diabetes mellitus
Others :-
Biochemical Parameters
Random blood sugar (RBS) Glycosylated Hemoglobin A1c (HbA1c) Cholesterol (CHOL) Triglyceride (TG) High density lipoprotein (HDL) Low density lipoprotein (LDL) Very low density lipoprotein (VLDL) Albumin (ALB) UREA Creatinine (CREA)
Adiponectin (ADP) Tumor Necrosis Factor Alpha (TNF-α) Malondialdehyde (MDA)

3.3 Chemicals

The kits applied in the study with their companies and the catalog number are shown in (Table 3.3).

Table 3.3 Shows the tests that were carried out in the research

NO	Chemicals	Supplied Company
1	Tumor Necrosis Factor Alpha	Elabscience /USA
2	ADP/Acrp30(Adiponectin)	Elabscience /USA
3	MDA (Malondialdehyde)	**-**
4	RBS (random blood sugar)	AGAPPE, Switzerland
5	HbA1c (hemoglobin A1c)	FINECARE ,Belgium
6	Urea	AGAPPE ,Switzerland
7	Creatinine	AGAPPE ,Switzerland

Table 3.3 (Continue)

8	Albumen	SPINREACT, Spain
9	Cholesterol	AGAPPE ,Switzerland
10	Triglyceride	SPINREACT, Spain
11	HDL (high density lipoprotein)	SPINREACT, Spain
12	LDL (low density lipoprotein)	SPINREACT, Spain
13	VLDL (very low density lipoprotein)	SPINREACT, Spain

3.4 Instruments

Several instruments were used to conduct tests in the thesis, and the origin and names of these instruments can be known in the (Table 3.4).

Table 3.4 Shows the devices that were used in conducting the tests

No	Instruments	Manufacturers
1	Centrifuge	KOKUSAN H-103N, JAPAN
2	Centrifuge	HETTICH EBA20 , Germany
3	Centrifuge	ALSLAB, China
4	Freezer	**----**
5	UV/VIS spectrophotometer	Model 721 , CHINA
6	UV/VIS spectrophotometer	CECIL CE7200, ENGLAND
7	Water bath	SKYTOU Digital Thermostatic, CHINA
8	Enzyme linked immunosorbent assay (ELIZA)	Human- Elisys Uno, Germany
9	Finecare (HbA1c)	Finecare FLA meter plus FS-113, China

3.5 Biochemical Parameters

3.5.1 TNF-α (Tumor Necrosis Factor Alpha)

Test principle

The Sandwich-ELISA theory is employed by this ELISA package. TNF-alpha

antibodies were pre-covered. Tests or standards are utilized to the wells of the micro-ELISA plate and blended with the suitable antibody. Following that, a biotinylated identifying antibody appointed for Human TNF-a and an Avid in-Horseradish Peroxidase (HRP) combine are progressively introduced. Each microplate well was individually incubated. The components that are no longer needed are thrown away. Every well receives the substrate solution. TNF-a, biotinylated identifying antibody, and Avid in-HRP conjugate would be the only wells that were blue. The color of the enzyme-substrate reaction changes to yellow as stop solution is introduced. The optical density (OD) is determined spectrophotometrically at 450 nm. The OD icon refers to the contain of TNF-a in blood. TNF-a concentration in the samples may be measured by comparing the samples OD to the normal curve.

Kit elements

An unopened kit can be kept for one month at 2-8 C°. If the kit isn't used within a month, separate the pieces and keep them individually according to the instructions below after the package arrives.

- ELISA plate (Dismountable) 12 strips × 8 wells
- Standard of reference Two vials
- Biotinylated detection(Concentrated Form) Ab (100) One vial, 120 µL
- HRP conjugate concentrated (100×) One vial, 120 µL
- Sample diluent and reference standard One vial, 20 mL
- Diluent for biotinylated detection Ab One vial, 14 mL
- Diluent for HRP conjugate One vial, 14 mL
- Wash buffer concentrate (25×) One vial, 30 mL
- Reagent for substrate One vial, 10 mL
- Solution to stop One vial, 10 mL
- Sealer for plates Five pieces
- Description of the product One copy
- Certificate One copy

Preparation of the reagent

1. Before using, get all reagents to 18-25 C°. Install the Microplate Reader based to the directives in the handbook, then warm it for 15 minutes before measurement.
2. Wash Buffer: To produce 750 mL of Wash Buffer, baulk 30 mL of fixed wash buffer with 720 mL distilled water. If crystals have developed in the concentrate, reheat it in a 40 C° water bath and pleasantly blend it until the crystals have dissolved.
3. Centrifuge the normal active solution for 1 minute at 10,000 RPM. Allow 1.0 mL of indication standard and sample diluent to sit for 10 minutes before gently inverting it many times. With a pipette, fully mix it once it has completely dissolved. This reconstitution outputs a 500 pg/mL active solution. Then, if needed, produce repeated dilutions. 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 0 pg/mL is the suggested dilution gradient.
Method of dilution: Fill 7 EP tubes with 500uL of indication standard and sample diluent each. 500Ul of the 500 pg/mL active solution should be pipetted into the 1st tube and blended to produce a 250 pg/mL active solution. Follow these procedures to pipette 500uL from the 1st tube to the 2nd. The graphic below serves as a guide. The last tube is treated as a blank.
4. Active solution for Biotinylated Detection Ab: Before you start the experiment, figure out how much you'll need (100 L per well). It's a good idea to prepare a little bit extra than you think you'll need. Before using, centrifuge the supplies tube and dilute the 100 concentrated biotinylated exposure Ab with biotinylated exposure Ab diluent to 1 active solution.
5. HRP combine active solution (concentrated): Determine the needed quantity (100 µL/well) previous to the experience. It's a well idea to prepare an extra than you think you'll need. Using concentrated HRP combine diluent, dilute the 100 concentrated HRP combine to 1 workable solution.

Procedure

1. Fill up the two columns in the first with the standard functioning solution: Each solution concentration is used to one well in repeat, next to each other (100 uL for each

well). Fill the remaining wells with the samples (100 uL for each well). Use the supplied sealant. At 37 C°, incubate for 90 minutes.

2. Remove the liquid from each well without washing it. Then immediately add 100 mL of biotinylated detection Ab active solution and incubate for an hour at the same temperature as before, making sure to cover it well.

3. Suction the solution from the well to remove it, then adding 350 uL of wash buffer to each well. Pour the solution after 2 minutes, let it dry and repeat the process.

4. To each well, add 100 mL of HRP combine active solution. Seal the plate and incubate for 30 minutes.

5. Remove the solution, then continue the wash procedure five times as directed in step 3.

6. Add 90 μL of substrate reagent to each well. Use a new plate sealer. Incubate for 15 minutes.

Note: based on the actual color change, the time of the reaction can be decreased or prolonged.

7. To each well, pour 50 μL of stop solution. It's important to apply the stop solution in the same sequence.

8. Determine the optical density (OD value) of each well at the same time.

The calculations

Subtract the average zero standard OD from the duplicate values for each standard and sample. On graph paper, make a log-log graph., draw a four-parameter logistic curve with OD values on the y-axis and standard concentration on the x-axis. The concentration estimated from the standard curve. If the samples have been diluted, multiply by the dilution factor. If the sample's OD exceeds the standard curve's top limit, it should be re-tested using a suitable dilution. The estimated concentration is multiplied by the dilution factor to get the actual concentration. As shown in (Figure 3.1).

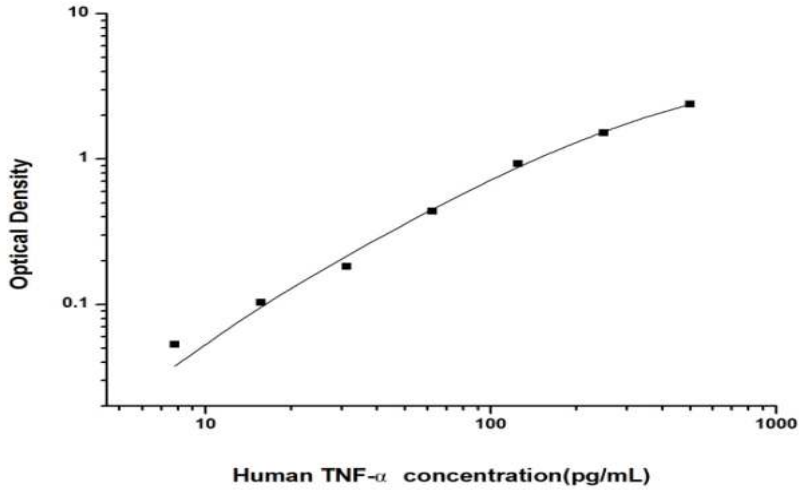

Figure 3.1 The standard curve's graph shows between OD and TNF-alpha

3.5.2 ADP/Acrp30 (Adiponectin)

Test principle

The Sandwich-ELISA concept used in this ELISA contains. This kit comprises a micro-ELISA plate that has been pre-covered with ADP/Acrp30-specific antibody. Samples (or Standards) are combined with the appropriate antibody and placed in the micro-ELISA plate wells. The biotinylated detection antibody specific for human ADP/Acrp30 and Avid in-horseradish peroxidase (HRP) conjugate is then introduced and incubated in each microplate well. The unneeded components are swept away. Every well receives the substrate solution. The only wells that will be blue are those containing human ADP/Acrp30, biotinylated detection antibody, and Avid in-HRP conjugate. The color of the enzyme-substrate reaction changes to yellow as stop solution is introduced. The OD is determined spectrophotometrically at 450 nm. The quantity of ADP/Acrp30 present is equivalent to the OD value. The concentration of human ADP/Acrp30 in the samples may be determined by comparing the OD of the samples to the normal curve.

Kit elements

An unopened kit can be kept for one month at 2-8 C°. If the kit isn't used within a month, separate the pieces and keep them individually according to the instructions below after the package arrives.

- ELISA micro plate (Dismountable) 12 strips × 8 wells
- Standard of reference Two vials
- Biotinylated detection Ab Concentrate (100×) One vial, 120 µL
- HRP conjugate concentrated (100×) One vial, 120 µL
- Sample diluent and reference standard One vial, 20 mL
- Diluent for biotinylated detection Ab One vial, 14 mL
- Diluent for HRP conjugate One vial, 14 mL
- Wash buffer concentrate (25 ×) One vial, 30 mL
- Reagent for substrate One vial, 10 mL
- Solution to stop One vial, 10 mL
- Sealer of plate Five pieces
- Description of the product One copy
- Certificate One copy

Preparation of the reagent

1. Before using, get all reagents to 18-25 C°. Install the Microplate Reader based to the directives in the handbook, then warm it for 15 minutes before measurement.
2. Wash Buffer: To produce 750 mL of Wash Buffer, baulk 30 mL of fixed wash buffer with 720 mL distilled water. If crystals have developed in the concentrate, reheat it in a 40 C° water bath and pleasantly blend it until the crystals have dissolved.
3. Centrifuge the standard at 10,000 RPM for 1 minute as a working solution. Allow 1.0 mL of indication standard and sample diluent to sit for 10 minutes before gently inverting it many times. With a pipette, fully blend it once it has completely dissolved. This reconstitution outputs a 10 ng/mL active solution (Alternatively, add 1.0mL of reference standard and sample diluent, let it sit for 1-2 minutes, and then thoroughly

mix it with a low-speed vortex meter) Bubbles produced during the vortex might be eliminated by centrifuging at a low speed). Then, if needed, produce repeated dilutions. The suggested dilution gradient is 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0 ng/mL.

Take 7 tubes of EP and add 500 micro-L of indication standard and sample diluent. To produce a 5ng/mL active solution, pipette 500L of the 10 ng/mL working solution into the 1st tube and mix thoroughly. After that, pipette 500 µL of the solution from the 1st tube into the 2nd. The graphic below serves as a guide. The final tube is treated as a blank. Do not pipette solution from the previous tube into it.

4. Biotinylated Detection is a technique for detecting proteins that have been biotinylated active solution: Prior to the experiment, calculate the needed amount (100L/well). Centrifuge the concentrated biotinylated exposure Ab for 1 minute at 3000 RPM, then dilute the 100× concentrated biotinylated exposure Ab to 1× active solution using biotinylated exposure Ab diluent (1: 99).

5. Determine the needed quantity of HRP combine active solution before the experience (100 µL/well). It's a good idea to prepare a little extra than you think you'll need. 1 minute of centrifugation at 3000 RPM with the concentrated HRP conjugate, then 7 dilute, with HRP conjugate diluent, dilute the 100 concentrated HRP combine to 1 active solution (concentrated HRP conjugate: HRP conjugate diluent= 1: 99).

Procedure

1. Separate the diluted standard, blank, and sample wells. Fill the relevant wells with 100µL of each dilution of standard, blank, and sample (It is recommended that all samples and standards be assayed in duplicate). Use the sealer that included with the package to seal the plate. At 37 C°, incubate for 90 minutes.

Note that solutions should be put to the bottom of the micro ELISA plate well as much as possible to prevent contacting the inner wall and generating foaming.

2. Do not wash the liquid from each well; instead, decant it. To each well, immediately add 100 µL of biotinylated detection Ab working solution. Replace the sealer on the plate. At 37 C°, incubate for 1 hour.

3. Add 350 µL of wash buffer to each well after decanting the solution from each well. Soak for 1-2 minutes before aspirating or decanting the solution from each well and

patting it dry with clean absorbent paper. This wash process should be repeated three times.

This and other wash processes can be done with a microplate washer. After the wash process, use the tested strips right away. Allowing wells to dry out is not a good idea.

4. In each well, pour 100L of HRP conjugate working solution. Replace the sealer on the plate. At 37 C°, incubate for 30 minutes.

5. Decant the solution from each well and repeat the wash procedure as directed in step 3 for a total of 5 times.

6. Fill each well with 90 μL of substrate reagent. Replace the sealer on the plate. At 37 C°, incubate for around 15 minutes. Light should be kept away from the plate. Note: The reaction time can be decreased or prolonged depending on the actual color shift, but not for more than 30 minutes in any of the following cases: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, and 0.8. Before taking an OD reading, preheat the microplate reader for about 15 minutes.

7. To each well, pour 50 μL of stop solution.

Note: The stop solution and the substrate solution should be added in the same sequence.

8. Using a microplate reader set to 450 nm, determine the OD value of each well at the same time.

The calculations

Subtract the average zero standard OD from the duplicate values for each standard and sample. On log-log graph paper, draw a four-parameter logistic curve with standard concentration on the x-axis and OD values on the y-axis. The concentration estimated from the standard curve must be multiplied by the dilution factor if the samples have been diluted. If the sample's OD exceeds the top limit of the standard curve, it should be re-tested using a suitable dilution. The estimated concentration is multiplied by the dilution factor to get the actual concentration. As shown in (Figure 3.2).

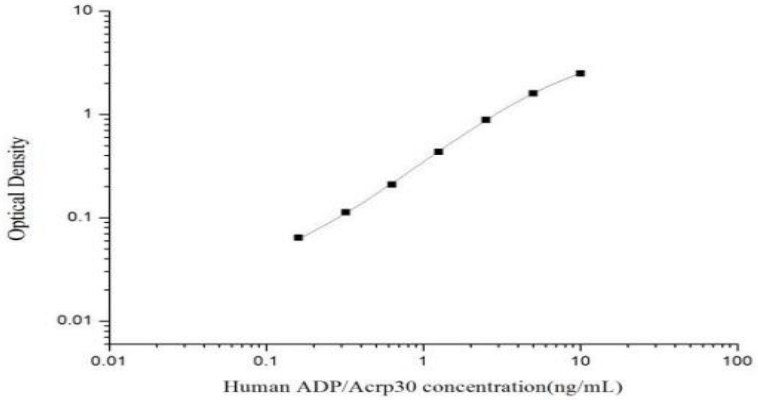

Figure 3.2 The standard curve's graph shows between OD and ADP

3.5.3 MDA (Malondialdehyde)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

Process was the thiobarbituric acid (TBA) used to evaluate lipid peroxidation. Malondialdehyde (MDA) derived Polyunsaturated fatty acids were broken down, was described as the LPO material that interacts with thiobarbituric acid to give a red chromophore absorption at 535 nm in the presence of trichloroacetic acid (TCA) (Fong *et al.* 1973). As shown in (Figure 3.3) the reaction equation below.

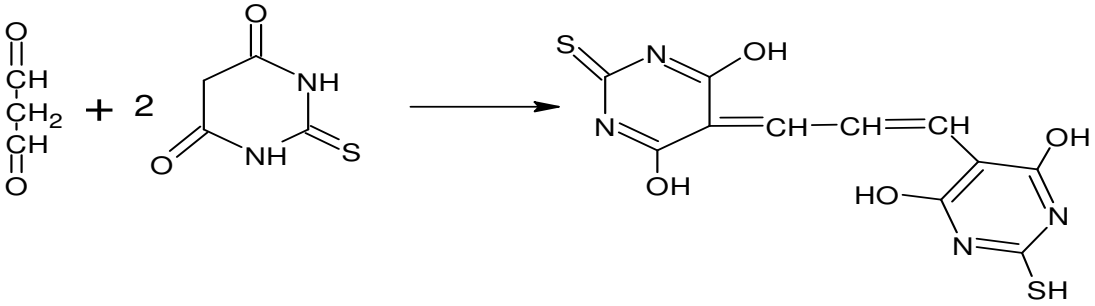

Figure 3.3 The reaction between thiobarbituric acid (TBA) and trichloroacetic acid (TCA)

The volume of serum MDA was shown to be calculated spectrophotometrically using a TBA solution. In summary, the next procedures were carried out on the 150 µL serum sample: 1 mL 17.5% TCA and 1 mL 0.66% TBA, well mixed with a vortex, incubated for 15 min in boiling water, after then it was allowed to cool. One mL of 70% TCA was added to the mixture. which was left for 20 min at 2 before being centrifuged for 15 min at 2000 rpm and the supernatant separated for spectrophotometric at room temperature, scan (532nm) (Muslih *et al.* 2002).

Calculation

$$MDA\ \left(\frac{\mu mol}{L}\right) = \frac{absorbance\ MDA\ at\ 532nm}{L\ x\ E_0} \longrightarrow x\ D\ x\ 10^6 \qquad (3.1)$$

L = (light path) (1cm)

E_0 = (extinction coefficient) 1.56 x 10^5 M .cm^{-1}

D = (dilution factor) = 1mL Vol. Used in Ref. /0.15 = 21

3.5.4 Urea

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

The following reaction is used to determine urea by enzymes (Weatherburn 1967)**.**

$$urea + H_2O \xrightarrow{urease} 2NH_3 + CO_2 \qquad (3.2)$$

$$NH_3 + Salicylate \xrightarrow[Hypochlorite]{Nitroprusside} 2.2 - Dicarboxy\ indophenol \qquad (3.3)$$

Kit components

- Urea color Reagent R1 2 x 53 mL
- Urea Reagent R2 10 x 10 mL
- Urea standard 1 x 4 mL

Procedure

This is a simple test done by drawing blood out of your body through a vein as shown in (Table 3.5). Urea is a waste product formed in the liver that travels through your blood to the kidneys, which then filters it out of the blood. It is then carried out of your body through urine.

Table 3.5 Shows how the urea test works

	blank	**standard**	**Sample**
Work solution	1mL	1mL	1mL
Standar	-	10µL	-
Sample	-	-	10µL
Incubate for 5 minutes at 37 C° after mixing. then add			
Color solution	1mL	1mL	1mL
Incubate for 5 minutes at 37 C° after mixing. then add			
distilled water	1mL	1mL	1mL
Measure the absorbance of the sample and standard against the reagent blank at 600 nm after thoroughly mixing.			

Calculation

$$urea\ con(mg\ /\ dL) = \frac{(absorbance\ of\ sample)}{absorbance\ of\ standard} X\ 40 \quad (3.4)$$

3.5.5 Creatinine (CREA)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Creatinine interacts with picric acid to produce creatinine alkaline picrate, a colorful molecule with an absorbance difference proportional to creatinine content (Tanganelli *et al.* 1982).

Kit components

- Creatinine reagent R1 2 x 50 mL
- Creatinine reagent R2 2 x 50 mL
- Creatinine standard 1 x 4 mL

Procedure

To test for creatinine in the blood, a blood sample is taken from a vein, and the working steps are taken on it as in (Table 3.6).

Table 3.6 Shows how the creatinine test works

	Standard	**Test**
Working reagent	1mL	1mL
Standar	100µL	-
Serum	-	100µL
Mix well and read the OD (T1) 60 seconds after the sample or standard addition, then take a second reading (T2) at 492 nm, exactly 60 seconds after the first measurement.		

Calculation

$$creatinine\ con(\frac{mg}{dL}) = \frac{(T_2-T_1)ofsample}{(T_2-T_1)of\ standard} \quad x\ 2 \tag{3.5}$$

3.5.6 Glycosylated hemoglobin A1c (HbA1c)

Note: This analysis was performed using a technique called Rapid Quantitative Test is based on fluorescence immunoassay technology

Principle

Fluorescence immunoassay technology is used in the Finecare HbA1c Rapid Quantitative Test. The Finecare HBA1 Rapid Quantitative Test employs a sandwich immune detection process, in which the fluorescence-labeled on the sample pad,

detector HBA1c antibodies and detector HB antibodies bind to HBA1c antigens. when the sample is placed in the sample well of the test cartridge, and Hb antigens in blood samples, which combine to form immune complexes. The detector antibodies and HbA1c are captured by immobilized HbA1c antibodies on the test strip, and the complexes of detector antibodies and Hb are trapped by immobilized Hb antibodies on the test strip, while the complexes move on the test strip's nitrocellulose matrix by capillary motion. As a result, the more higher the concentration of HbA1c and Hb antigens in a blood sample, On the test strip, more complexes develop. The sum of HbA1c and Hb captured is reflected in the signal strength of detector antibodies, and the Finecare FIA System displays the HbA1c ratio in blood specimen (Jeppsson *et al.* 2002)

Kit components

- Test cartridge 25
- ID chip 1
- Buffer for detection 25
- Tip of the pipette 25
- Capillary tube 25
- Capillary tube clip 1
- Leaflet with instruction for use 1
- Quick operation instruction for finegerstick whole blood 1

Test procedure

1. Preparation: Before running the test, turn on "use" in the settings and save it. Insert ID Chip into Fine Care FIA System.
2. Sampling: draw 10 µL of whole blood and place it in the detection buffer tube.
3. Shake the sample mixture well for 1 minute after closing the cover of the detection buffer tube.
4. take 75 µL of sample mixture and load it into the test cartridge.
5. Testing: There are two test modes for Finecare FIA System, Standard Test mode and Quick Test mode.

Interpretation of results

The Finecare System automatically calculates HbA1c test results the form of XXX.X percent.
Normal reference value: <6.5%

3.5.7 Random blood sugar (RBS)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

The following reaction is used to determine glucose by enzymatic colorimetry (Trinder 1969).

$$glucose + O_2 + H_2O \xrightarrow{glucose\ oxidase} gluconic\ acid + H_2O_2 \quad (3.6)$$

$$2H_2O_2 + phenol + 4 - aminoantipyrine \xrightarrow{peroxidase} quinonimine + 4H_2O \quad (3.7)$$

Kit component

Glucose reagent R1	5 x 100 mL
standard	1 x 4 mL

Procedure

The procedure for random blood sugar test is pretty simple and does not even require fasting unlike Fast Blood Glucose test. RBS test involves drawing of blood out from the veins by means of an injection. Apart from a tiny prick not much discomfort is felt during the RBS test (See Table 3.7).

Table 3.7 Shows how the RBS test works

	blank	standard	Sample
Glucose reagent	1mL	1mL	1mL
standard	-	10µL	-
Sample	-	-	10µL
Read the absorbance of the standard and sample against the reagent blank after mixing and incubating for 10 minutes at 37 C° at wavelength 505 nm.			

Calculation

$$glucose\ concentration\ \frac{mg}{dL} = \frac{absorbance\ of\ sample}{absorbance\ of\ standard} X\ 100 \quad (3.8)$$

3.5.8 Cholesterol (CHOL)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

The amount of cholesterol in the sample results in the formation of a colored compound, and the intensity of the color is proportional to the amount of cholesterol in the sample (Charles *et al*. 1974).

$$cholesterol\ esters + H_2O \xrightarrow{CHE} cholesterol + fatty\ acids \quad (3.9)$$

$$cholesterol + O_2 \xrightarrow{CHOD} 4 - cholestenona + H_2O_2 \quad (3.10)$$

$$2H_2O_2 + phenol + 4 - aminophenazone \xrightarrow{POD} Quinonimine + 4H_2O \quad (3.11)$$

Kit component

- R1 Buffer — PH 6.9 — 90 mmol/L
  Phenol — 26 mmol/L
- R2 enzymes — Cholesterol esterase CHE — 300 U/L
  Cholesterol oxidase CHOD — 300 U/L

	Peroxidase POD	1250 U/L
	4-aminophenazone 4-AP	0.4 mmol/L
• Cholesterol standard	Cholesterol aqueous primary standard	200 mg/dL

Procedure

The Kit are prepared at room temperature, after which the blood sample is drawn from the patient and the work steps are taken on it as in the (Table 3.8).

Table 3.8 Shows how the CHOL test works

	Blank	**Standard**	**Sample**
Work solution	1mL	1mL	1mL
Standard	-	10µL	-
Sample	-	-	10µL
5 minutes at 37 C°, mix and incubate			
Read the absorbance A of the sample and standard against the blank at 505 nm			

Calculation

$$cholesterol\,\frac{mg}{dL} = \frac{absorbance\ of\ sample}{absorbance\ of\ standard} \longrightarrow X\ 200 \qquad (3.12)$$

3.5.9 Triglyceride (TG)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

Glycerol and free fatty acids are liberated from triglycerides incubated with lipoprotein lipase (LPL). Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol phosphate dehydrogenase converts glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DAP) and

hydrogen peroxide (H_2O_2) (GPO). In the presence of peroxidase (POD), hydrogen peroxide (H_2O_2) reacts with 4-aminophenazon (4-AP) and p-chlorophenol to create a red color. The color intensity is related to the amount of triglycerides present in the sample (Giovanni and Harold 1973).

$$triglycerides + H_2O \xrightarrow{LPL} glycerol + free\ fatty\ acids \quad (3.13)$$

$$glycerol + ATP \xrightarrow{glycerolkinase} G3P + ADP \quad (3.14)$$

$$G3P + O_2 \xrightarrow{GPO} DAP + H_2O_2 \quad (3.15)$$

$$H_2O_2 + 4 - AP + P - chlorophenol \xrightarrow{POD} Quinone + H_2O \quad (3.16)$$

Kit component

• R1	GOOD PH 7,5	50 mmol/L
	p- Chlorophenol	2 mmol/L
• R2	Lipoprotein lipase (LPL)	150000 U/L
	Glycerol kinase (GK)	500 U/L
	Glycerol-3-oxidasa (GPO)	2500 U/L
	Peroxidase (POD)	440 U/L
	4-Aminophenazone (4-AP)	0.1 mmol/L
	ATP	0.1 mmol/L
• Triglycerides standar	Triglycerides aqueous primary standard 200 mg/Dl	

Procedure

When conducting this test, the sample owner must be fasting for a period of no less than 8 hours. Blood is drawn and kit are prepared for the test as shown in the (Table 3.9).

Table 3.9 Shows how the TG test works

	Blank	Standard	Sample
Work solution	1mL	1mL	1mL
Standard	-	10µL	-
Sample	-	-	10µL
5 minutes at 37 C°, mix and incubate			
at a wavelength of 505nm Compare the sample's and standard's absorbance A to the blank.			

Calculation

$$triglycerides\ \frac{mg}{dL} = \frac{absorbance\ of\ sample}{absorbance\ of\ standard} X\ 200 \quad (3.17)$$

3.5.10 High density lipoprotein (HDL)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Test principle

In the presence of magnesium ions, by phosphotungstate precipitates VLDL and LDL lipoproteins from serum or plasma. HDL is present in the supernatant after centrifugation. The total cholesterol enzymatic reagent is used to determine the HDL cholesterol fraction (Grove 1979)

Kit component

- Reagent — Phosphotungstic 14 mmol/L; Magnesium 2 mmol/L
- Optional STD — HDL Aq. prim. Std 50 mg/dL
- Optional reagent — Cholesterol CHOD-POD

Procedure

Take 1mL of working solution with 100 µL blend well and let aside for ten min. For 20 minutes, centrifugation at 4000 rpm. Collect the supernatant and use it as a sample for determining total cholesterol.

Calculation

$$HDL\,\frac{mg}{dL} = \frac{(A2-A1)Sample - (A2-A1)Blank}{(A2-A1)standared - (A2-A1)Blank} \rightarrow X\ standared\ concentration \quad (3.18)$$

3.5.11 LDL and VLDL

In a lipoprotein estimation test, the Friedewald equation was applied to determine VLDL and LDL values. The method used reagents to quantify total cholesterol, triglycerides, and HDL. The formula that was released by (Friedewald *et al.* 1972).

VLDL-c= Triglycerides/5 when units mg/dL

VLDL-c= Triglycerides/2.2 when units mmol/L

When plasma triglyceride concentrations reach 400 mg/dL, the Friedewald equation should not be utilized.

LDL-c = Total cholesterol – HDL c – VLDL c

LDL-c = Total cholesterol – HDL c – VLDL c

3.5.12 Albumin (ALB)

Note: This analysis was performed using a technique called UV/VIS spectrophotometer

Principle

Albumin replace the color from yellow-green to green-blue in the being of bromocresol green at a slightly acid pH (Webster 1974, Doumas *et al.* 1971).

Kit component

R	Bromocresol green PH 4.2	0.12 mmo/L
Albumin standar	Albumin aqueous primary standard	5 g/dL

Procedure

The kits are prepared at room temperature and the blood sample is prepared for the purpose of testing (see Table 3.10).

Table 3.10 Shows how the ALB test works

	Blank	**Standard**	**Sample**
Work solution	1mL	1mL	1mL
Standard	-	5µL	-
Sample	-	-	5µL
for 5 minutes at 37 C°. Mix and incubate			
Read the absorbance A of the sample and standard against the blank at wavelength 630 nm; the color is stable for 1 hour at room temperature 15-25 C°.			

Calculation

$$albumin\ \frac{g}{dL} = \frac{absorbance\ of\ sample}{absorbance\ of\ standard} \longrightarrow X\ 5 \tag{3.19}$$

4. RESULTS

4.1 Anthropometric Analysis

In this study, 57 patients were studied and they were divided into 5 stages based on the (GFR) equation, stages (1 and 2) were excluded as they could not be considered from the end stage renal disease (ESRD), stages (3, 4 and 5) were taken and compared with the healthy 30 participants. Where we will review the results according to each stage, the number of patients in stage 3 is 9 patients, stage 4 is 19 patients, and stage 5 is 29 patients.

NOT The clinical characteristics of the subjects' study participants are shown using IBM SPSS Statistics version 26 (Independent T-test).

4.2 Comparison of Studied Parameters for Stages (3, 4 and 5)

4.2.1 Urea and creatinine (mg/dL)

In this study, (Table 4.1) shows a significant difference in the mean of urea in stage 3 ($P < 0.001$) between control (24.16 ± 3.86) compared with the DN patients (93.11 ± 31.91), and also there was a significant difference in the mean of creatinine in stage 3 ($P < 0.001$) between control (0.67 ± 0.11) compared with the DN patients (1.88 ± 0.38) as shown in (Table 4.2).

As for stage 4 was also a significant difference in the mean of urea in stage 4 ($P < 0.001$) between control (24.16 ± 3.86) compared with the DN patients (101.94 ± 40.29) as shown in (Table 4.1). In addition, there was a significant difference in the mean of creatinine in stage 4 ($P < 0.001$) between control (0.67 ± 0.11) compared with the DN patients (2.87 ± 0.54) as shown in (Table 4.2).

For stage 5, was found a significant difference in the mean of urea in stage 5 ($P < 0.001$) between control (24.16 ± 3.86) compared with the DN patients (162.27 ± 48.03) as shown in (Table 4.1). Also, there was a significant difference in the mean of creatinine

in stage 5 (P < 0.001) between control (0.67 ± 0.11) compared with the DN patients (7.66 ± 3.62) as shown in (Table 4.2).

Table 4.1 Mean and S.D of urea in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
Urea (mg/dL)	3	24.16	3.86	93.11	31.09	< 0.0001
	4	24.16	3.86	101.94	40.29	< 0.0001
	5	24.16	3.86	162.27	48.03	< 0.0001

Table 4.2 Mean and S.D of creatinine in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
Creatinine (mg/dL)	3	0.67	0.11	1.88	0.38	< 0.001
	4	0.67	0.11	2.87	0.54	< 0.001
	5	0.67	0.11	7.66	3.62	< 0.001

4.2.2 Lipid profile

4.2.2.1 Cholesterol and triglyceride (mg/dL)

Table (4.3) shows a significant difference in the mean of cholesterol in stage 3 (P < 0.001) between control (131.93 ± 10.17) compared with the DN patients (103.44 ±14.74). While there were insignificant differences in the mean of TG in stage 3 (P = 0.067) between control (110.93 ± 8.64) compared with the DN patients (123.33 ± 33.31).

Stage 4 revealed no significant in the mean of cholesterol in stage 4 (P = 0.820) between control (131.93 ± 10.17) compared with the DN patients (133.73 ± 41.58) as shown in (Table 4.3). No significant differences in the mean of TG in stage 4 (P = 0.085) was found between control (110.93 ± 8.64) compared with the DN patients (135.47 ± 66.17).

Mean of cholesterol in stage 5 was significantly increasing (P = 0.024) between control (131.93 ± 10.17) compared with the DN patients (118.48 ± 30.00) as shown in (Table

4.3). No significant differences in the mean of TG in stage 5 (P = 0.617) found between control (110.93 ± 8.64) compared with the DN patients (115.68 ± 50.99)**.**

Table 4.3 Mean and S.D of CHOL and TG in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
CHOL (mg/dL)	3	131.93	10.17	103.44	14.74	< 0.0001
	4	131.93	10.17	133.73	41.58	0.820
	5	131.93	10.17	118.48	30.00	0.024
TG (mg/dL)	3	110.93	8.64	123.33	33.31	0.067
	4	110.93	8.64	135.47	66.17	0.085
	5	110.93	8.64	115.68	50.99	0.617

4.2.2.2 HDL (mg/dL)

There were no significant differences in the mean of HDL in stage 3 (P = 0.257), 4 (P = 0.538) and **5** (P = 0.997) between control (56.20 ± 6.88) compared with the DN patients (53.00 ± 8.71), (54.73 ± 9.61) and (56.20 ± 8.60), respectively, as shown in (Table 4.4).

Table 4.4 Mean and S.D of HDL in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
HDL (mg/dL)	3	56.20	6.88	53.00	8.71	0.257
	4	56.20	6.88	54.73	9.61	0.538
	5	56.20	6.88	56.20	8.60	0.997

4.2.2.3 LDL (mg/dL)

As shown in (Table 4.5), LDL mean value showed a significant increasing in stage 3 (P = < 0.001) between control (53.58 ± 12.53) compared with the DN patients (55.86 ± 13.78). While in stage 4 there was no significant in the mean of LDL in stage 4 (P = 0.832) between control (53.58 ± 12.53) compared with the DN patients (54.92 ± 39.71). In addition, there was no significant in the mean of LDL in stage 5 (P = 0.964) between control (53.58 ± 12.53) compared with the DN patients (54.32 ± 87.88).

Table 4.5 Mean and S.D of LDL in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
LDL(mg/dL)	3	53.58	12.53	55.86	10.78	< 0.001
	4	53.58	12.53	54.92	25.71	0.832
	5	53.58	12.53	54.32	17.88	0.964

4.2.2.4 VLDL (mg/dL)

The differences in the VLDL mean value in stage 3 (P = 0.067) was insignificantly between control (22.18 ± 1.72) compared with the DN patients (24.66 ± 6.66). While in stage, 4 there was also insignificant differences in the mean of VLDL (P = 0.085) between control (22.18 ± 1.72) compared with the DN patients (27.09 ± 15.23). No significant in the mean of VLDL in stage 5 (P = 0.617) between control (22.18 ± 1.72) compared with the DN patients (23.13 ± 10.19) was found as shown in (Table 4.6).

Table 4.6 Mean and S.D of Lipid Profile in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
VLDL (mg/dL)	3	22.18	1.72	24.66	6.66	0.067
	4	22.18	1.72	27.09	15.23	0.085
	5	22.18	1.72	23.13	10.19	0.617

4.2.3 Albumin

Table (4.7) shows a significant increasing in the mean of albumin in stage 3 (P < 0.001) between control (4.57 ± 0.36) compared with the DN patients (3.30 ±0.76). In addition, there was a significant difference in the mean of albumin in stage 4 (P < 0.001) between control (4.57 ± 0.36) compared with the DN patients (3.22 ± 0.79). Furthermore, in stage 5 also there was a significant difference in the mean of albumin (P < 0.001) between control (4.57 ± 0.36) compared with the DN patients (3.27 ± 0.69).

Table 4.7 Mean and S.D of ALB in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
ALB (mg/dL)	3	4.57	0.36	3.30	0.76	< 0.001
	4	4.57	0.36	3.22	0.79	< 0.001
	5	4.57	0.36	3.27	0.69	< 0.001

4.2.4 Random blood sugar

In this study, the mean value of RBS showed a significant increasing ($P < 0.001$) in stage 3, 4 and 5 between control (106.46 ± 6.76) compared with the DN patients (270.66 ± 118.98), (220.47 ± 79.26) and (211.75 ± 66.22), respectively, as shown in (Table 4.8).

Table 4.8 Mean and S.D of RBS in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
RBS (mg/dL)	3	106.46	6.76	270.66	118.98	< 0.001
	4	106.46	6.76	220.47	79.26	< 0.001
	5	106.46	6.76	211.75	66.22	< 0.001

4.2.5 Glycosylated hemoglobin A1c (HbA1c)

A significant increasing ($P < 0.001$) in the mean of HbA1c in stage 3, 4 and 5 between control (5.06 ± 0.24) compared with the DN patients (10.43 ± 1.48), (10.52 ± 1.47) and (9.99 ± 1.07), respectively, as shown in (Table 4.9).

Table 4.9 Mean and S.D of HbA1c in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
HbA1c (%)	3	5.06	0.24	10.43	1.48	< 0.001
	4	5.06	0.24	10.52	1.47	< 0.001
	5	5.06	0.24	9.99	1.07	< 0.001

4.2.6 Malondialdehyde (MDA)

A significant increasing ($P < 0.001$) in the mean of MDA in stage 3, 4 and 5 between control (1.94 ± 0.50) compared with the DN patients (4.25 ± 1.19), (3.71 ± 1.27) and (3.72 ± 0.81), respectively, as shown in (Table 4.10).

Table 4.10 Mean and S.D of MDA in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
MDA (nmol/mg)	3	1.94	0.50	4.25	1.19	< 0.001
	4	1.94	0.50	3.71	1.27	< 0.001
	5	1.94	0.50	3.72	0.81	< 0.001

4.2.7 Adiponectin (ADP)

This study has shown no significant difference in the mean of ADP in stage 3 ($P = 0.249$) between control (7.200 ± 1.167) compared with the DN patients (7.660 ± 0.088). While in stage 4 there was insignificant differences in the mean of ADP ($P = 0.099$) between control (7.200 ± 1.167) compared with the DN patients (7.654 ± 0.098). Also, there was insignificant differences in the mean of ADP in stage 5 ($P = 0.052$) between control (7.200 ± 1.167) compared with the DN patients (7.632 ± 0.126) as shown in (Table 4.11).

Table 4.11 Mean and S.D of ADP in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
ADP (pg/mL)	3	7.200	1.167	7.660	0.088	0.249
	4	7.200	1.167	7.654	0.098	0.099
	5	7.200	1.167	7.632	0.126	0.052

4.2.8 Tumor necrosis factor alpha(TNF-α)

The current study has shown no significant difference in the mean of TNF-α in stage 3 (P = 0.07) between control (1.13 ± 0.39) compared with the DN patients (1.59 ± 1.18). Also, there was no significant difference in the mean of TNF-α in stage 4 (P = 0.18) between control (1.13 ± 0.39) compared with the DN patients (1.37 ± 0.84). In addition, in stage **5** there was no significant difference in the mean of TNF-α (P = 0.43) between control (1.13 ± 0.39) compared with the DN patients (1.26 ± 0.78) as shown in (Table 4.12).

Table 4.12 Mean and S.D of TNF-α in DN patients and control

Parameter	Stages	Healthy control		DN patients		P value
		Mean	S. D.	Mean	S. D.	
TNF-α (pg/mL)	3	1.13	0.39	1.59	0.18	0.07
	4	1.13	0.39	1.37	0.84	0.18
	5	1.13	0.39	1.26	0.78	0.43

4.3 Pearson Correlation Analysis

The clinical characteristics of the subject's study participants are shown using IBM SPSS Statistics version 26 (Pearson Correlation).

4.3.1 The Correlation of TNF-alpha with biochemical parameters

As shown in (Table 4.13), (Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8) no significant correlation was observed between TNF-alpha with urea, creatinine, cholesterol, TG, HDL, VLDL, albumin, and MDA (r = 0.074, P = 0.493), (r = 0.036, P = 0.737) , (r = 0.073, P = 0.500), (r = -0.007, P = 0.950), (r = -0.076, P = 0.483), (r = -0.007, P = 0.950) , (r = 0.029, P = 0.788), (r = 0.051, P = 0.641) respectively. In addition, non-significant correlation was observed between TNF-alpha with ADP and HbA1c (r = 0.100, P = 0.359), (r = 0.163, P = 0.132) respectively as shown in (Table 4.13), (Figures 4.9, 4.10). Finally (Table 4.13), (Figure 4.11) showed TNF- alpha a significant positive correlation with RBS (r = 0.214, P = 0.046).

Table 4.13 The correlation of TNF-alpha with biochemical parameters

	Biochemical Parameters	Pearson Correlation	Sig. (2tailed)
TNF-alpha	**ADP**	0.100	0.359
	MDA	0.051	0.641
	HbA1c	0.163	0.132
	RBS	0.214*	0.046
	ALB	0.029	0.788
	VLDL	-0.007-	0.950
	LDL	0.101	0.351
	HDL	-0.076	0.483
	TG	-0.007	0.950
	CHOL	0.073	0.500
	CREA	0.036	0.737
	UREA	0.074	0.493

NOT: ADP (adiponectin); TNF-alpha (tumor necrosis factor alpha); MDA (malondialdehyde); HbA1c (Glycosylated Hemoglobin A1c); RBS (random blood sugar); ALB (albumin); VLDL (very low density lipoprotein); LDL (low density lipoprotein); HDL (high density lipoprotein); TG (triglyceride); CHOL (cholesterol); CREA (creatinine); and UREA;* a significant

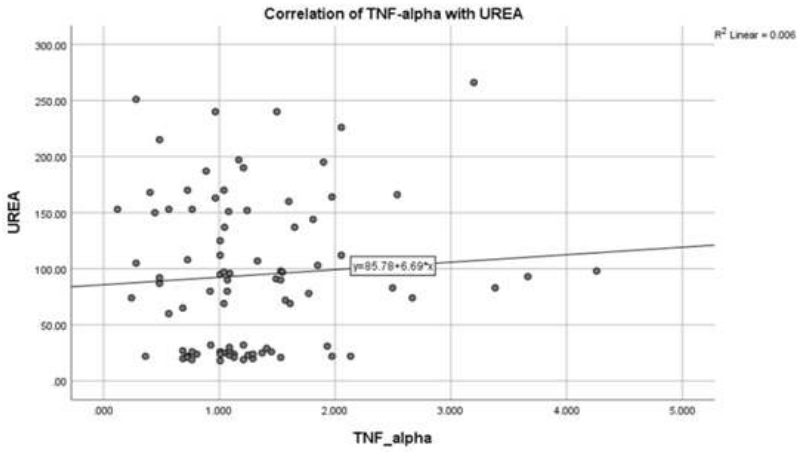

Figure 4.1 Correlation of TNF-alpha showed no significant with UREA

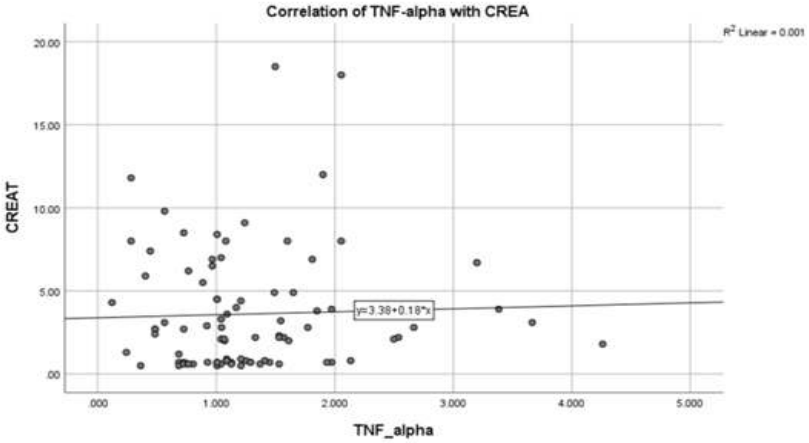

Figure 4.2 Correlation of TNF-alpha showed no significant with CREA

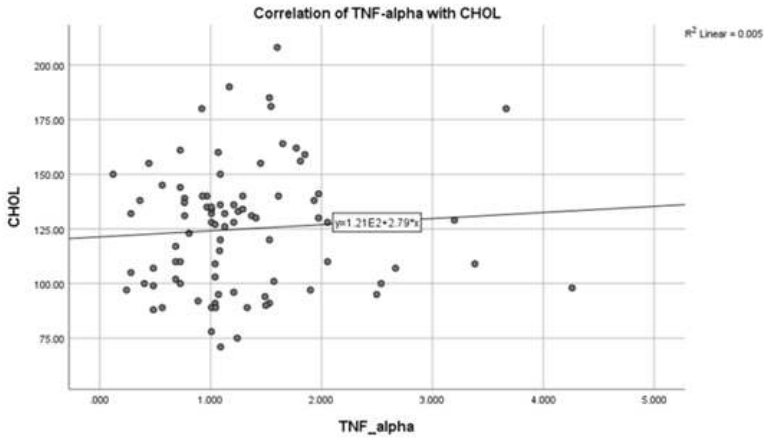

Figure 4.3 Correlation of TNF-alpha showed no significant with CHOL

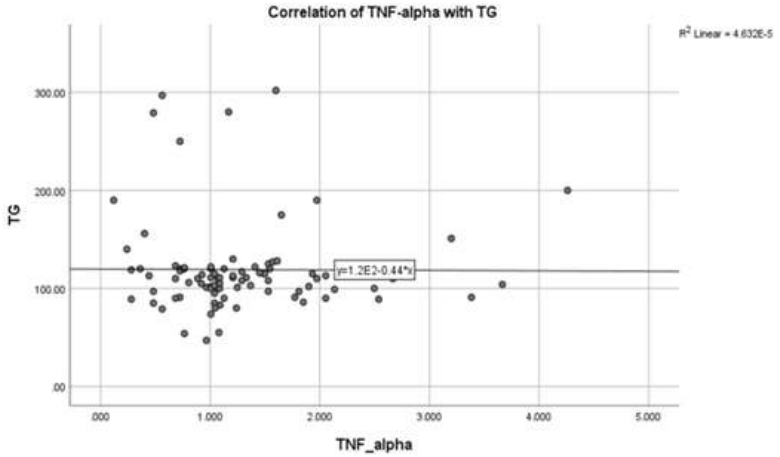

Figure 4.4 Correlation of TNF-alpha showed no significant with TG

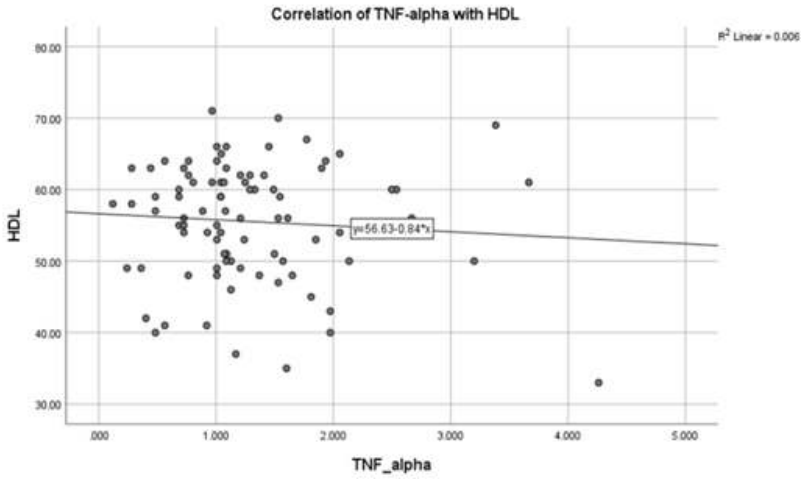

Figure 4.5 Correlation of TNF-alpha showed no significant with HDL

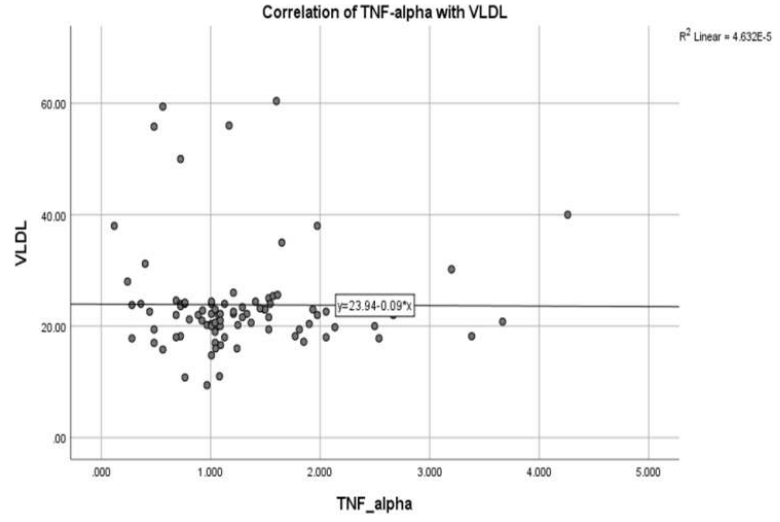

Figure 4.6 Correlation of TNF-alpha showed no significant with VLDL

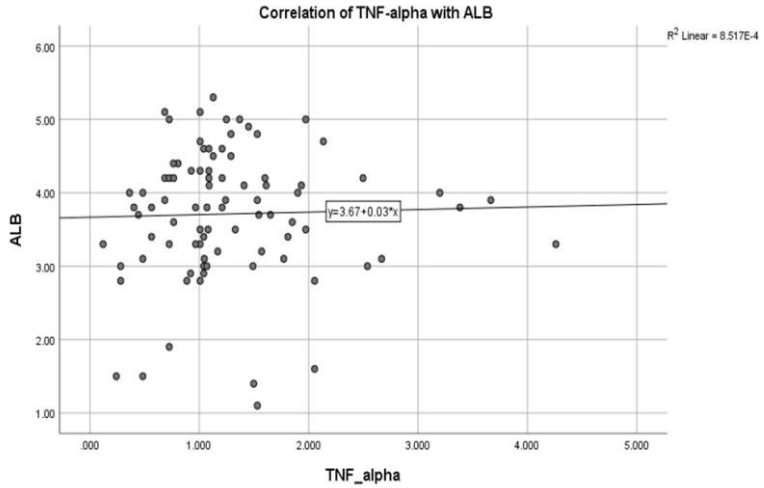

Figure 4.7 Correlation of TNF-alpha showed no significant with ALB

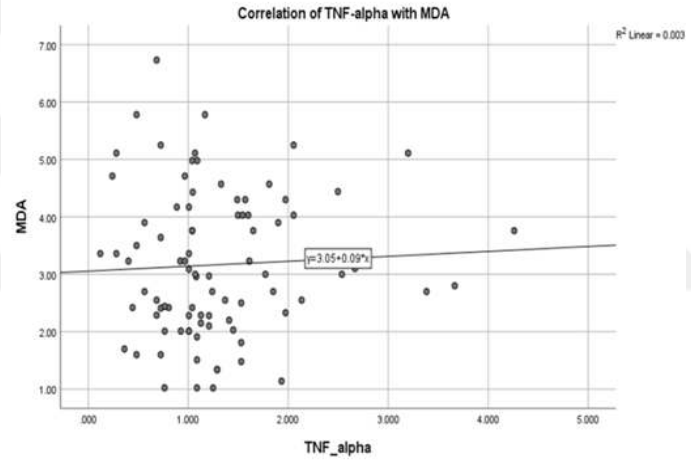

Figure 4.8 Correlation of TNF-alpha showed no significant with MDA

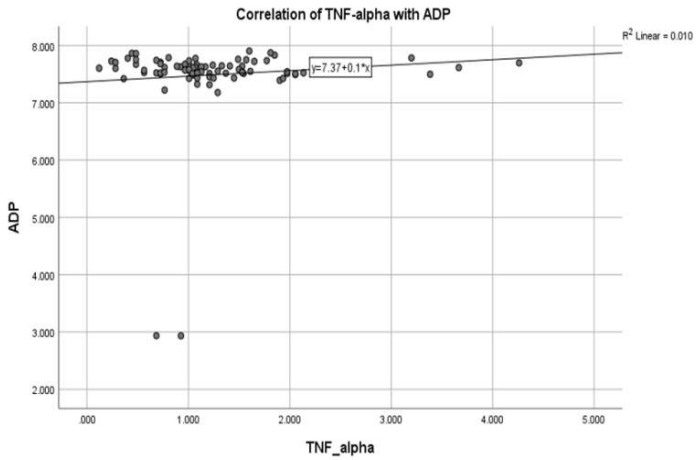

Figure 4.9 Correlation of TNF-alpha showed non-significant with ADP

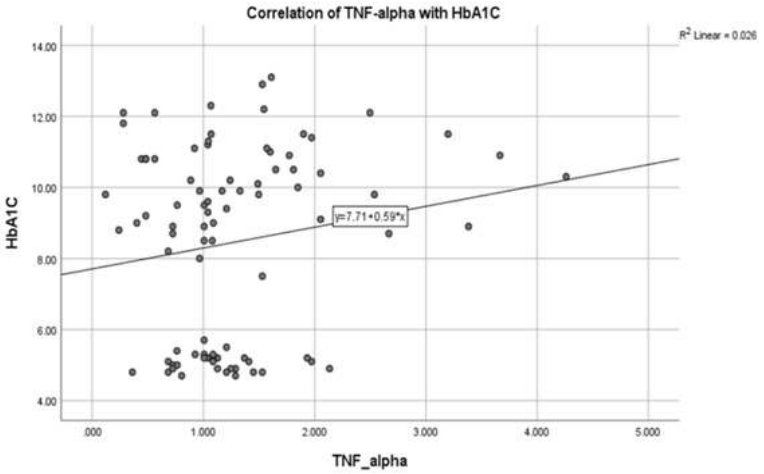

Figure 4.10 Correlation of TNF-alpha showed non-significant with HbA1c

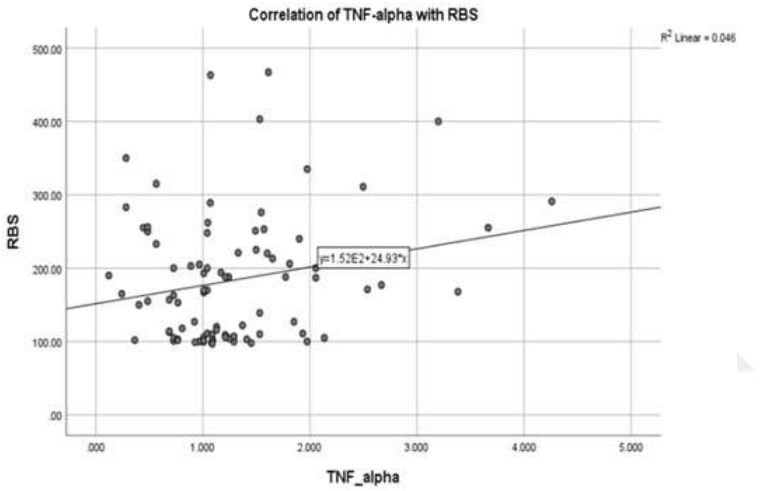

Figure 4.11 Correlation of TNF-alpha showed a significant positive with RBS

4.3.2 The Correlation of ADP with biochemical parameters

The results in the present study have indicated that there was a significant positive correlation of ADP with HbA1c and urea (r = 0.266, P = 0.013), (r = 0.212, P = 0.048) respectively as shown in (Table 4.14), (Figures 4.12, 4.13), while a significant negative correlation of ADP with ALB (r = -0.223, P = 0.038) as shown in (Table 4.14), (Figure 4.14). As well, non-significant correlation was observed between ADP with TNF-alpha, MDA, RBS and CREA (r = 0.100, P = 0.359), (r = 0.180, P = 0.095), (r = 0.192, P = 0.075), (r = 0.153, P = 0.158) as shown in (Table 4.14), (Figures 4.15, 4.16, 4.17 and 4.18). Likewise, no significant correlation between ADP with VLDL, LDL, HDL, TG and CHOL (r = 0.031, P = 0.775), (r = 0.011, P = 0.920), (r = -0.085, P = 0.436), (r = 0.031, P = 0.775), (r = -0.004, P = 0.972) respectively as shown in (Table 4.14), (Figures 4.19, 4.20, 4.21, 4.22 and 4.23).

Table 4.14 The correlation of ADP with biochemical parameters

	Biochemical Parameters	Pearson Correlation	Sig. (2tailed)
ADP	**TNF-alpha**	0.100	0.359
	MDA	0.180	0.095
	HbA1c	0.266*	0.013
	RBS	0.192	0.075
	ALB	-0.223*	0.038
	VLDL	0.031	0.775
	LDL	0.011	0.920
	HDL	-0.085	0.436
	TG	0.031	0.775
	CHOL	-0.004	0.972
	CREA	0.153	0.158
	UREA	0.212*	0.048

NOT: ADP (adiponectin); TNF-alpha(tumor necrosis factor alpha); MDA(malondialdehyde); HbA1c(Glycosylated Hemoglobin A1c); RBS(random blood sugar); ALB(albumin); VLDL(very low density lipoprotein); LDL(low density lipoprotein); HDL(high density lipoprotein); TG(triglyceride); CHOL(cholesterol); CREA(creatinine); and UREA; * significant; r = Pearson Correlation

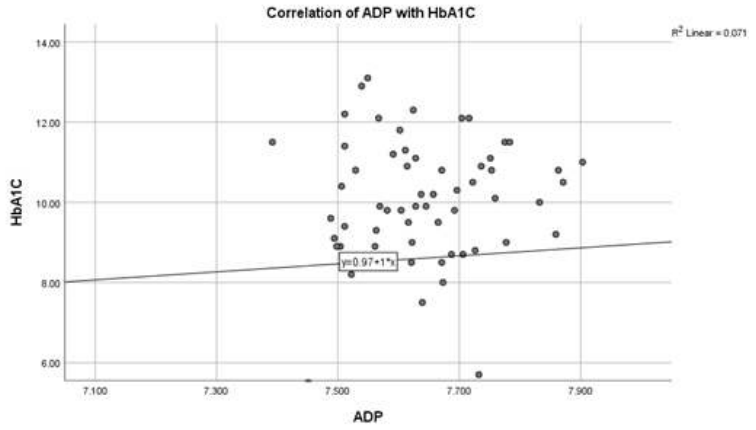

Figure 4.12 Correlation of ADP showed a significant positive with HbA1c

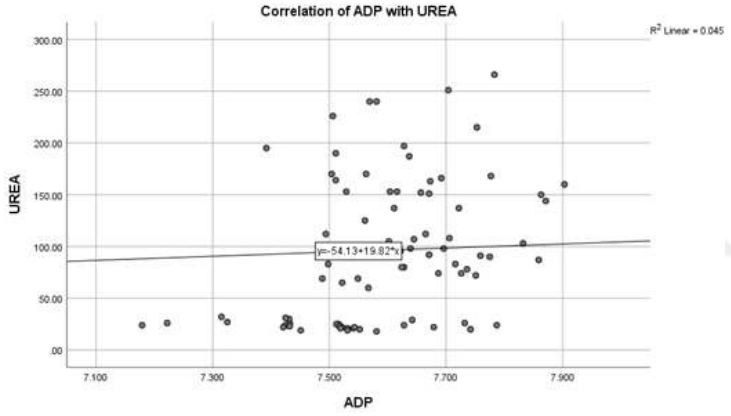

Figure 4.13 Correlation of ADP showed a significant positive with urea

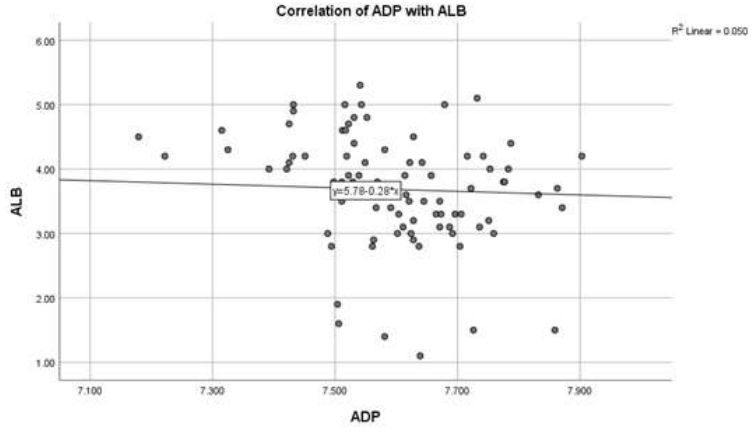

Figure 4.14 Correlation of ADP showed a significant negative with ALB

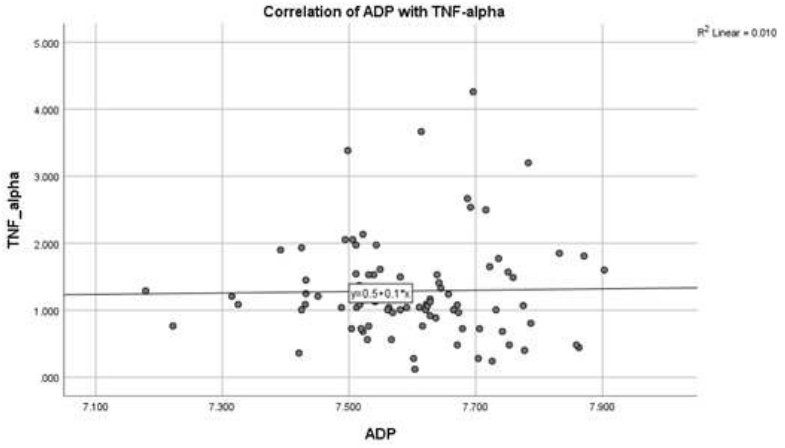

Figure 4.15 Correlation of ADP showed non-significant with TNF-alpha

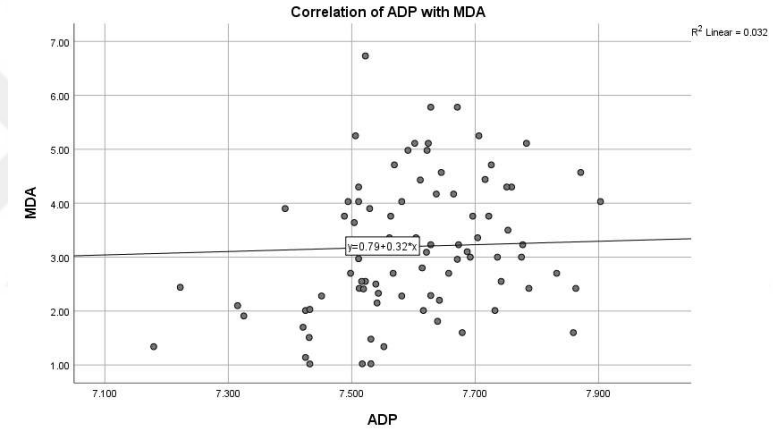

Figure 4.16 Correlation of ADP showed non-significant with MDA

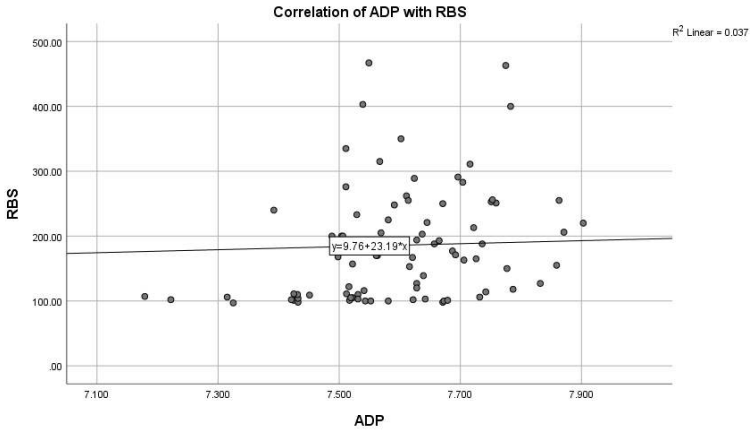

Figure 4.17 Correlation of ADP showed non-significant with RBS

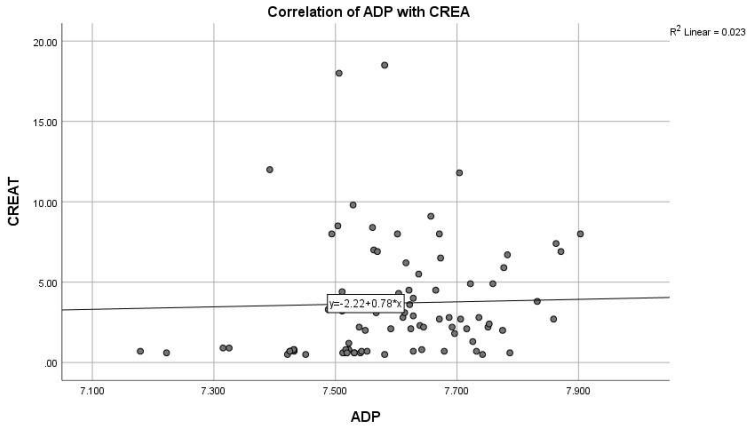

Figure 4.18 Correlation of ADP showed non-significant with CREA

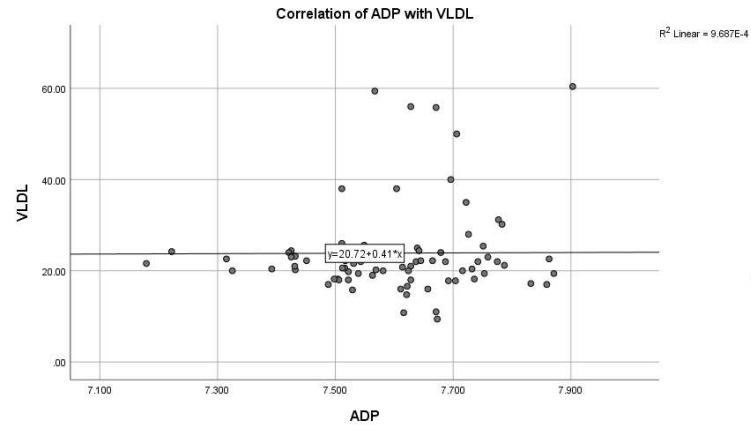

Figure 4.19 Correlation of ADP showed non-significant with VLDL

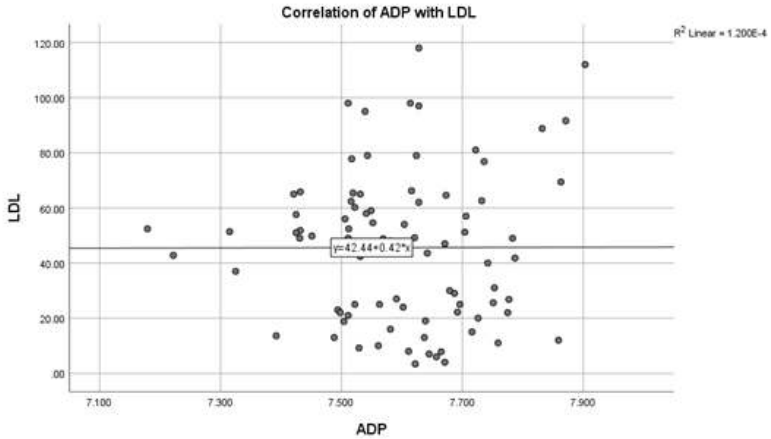

Figure 4.20 Correlation of ADP showed non-significant with LDL

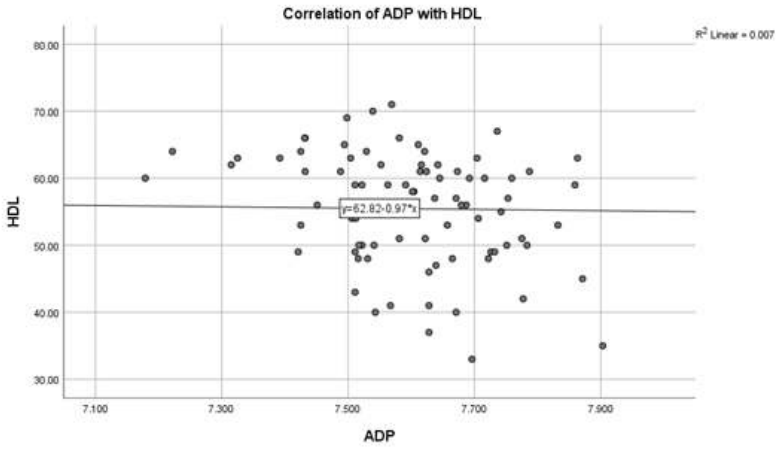

Figure 4.21 Correlation of ADP showed non-significant with HDL

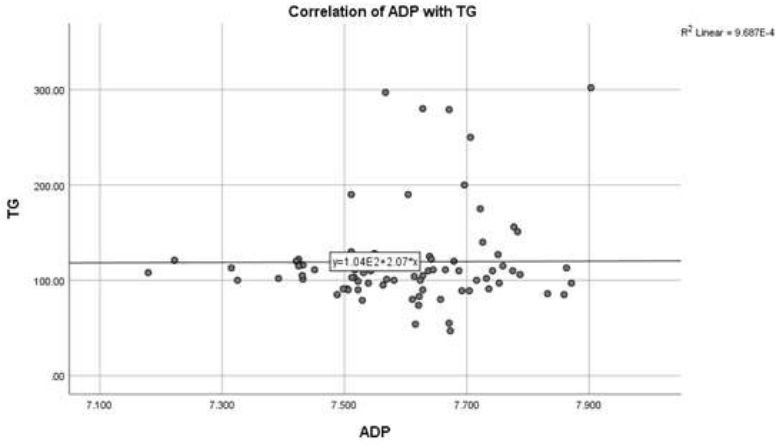

Figure 4.22 Correlation of ADP showed non-significant with TG

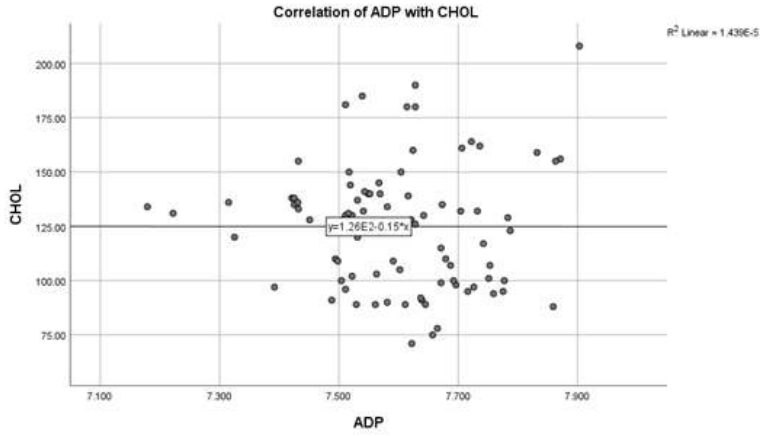

Figure 4.23 Correlation of ADP showed non-significant with CHOL

4.3.3 The Correlation of MDA with biochemical parameters

The results in the present study have indicated that there was a significant positive correlation of MDA with HbA1c, RBS, VLDL, TG, CREA and UREA (r = 0.673, P < 0.001), (r = 0.511, P < 0.001), (r = 0.273, P = 0.011), (r = 0.273, P = 0.011) , (r = 0.431, P < 0.001), (r = 0.580, P < 0.001) respectively as shown in (Table 4.15), (Figures 4.24, 4.25, 4.26, 4.27, 4.28 and 4.29), while a significant negative correlation of ADP with ALB and LDL (r = -0.473, P < 0.001), (r = -0.222, P = 0.039) respectively as shown in (Table 4.15), (Figures 4.30, 4.31), finally there was no significant correlation between MDA with TNF-alpha, ADP, HDL and CHOL (r = 0.051, P = 0.641), (r = 0.180, P = 0.095), (r = -0.160, P = 0.139) , (r = -0.168, P = 0.119) respectively as shown in (Table 4.15), (Figures 4.32, 4.33, 4.34 and 4.35).

Table 4.15 The correlation of MDA with biochemical parameters

	Biochemical Parameters	Pearson Correlation	Sig. (2tailed)
MDA	**TNF-alpha**	0.051	0.641
	ADP	0.180	0.095
	HbA1c	0.673**	< 0.001
	RBS	0.511**	< 0.001
	ALB	-0.473**	< 0.001
	VLDL	0.273*	0.011
	LDL	-0.222*	0.039
	HDL	-0.160	0.139
	TG	0.273*	0.011
	CHOL	-0.168-	0.119
	CREA	0.431**	< 0.001
	UREA	0.580**	< 0.001

NOT: ADP (adiponectin); TNF- alpha (tumor necrosis factor alpha); MDA (malondialdehyde); HbA1c (Glycosylated Hemoglobin A1c); RBS (random blood sugar); ALB (albumin); VLDL (very low density lipoprotein); LDL (low density lipoprotein); HDL(high density lipoprotein); TG(triglyceride); CHOL (cholesterol); CREA (creatinine); and UREA; * significant; r = Pearson Correlation; ** high significant.

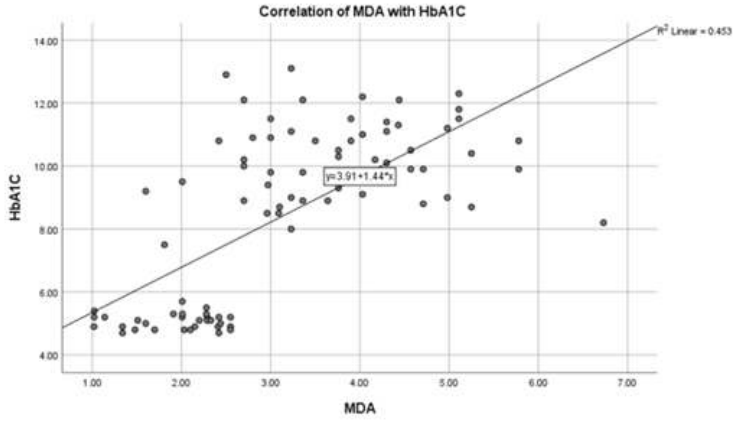

Figure 4.24 Correlation of MDA showed a significant positive with HbA1c

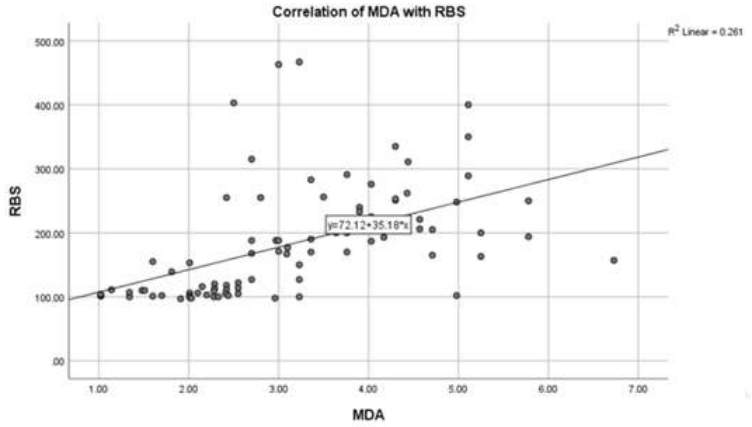

Figure 4.25 Correlation of MDA showed a significant positive with RBS

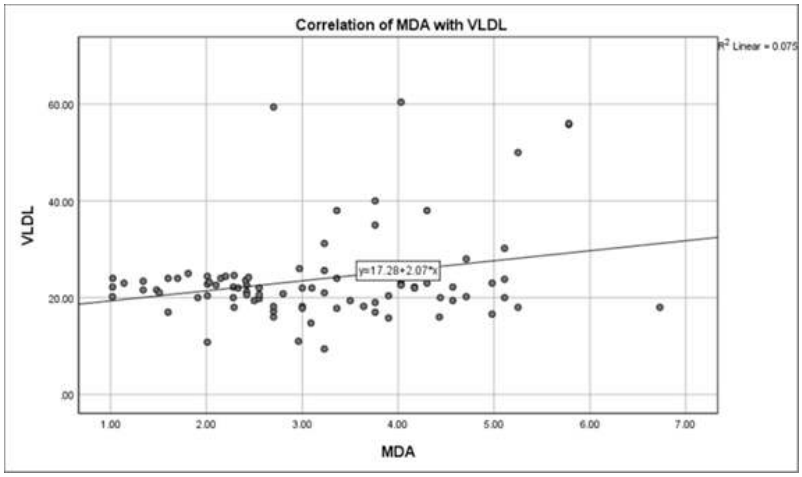

Figure 4.26 Correlation of MDA showed a significant positive with VLDL

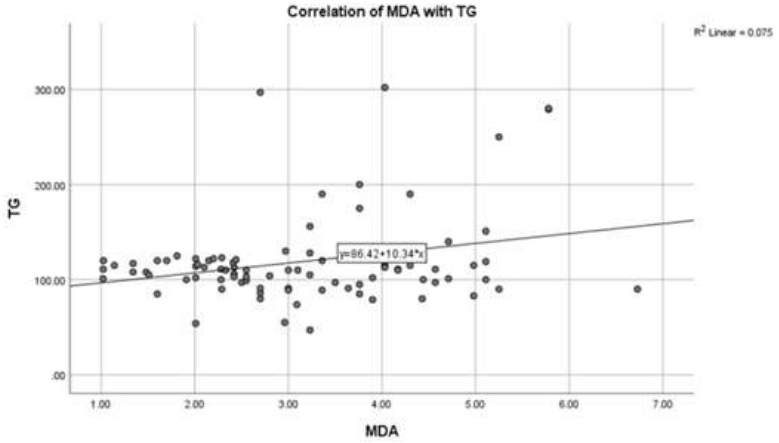

Figure 4.27 Correlation of MDA showed a significant positive with TG

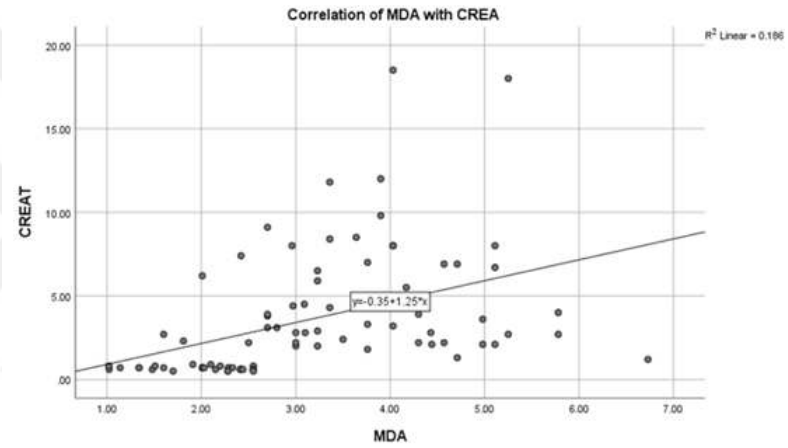

Figure 4.28 Correlation of MDA showed a significant positive with CREA

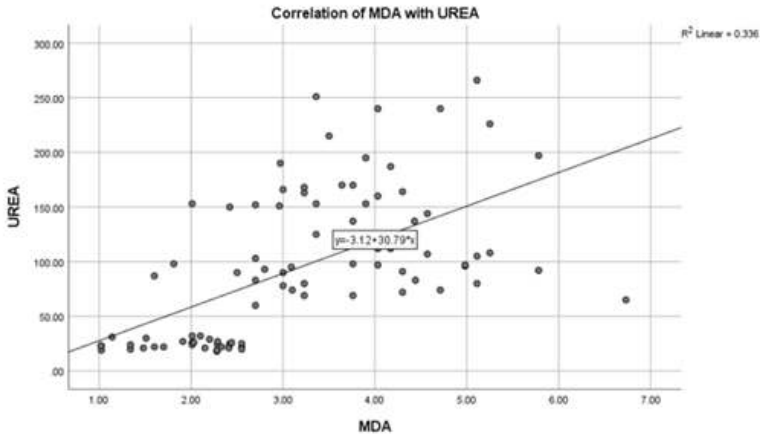

Figure 4.29 Correlation of MDA showed a significant positive with UREA

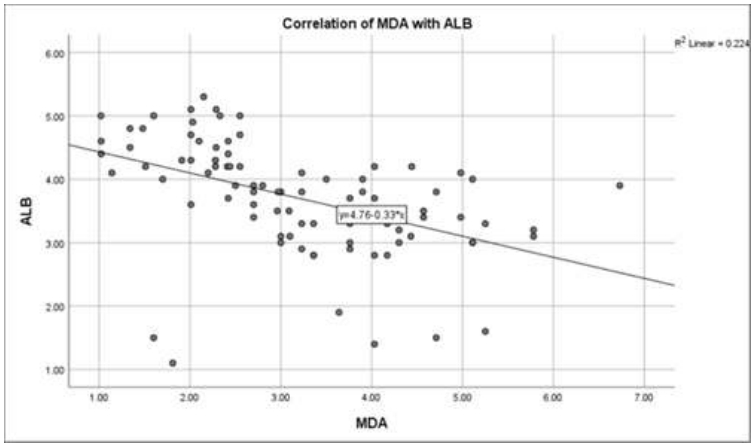

Figure 4.30 Correlation of MDA showed a significant negative with ALB

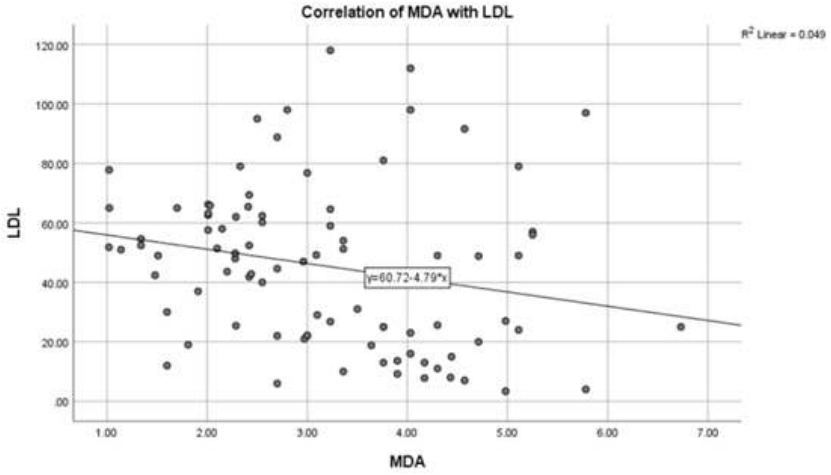

Figure 4.31 Correlation of MDA showed a significant negative with LDL

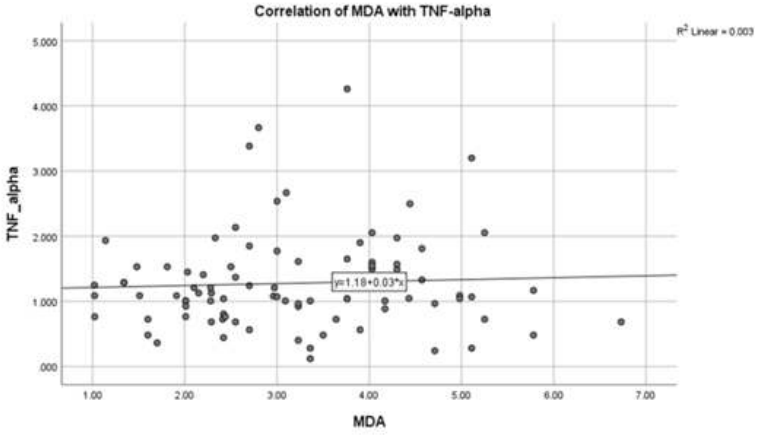

Figure 4.32 Correlation of MDA showed no significant with TNF-alpha

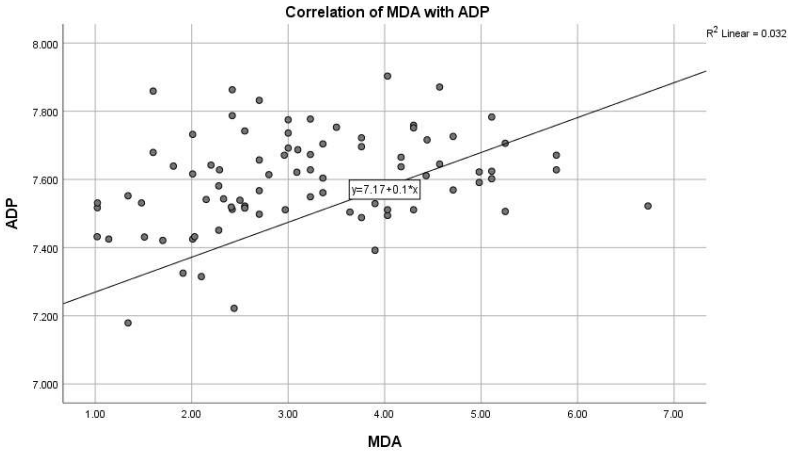

Figure 4.33 Correlation of MDA showed no significant with ADP

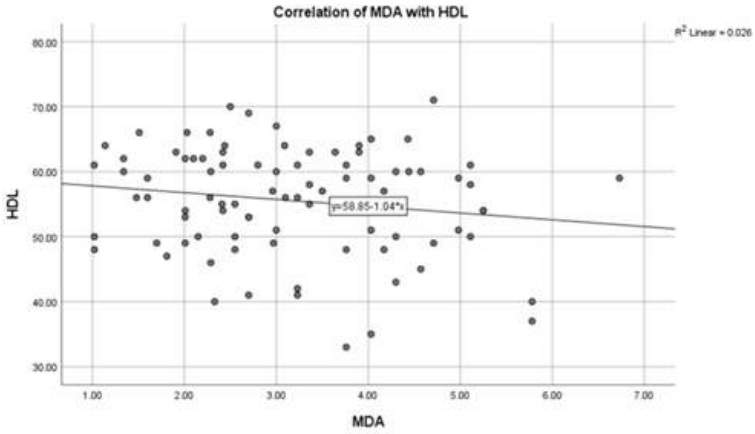

Figure 4.34 Correlation of MDA showed no significant with HDL

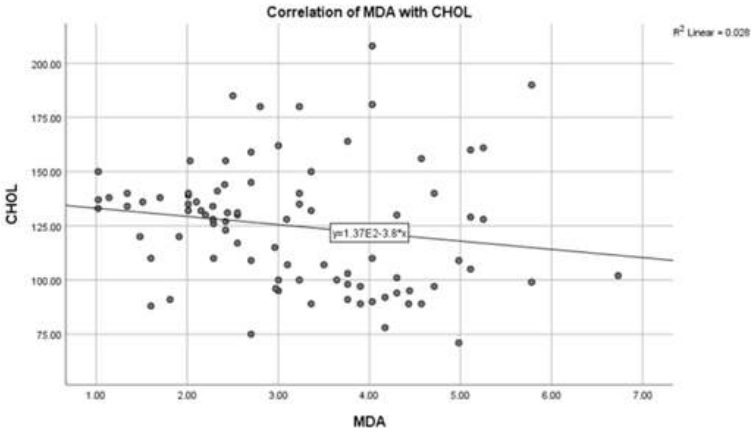

Figure 4.35 Correlation of MDA showed no significant with CHOL

4.4 Receiver Operating Characteristic Curve Analysis (ROC)

In clinical epidemiology, ROC analysis is used to measure how well medical diagnostic tests (or systems) can distinguish between two patient states, commonly referred to as "diseased" and "non-diseased." The concept of a "separator" scale, on which the findings for the diseased and non-diseased form a pair of overlapping distributions, is the foundation of a ROC curve. A completely discriminating test is implied by total separation of the two underlying distributions, whereas total overlap suggests no discrimination. As the threshold for positivity is changed, the ROC curve depicts the trade-off between the true positive fraction (TPF) and the false positive fraction (FPF) (Kumar and Indrayan 2011).

Not The clinical characteristics of the subjects' study participants are shown using IBM SPSS Statistics version 26

4.4.1 ROC for MDA

The ROC curve demonstrated that the MDA in stage 3 have exhibited an excellent method for discriminating' between healthy individuals and patients with DN [AUC =1.000; 95% Confidence Interval (CI):1.000 to 1.000; Std. Error =<0.0001; P ≤ 0.001] as shown in (Table 4.16), (Figure 4.36). Therefore, it is possible to state that MDA in stage 3 is definitely functional for the diagnosis of DN disease, and also MDA in stage 4 value is also very important parameter with a value of [AUC = 0.922; 95% Confidence Interval (CI): 0.824 to 1.000; Std. Error = 0.050; P ≤ 0.001] as shown in (Table 4.16), (Figure 4.37). In addition, in stage 5 MDA value very important parameter with a value of [AUC = 0.974; 95% Confidence Interval (CI): 0.931 to 1.000; Std. Error = 0.022; P ≤ 0.001] was found as shown in (Table 4.16), (Figure 4.38).

Table 4.16 Area under the ROC curve for MDA

Test	STAGES	Area	Std. Error	Asymptotic Sig.	Lower Bound	Upper Bound
MDA	3	1.000	0.000	< 0.001	1.000	1.000
	4	0.922	0.050	< 0.001	0.824	1.000
	5	0.974	0.022	< 0.001	0.931	1.000

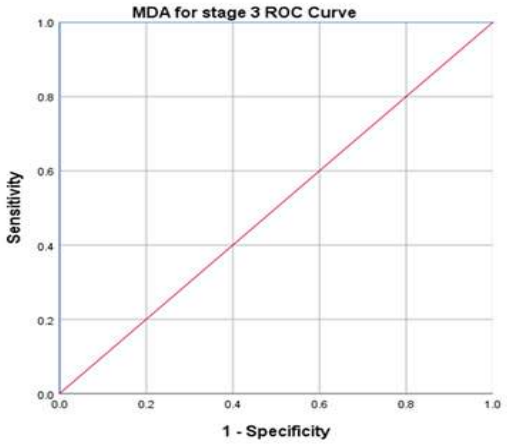

Figure 4.36 ROC for MDA stage 3

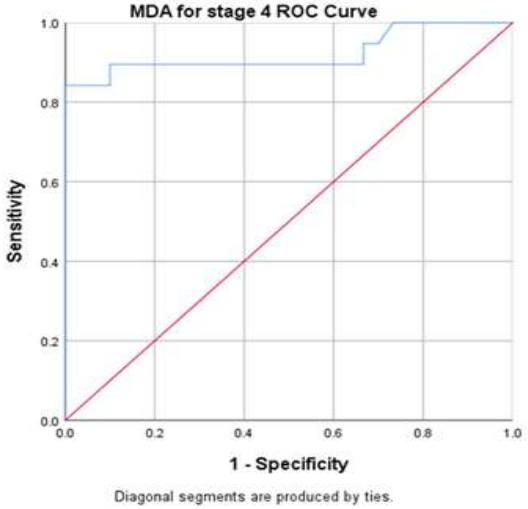

Figure 4.37 ROC for MDA stage 4

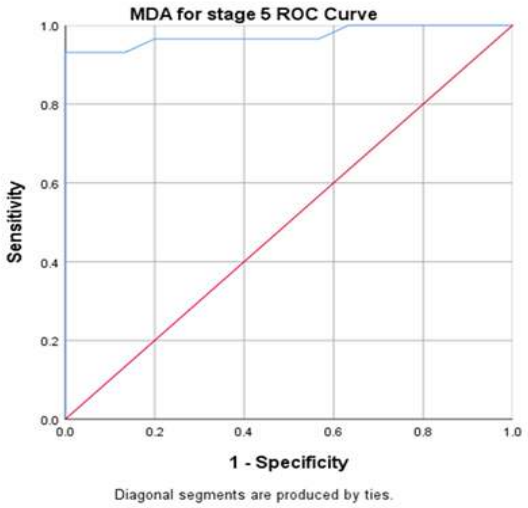

Figure 4.38 ROC for MDA stage 5

4.4.2 ROC for ADP

With a value of [AUC =0.846; P = 0.002; 95% CI: 0.723 to 0.970 and SE:0.063] as shown in (Table 4.17), (Figure 4.39). ADP in stage 3 is considered as a highly significant parameter for DN disease. In addition, ADP in stage 4 shows a significant parameter a value of [AUC =0.812; P = < 0.001; 95% CI: 0.691 to 0.933 and SE:0.062] as shown in (Table 4.17), (Figure 4.40). Moreover, ADP in stage 5 shows a significant parameter a value of [AUC =0.748; P = 0.001; 95% CI: 0.622 to 0.873 and SE:0.064] as shown in (Table 4.17), (Figure 4.41).

Table 4.17 Area under the ROC curve for ADP

Test	STAGES	Area	Std. Error	Asymptotic Sig.	Lower Bound	Upper Bound
ADP	3	0.846	0.063	0.002	0.723	0.970
	4	0.812	0.062	< 0.001	0.691	0.933
	5	0.748	0.064	0.001	0.622	0.873

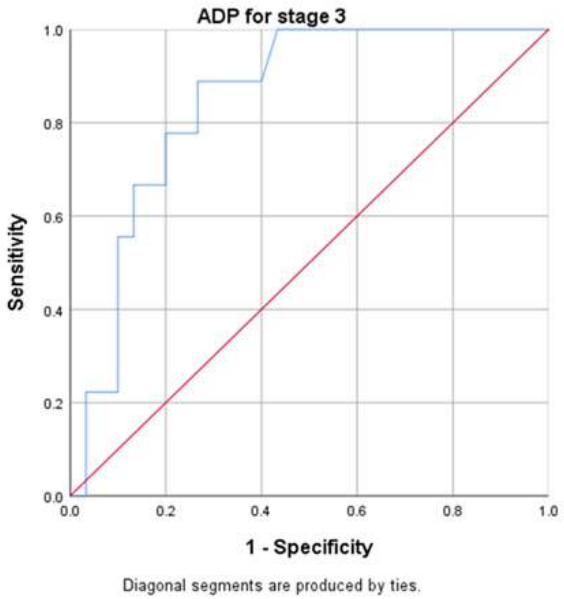

Figure 4.39 ROC for ADP stage 3

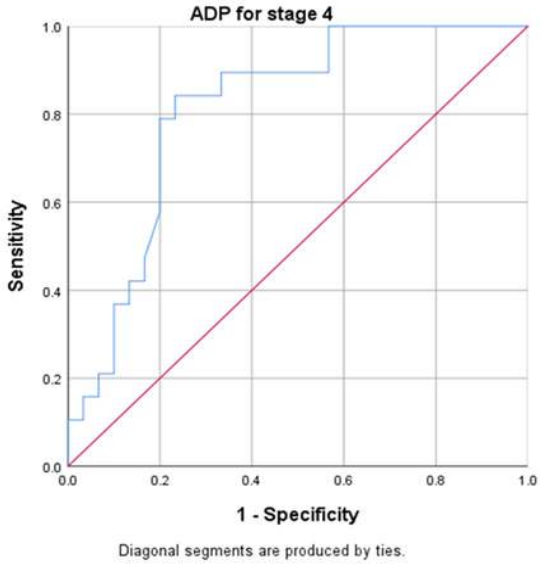

Figure 4.40 ROC for ADP stage 4

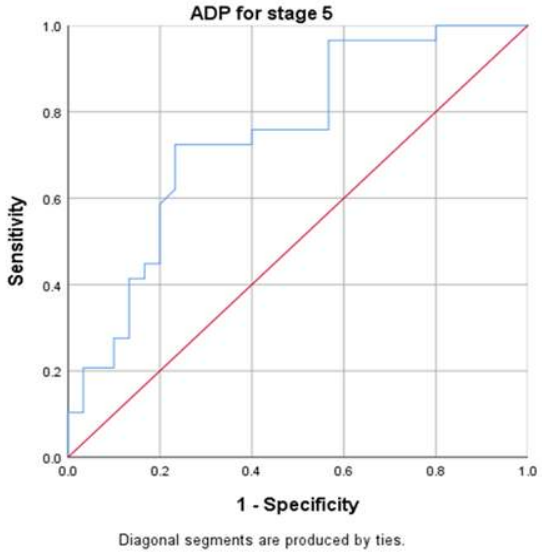

Figure 4.41 ROC for ADP stage 5

4.4.3 ROC for TNF-alpha

TNF-alpha in stage 3 showed low validity in predicting of DN with a value of [AUC =0.311; P = 0.089; 95% CI: 0.068 to 0.554 and SE:0.124] as shown in (Table 4.18), (Figure 4.42). Also, TNF-alpha in stage 4 shows no significant a value of [AUC =0.258; P = 0.005; 95% CI: 0.096 to 0.419 and SE:0.082] as shown in (Table 4.18), (Figure 4.43). Finally, TNF-alpha revealed no significant a value of [AUC =0.191; P < 0.001; 95% CI: 0.077 to 0.306 and SE:0.059] in stage 5 as shown in (Table 4.18), (Figure 4.44).

Table 4.18 Area under the ROC curve for TNF-alpha

Test	STAGES	Area	Std. Error	Asymptotic Sig.	Lower Bound	Upper Bound
TNF-α	3	0.311	0.124	0.089	0.068	0.554
	4	0.258	0.082	0.005	0.096	0.419
	5	0.191	0.059	< 0.001	0.077	0.306

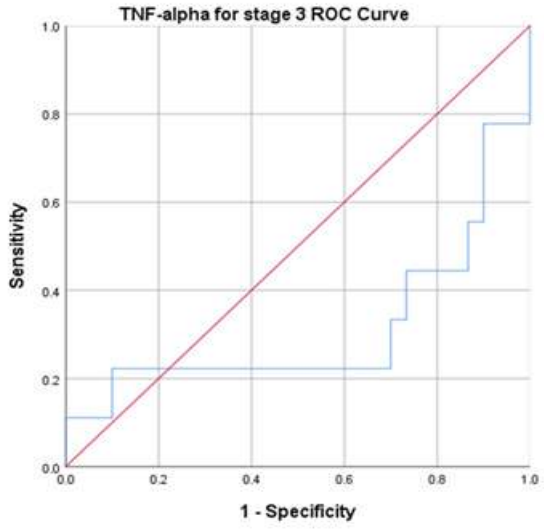

Figure 4.42 ROC for TNF-α stage 3

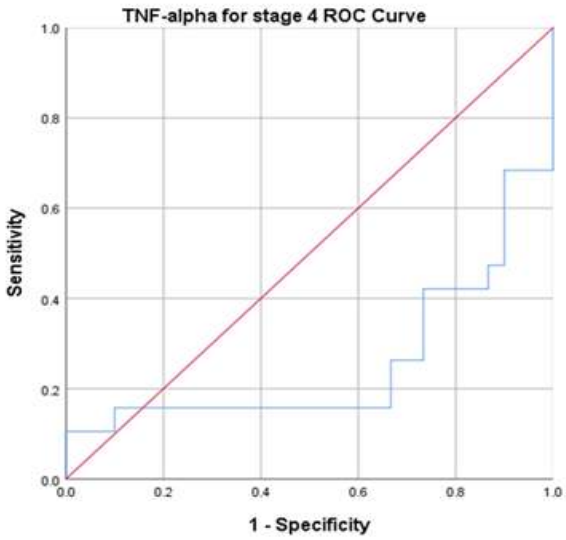

Figure 4.43 ROC for TNF- α stage 4

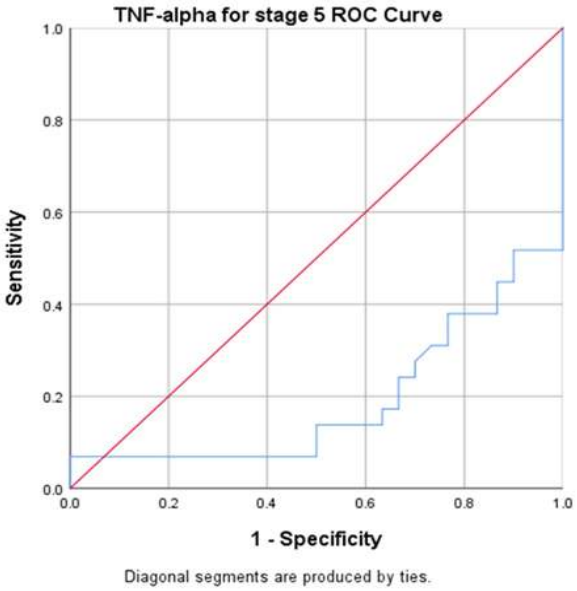

Figure 4.44 ROC for TNF- α stage 5

5. DISCUSSION

DM are more most likely to occur renal disease owing to a variety of risk factors. One of the factors is a lack of blood glucose regulation. In addition, hypertension or a family history of hypertension is more likely than those without a family history to develop renal disease. The length of time that diabetes has been present is also essential; the longer diabetes has been present, the greater the risk of kidney damage. The significant growth in the number of diabetics may result in a high risk of DN. As a result, reducing diabetic complications is critical in preventing the development of DN (Woo *et al.* 2012).

5.1 Adiponectin

Is a peptide adipokine having a molecular weight of 30 kDa and 244 amino acids (Scherer *et al.* 1995). Adipose tissue is a critical endocrine organ that secretes a vast amount of endocrine hormones substances that govern a diverse set of physiological functions. Adiponectin is one such factor, which is predominantly released by adipose tissue in considerable amounts into the circulation after being From a 30 kDa monomeric protein to different multimers after post-translational modification (low molecular weight or trimer, intermediate molecular weight or hexamer, and high molecular weight) (Fang and Judd 2018).

In this study there was have higher adiponectin levels, blood the levels of adiponectin were used to predict ESRD in diabetic patients were classified into three phases according to the GFR formula MDRD (Houlind *et al.* 2018). Clinical changes were studied for several factors, including ADP.

The current study showed an increase in ADP rates for the participants more than the healthy controls. The ADP in the 3rd stage was high, while in the 4th and 5th stages was slightly decreasing and this is due to albuminuria, where the more albumin extracted in urine and its decrease in the blood led to a decrease in ADP, which agrees with (Georgoulidou *et al.* 2017) results that lower plasma adiponectin levels suggest a greater likelihood of future albuminuria deterioration.

In another study, consistent with our study, the participants had higher levels of ADP compared to healthy control; ADP is a kind of adipocytokine that has anti-oxidant and anti-inflammatory properties. This study explained, adiponectin exerts its action on the renal glomerulus by activating adenosine monophosphate activated protein kinase (Georgoulidou *et al.* 2017). In another study, the following was shown, High blood adiponectin levels were found in DN patients, and some investigations have high ADP levels have been mentioned as a possible cause, might predict mortality and the progression of CKD to dialysis (Menon *et al.* 2006). Higher levels of adiponectin may be attributed to an increase in breakdown or increased synthesis from adipose tissue as a result of renal insufficiency. In DN, a rise in adiponectin may be a preventive mechanism aimed at endothelial function is improved, oxidative stress is reduced, and nitric oxide synthase is increased (Alnaggar *et al.* 2019, Zha *et al.* 2017). ADP levels of serum were shown to be remarkably greater in individuals with renal insufficiency and to be inversely related to e GFR (Cha *et al.* 2018), this result is agree with our study, the lower the GFR, the higher the levels of ADP.

Current study revealed a relationship between ADP, urea, creatinine, albumin and HbA1c which are indicators of kidney deterioration, and the more deterioration of the kidney in its last stages leads to high levels of ADP which agrees with study had been done by (Alnaggar *et al.* 2019).

5.2 Tumor Necrosis Factor-Alpha(TNF-α)

Monocytes, macrophages, and T lymphocytes are the primary producers of TNF-alpha. Resident renal cells, such as , glomerular, dendritic, endothelial, mesangial, and renal tubular cells, may all generate TNF-alpha (Dong *et al.* 2007). TNF-alpha is involved in the recruitment of monocyte-macrophages, which results in a decrease in GFR due to alterations in hemodynamics (Lim and Tesch 2012). Individuals with T2DM have 3-4 times higher blood levels of TNF-alpha than patients that are not diabetic, according to experimental evidence, in addition diabetic patients with microalbuminuria had greater levels than diabetic individuals with normoalbuminuria (Moriwaki *et al.* 2003). Furthermore, TNF-alpha excretion in the urine also correlated strongly with DN clinical indicators and disease progression (Navarro *et al.* 2006).

This study showed increased levels of TNF-alpha at all stages of disease development compared with healthy control which was agree with (Lim and Tesch 2012), finding, who explains that a variety of inflammatory cytokines have become well-known as key players in the disease's etiology. Some of the key inflammatory cytokines that are thought to take part in a role in DN.

In addition, current study demonstrated a slight decrease in the levels of TNF-alpha through stages of disease progression 3, 4 and 5, respectively, TNF-alpha has an inverse relationship with ADP, as the latter is an anti-inflammatory, which explains the increase in ADP and the decrease in TNF-alpha This agrees with (Wang *et al.* 2014) who showed ADP is anti-inflammatory and antioxidant, it inhibits leukocyte rolling and adherence by suppressing TNF-alpha induced elevation of endothelial cell adhesion molecules. Also, in another study, it was shown ADP inhibits the development of leukocyte colonies and lowers macrophage TNF-alpha production (Ouchi *et al.* 2001).

In the current study, there was a relationship between TNF-alpha with HbA1c and RBS, which is agree with (Avci *et al.* 2014), finding. TNF-alpha levels in the blood had a substantial positive relationship with HbA1c and RBS levels. This link was seen in individuals who had diabetes for more than 5 years as well as those who had the condition for a shorter time. TNF-alpha plasma levels are linked to diabetes and poor glycemic control.

5.3 Malondialdehyde (MDA)

Lipid peroxidation is the free radicals oxidize polyunsaturated fatty acids (linoleic acid, arachidonic acid, etc.), which can cause substantial tissue damage (Fatani *et al.* 2016). Peroxidation changes the characteristics of lipids, which affects their function. Lipids constitute the major peroxidation and component of cellular membranes changes their characteristics (Bigagli and Lodovici 2019). MDA, 4-hydroxynonenal (HNE), thiobarbituric acid reactive substances (TBARS), and isoprostanes such as 8-iso-prostaglandin F2 (8-iso-PGF2) are the most often researched lipid peroxidation indicators. MDA is produced via lipid peroxidation as well as the creation of

prostaglandins and thromboxane, it has the ability to assault macromolecules, causing changes in their functionality (Bigagli and Lodovici 2019).

There was a notable rise in MDA levels in our study in all stages 3, 4 and 5 among patients compared to healthy controls, as shown in (Table 4.10). This finding is similar to several studies, including, CKD patients had greater blood MDA levels than healthy control persons (Vecchi *et al*. 2009).

In another study, MDA had a negative correlation with CKD and GFR patients in stages 2, 3, 4, and 5 has significantly varied outcomes (Hojs *et al.* 2020). This is agreed with our result that the lower the GFR, the higher the MDA level.

In a study similar to ours, MDA levels are increased in dialysis patients (Kaya *et al.* 2012). MDA levels in the blood were also found to be greater in hemodialysis patients.

Furthermore levels of MDA in the blood were much lower in transplant patients than in dialysis patients (Hojs *et al.* 2020).

The most frequent cause of CKD is diabetes. The exact etiology of DN and its development to ESRD is complicated, although oxidative stress plays an important have a role in the DN pathogenesis and its development to ESRD. A number of oxidative stress indicators related with DN have been discovered in recent years Among them is MDA (Noce *et al.* 2014).

In the current study, there was a significant positive correlation of MDA with HbA1c, RBS, this result agrees with (Fatani *et al.* 2016). Lipid peroxidation, as measured by MDA, was considerably higher in the uncontrolled T2DM group than in the managed T2DM group, with both groups showing substantial increases above healthy participants. Between MDA and HbA1c, there is a substantial positive association.

5.4 Receiver Operating Characteristic Curve Analysis (ROC) FOR ADP, TNF-alpha and MDA

5.4.1 ADP

The Receiver Operating Characteristic (ROC) curve for ADP in all renal stages was also studied. There was a clearly increase in AUC at all stages as shown in Table 4.17 and This is agree with this study which showed, Patients with T1DM had a higher blood ADP level, which was linked to renal insufficiency (Saraheimo *et al.* 2005). In addition, other study which excluded patients with DN, serum ADP in patients with T2DM showed significantly lower level than in healthy people (Eynatten *et al.* 2009), while according to our patients with nephropathy had greater levels of ADP than those without nephropathy, according to the data. Serum ADP levels were shown to be elevated using the ROC-AUC method. These conclusions were drawn from the findings were observed by (Koshimura *et al.* 2004).

Given that AUC levels are elevated at all stages of the disease, ADP can be considered as an indicator of DN and this agrees with (Lee *et al.* 2019), who showed adiponectin's antioxidant ability is critical for decreasing metabolic damage in people with DN. Through the AMPK pathway, adiponectin decreases oxidative stress by decreasing Nox4 is a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is abundant in the kidney.

5.4.2 TNF- alpha

The ROC curve for TNF-alpha in all renal stages 3, 4 and 5 was also studied. AUC levels were below the line, it can be considered as an inverse relationship with increasing ADP as in study (Liu *et al.* 2016). As reported evidence, adiponectin appears reduces the production of pro-inflammatory cytokines including IL-6 and TNF-a, making it anti-inflammatory. in macrophages and/or lowering their phagocytic capability. The capacity of ADP to decrease pro-inflammatory cytokine production appears to be a key element in correcting metabolic dysfunction in this regard.

5.4.3 MDA

The ROC curve for MDA in all renal stages 3,4 and 5 was also examined. There was a clearly increase in AUC at all stages as shown in (Table 4.16), therefore, it can be considered a good indicator for predicting DN in its early stages. We noticed that the levels of AUC in stage 3 rise to the highest level as shown in (Table 4.16), as stage 3 can be considered as the beginning of DN. This agrees with (Sauriasari *et al.* 2015), who found that presence of MDA in the erythrocytes of DM patients with evident nephropathy was shown to be significantly higher than in the involvement of lipid peroxidation in nephropathy was confirmed in a group without nephropathy.

Furthermore, this study's findings are in line with those of other researchers who have shown significantly greater levels of MDA in difficult diabetes with nephropathy and non-complicated diabetes as compared to healthy controls (Rani *et al.* 2019, Mahreen *et al.* 2010) The current study also shows an increase in MDA levels with an increase in the duration of DM, which agrees (Rani *et al.* 2019).

5.5 Renal Function Test

Blood tests is the easiest technique to assess kidney function via measuring blood urea (BU) and serum creatinine. These chemicals are common metabolic waste products that the kidneys eliminate. Protein degradation produces urea as a byproduct. Serum creatinine is primarily a creatine metabolite found almost entirely in skeletal muscle. Because women have less muscle mass than men, thus creatinine levels are generally lower than creatinine in men (Dabla 2010).

There was a notable rise in urea and creatinine levels in the current study. in all stages of DN 3, 4 and 5 stages in comparison to healthy controls. Urea and creatinine can be considered as the primary initial diagnosis of DN. This agrees with (Ahmed *et al.* 2010), who showed Increases in urea are induced by a decreased ability to eliminate protein catabolites due to a substantial drop in GFR in renal failure. Urea is proportional to the amount of protein in the diet. Gastrointestinal bleeding, as well as increased protein catabolism, can cause a rise in urea. Creatinine levels rise as a result of reduced renal

excretion. Because urea concentration can be affected by a variety of external variables, creatinine is frequently utilized as a more accurate measure of GFR in individuals with renal disease.

5.6 Serum Albumin

Serum albumin, the most abundant plasma protein, is produced in the liver and released into the vascular space, where it is distributed throughout the body. It is critical for maintaining homeostasis and achieving inside vessels, a balance between hydrostatic and colloid osmotic pressure serum albumin has a variety of physiological activities, including binding to hormones, ions, and medicines (Zhang *et al.* 2019).

The present study revealed a clearly difference in the decrease of serum albumin in all stages 3, 4 and 5 of DN patients compared to the healthy controls. The decrease can be observed with the increase in the risk of disease progression. As albumin level in the 5th stage is lower than in the 3rd stage. Evidence for the inability of the kidneys to preserve albumin molecules in agreement with a study revealed a decrease in serum albumin can be considered as a predictive sign of nephropathy (Dabla 2010), who showed total blood protein, serum albumin, and the A/G ratio all decreased in the patients' group. Whereas serum globulin increased significantly when compared to the controls indicates that such individuals have nephropathies due to severe malnutrition.

In other study (Zhang *et al.* 2019) in individuals with T2DM and DN, a decreased serum level of albumin was linked to impaired kidney function and a worse renal prognosis, regardless of clinical or histological characteristics.

5.7 Lipid Profile

Many lipoprotein abnormalities have been linked to DN patients, including increased plasma levels of VLDL, TG, and LDL, as well as decreased HDL levels. lipids can cause glomerular and tubulointerstitial damage via mediators such cytokines, ROS, and chemokines, as well as hemodynamic alterations (Chen *et al.* 2005).

This study showed a decrease in cholesterol compared to healthy controls, while there was a raise in TG, VLDL, and LDL, and a reduction in HDL in all studied stages. This is attributed to dyslipidemia. It occurs in DN patients,

In diabetes, dyslipidemia is defined by a raise in VLDL, TG, and LDL, as well as a reduction in HDL. Hormone-sensitive lipase is activated in the diabetic environment, resulting in the release of free fatty acid (FFA) from adipose tissue, FFA flow stimulates hepatic TG formation, resulting in an excess of Apo B and VLDL. In addition, insulin resistance leads to decrease lipoprotein lipase (LPL) activity. The blood levels of TG and residual particles rise as a result of these alterations (Kawanami *et al.* 2016).

Diabetic individuals frequently have dyslipidemia. Serum cholesterol appears to have an essential role in the progress and development of DN in a number of clinical and experimental investigations. In the prevention and treatment of DN, cholesterol management appears to be crucial (Chen *et al.* 2005).

Another study showed increased plasma levels of VLDL, LDL, and TG which are commonly detected in individuals with microalbuminuria and overt proteinuria. However, people with normoalbuminuria had lower HDL levels in their blood (Jenkins *et al.* 2003).

DN can be accelerated by dyslipidemia. DN promotes abnormal lipoprotein metabolism, which leads to additional renal damage, ESRD, and CV events (Kawanami *et al.* 2016).

5.8 Glycosylated Hemoglobin A1c (HbA1c) and Sugar

Hyperglycemia causes glycosylated end products to adhere to numerous surfaces of cells in one sense, it covers the adhesion molecules found in inflammatory cells, causing an immune-deficient condition. It also attaches to RBC hemoglobin (Hb). RBCs in a normal healthy adult include roughly % HbA 97 % HbA_2 2.5%, and 0.5 % HbF. HbA_1 accounts for roughly 6% of total HbA. HbA_1 is divided into HbA_1a_1, HbA_1a_2, HbA_1b, and HbA1c fractions based on electrophoretic characteristics, with HbA1c being the most common and accounting for 5% of total HbA. The principal glucose binding sites in hemoglobin are β-Val-1, α-Lys-61, and β-Lys-66. Glycation of Hb is a natural

occurrence. Aldimine is generated through the glycation of Hb, it's a reversible procedure. Aldimine is then gradually transformed becomes the irreversible form of ketoamine. The HbA1c levels rises in tandem with the amount of blood glucose (Acharya *et al.* 1991)**.**

Current study revealed an increase in RBS levels in all stages, as well as a clear increase in HbA1c levels. The duration of diabetes is important in the DN's development, as the control levels of blood sugar and the amount of HbA1c control are important in controlling the disease. As in some studies, T1DM individuals with related risk factors such as hereditary genetics, hypertension, and poor metabolic management have a greater prevalence of DN. metabolic control is a risk factor that may be altered. The diabetic control and complication trial (DCCT) found that intensive diabetes treatment, defined as a mean HbA1C of around 7%, can lower the chance of developing microalbuminuria by 34% as compared to those on standard care, whose HbA1C was 8.5 percent. It also shown that improving metabolic control reduces the likelihood of problems regardless of previous management (Fares *et al.* 2010).

Other study demonstrated that a person's HbA1c level is less than 6.5 % but greater than 5.7 % diagnosed as Prediabetes. DM is more likely in those with prediabetes. As a result, lifestyle changes, dietary changes, and treatment of other CVD risk factors are being treated. are suggested. Prediabetes patients should be checked at least once a year (Association 2018).

Because DM has so many consequences, early identification and good care may help to lessen the societal cost burden. It also reduces the number of people who die as a result of diabetic complications. Since 2011, the WHO has added HbA1c, along with FBG and RBS, as part of the diagnostic criteria for DM (Shiva 2018).

6. CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. The current study showed that the lack of control of HbA1c and RBS, as the diabetes for a longer period leads to the DN's fast development to its final stages.
2. This study supported the use of ADP as a good indicator for predicting diabetic nephropathy in its early stages, as we note high levels of ADP in all stages of DN, as it was observed through ROC curves.
3. ADP can be considered as an anti-inflammatory by observing its high levels at all stages of the DN's development due to the presence of inflammatory TNF-alpha cells.
4. The current study showed that DN is associated with oxidative stress and this can be seen by the high levels of MDA at all stages, which can be considered as a good indicator of oxidative stress associated with DN.
5. Current study revealed a relationship between ADP, urea, creatinine, albumin and HbA1c which are indicators of kidney deterioration, TNF-alpha levels in the blood had a substantial positive relationship with HbA_1c and RBS levels. and also, there was correlation of MDA with HbA_1c, RBS.

6.2 Recommendations

1. The future study should include extensive sample collection.
2. A study of ADP and MDA changes in patients with T1DM and T2DM before reaching ESRD.
3. Further studies are required of DN patients to find out the changes in ADP receptors ($AdipoR_1$ and $AdipoR_2$).
4. Future studies to look into the possible role of other cytokines in DN patients.
5. Investigate molecular mechanisms of ADP genetic studies.

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