University of Basrah
College of Science
Department of Chemistry

Study the Impact of Insulin Resistance on Specific Blood and Urinary Biomarkers for Early Predicting of Nephropathy in Type 2 Diabetic Obese-Patients / Basrah-Iraq

A Thesis

Submitted to the Council of the College of Sciences / University of Basrah in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

صَدَقَ اللّٰهُ العَلِيُّ العَظِيم

سورة البقرة – آية (٣٢)

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Dedication

To whom guided me in the whole of my life

To whom made all my dreams real

To whom never let me down

To whom gave me everything

To the darling Imam Hussein

(Allah blesses him and his household)

I dedicate this work to him with my unending

and deepest love and thankfulness

Yours Sincerely...

Sadoun…

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Summary

Diabetic nephropathy (DN) is a common and severe microvascular complication of type 2 diabetes mellitus (T2DM) that can result in end-stage renal disease (ESRD). Hence, the aim of the present study is to study the impact of insulin resistance (IR) on specific blood and urinary biomarkers for early predicting of DN in men and women of T2DM obese-patients of the population of Basrah province (southern of Iraq). In this study, 186 men and women volunteers were participated. Sixty-three of patients' with T2DM [31 patients who suffering from DN (15 men and 16 women) and 32 patients without DN (15 men and 17 women)] and 33 healthy controls (16 men and 17 women) were followed up for 8 months, till end the study while 90 of volunteers (50 patients and 40 healthy controls) were excluded from the study because enable to follow up study. The three groups were matched for some blood (glucose, insulin, urea, creatinine, BMI, GFR, CrCl, IR, Hcy, FA, Cys C, LC, OPG, SA, Se, Zn, Mg) and urine (Alb, NGAL, 8-OHdG and FN) biomarkers. Compared with normal controls, the results indicated that T2DM patients with and without DN had a significantly ($p<0.01$) increased levels of glucose, insulin, IR, Hcy, FA, SA, NGAL and 8-OHdG. Furthermore, patients with T2DM with DN had a significant increase ($p<0.01$) and patients with T2DM without DN had a significant increase ($p<0.05$) in the levels of serum urea, creatinine, Cys C, OPG, Alb and FN, compared to healthy control. On the other hand, our data reported that GFR, CrCl as well as LC were decreased significantly in T2DM patients ($p<0.01$ in patients with DN and $p<0.05$ in patients without DN), while levels of Se, Zn and Mg were significantly ($p<0.01$) lower in T2DM patients with and without DN as compared to control group. Moreover, the results obtained indicated that there was a non-significant change ($p>0.05$) in BMI level in T2DM patients with and without DN as compared to control group. We conclude that insulin resistance is strongly associated with obesity and had an important role in the pathogenesis and

increased complicating of many human diseases such as diabetes which can be used as a biomarker of renal dysfunction in DN thus decreasing the mortality and morbidity. Hence, taking into consideration the combination of obesity, IR and T2DM may demonstrated excellent indicator for early-stage DN in patients with T2DM.

Glossary of Abbreviations

8-OHdG	8-Hydroxy-2′-deoxyguanosine
ACR	Albumin Creatinine Ratio
ADA	American Diabetes Association
ADP	Adenosine Diphosphate
AGEs	Advanced Glycation End-products
Alb	Albumin
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CAD	Coronary Artery Disease
CI	Confidence Interval
CKD	Chronic Kidney Disease
CrCl	Creatinine Clearance
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
Cys C	Cystatin C
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-linked immune sorbent assay
ESRD	End Stage Renal Disease
FA	Fructosamine
FAAS	Flame Atomic Absorption Spectrometer
FFA	Free Fatty Acid
FN	Fibronectin
GBM	Glomerular Basement Membrane
GFR	Glomerular Filtration Rate
GOD	Glucose oxidase
GPx	Glutathion peroxidase
HbA1c	Glycated hemoglobin
Hcy	Homocysteine

HIV	Human Immunodeficiency Virus
HMG CoA	Hydroxymethylglutaryl Coenzyme A
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
HRP	Horseradish Peroxidase
IDDM	Insulin Dependent Diabetes Mellitus
IGF-1	Insulin-like Growth Factor-1
IL-6	Interleukin-6
IR	Insulin Resistance
LC	L-Carnitine
MAPK	Mitogen-Activated Protein Kinases
MAU	Microalbuminuria
MDRD	Modification of Diet in Renal Disease
mRNA	Messenger Ribonucleic Acid
NAD	Nicotinamide Dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NGAL	Neutrophil Gelatinase Associated Lipocalin
NIDDM	Non-insulin Dependent Diabetes Mellitus
OD	Optical Density
OPG	Osteoprotegerin
OS	Oxidative Stress
PBS	Phosphate Buffer Saline
PKC	Protein kinase C
POD	Peroxidase
PPAR	Peroxisome Proliferator-Activated Receptor
RAAS	Renin Angiotensin Aldosterone System
RANK	Receptor activator of necrosis factor - kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive Oxygen Species
SA	Sialic Acid
SD	Standard deviation
SE	Standard Errors
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TBM	Tubular Basement Membrane
TG	Triglycerides
TGF-β	Transforming Growth Factor-Beta

TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TXA2	Thromboxane A2
UACR	Urinary Albumin Creatinine Ratio
UAE	Urinary Albumin Excretion
UAER	Urinary Albumin Excretion Rate
VSMC	Vascular Smooth Muscle Cells
WHO	World Health Organization

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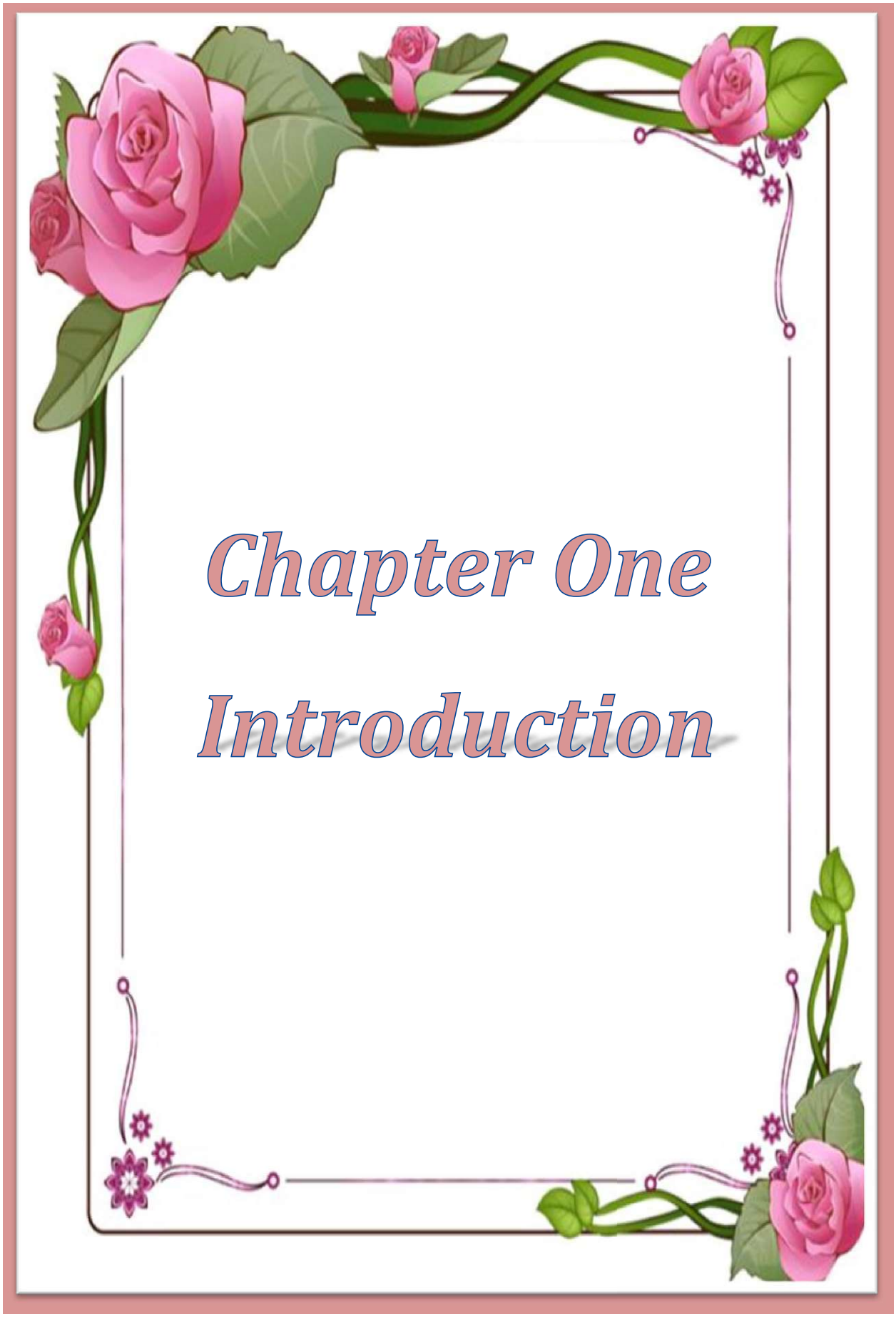


Chapter One

Introduction

Chapter One

1.1. Diabetes Mellitus (DM)

The term diabetes mellitus (DM) describes a metabolic disorder of multiple etiologies and it is a group of metabolic diseases in which a person has a high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Obaid, 2019). Diabetes mellitus is characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death (Al-Fartosy and Mohammed, 2017a). Often symptoms are not severe, or may be absent, and consequently hyperglycemia is enough to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long–term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (WHO, 2019). Several pathogenetic processes are involved in the development of diabetes. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat

and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (ADA, 2018).

1.2. Classification of Diabetes Mellitus:

1.2.1. Type 1 Diabetes Mellitus (T1DM): it results from the deficiency of insulin secretion from β-cells of pancreas, and currently requires the person to inject insulin or wear an insulin pump. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes" (Obaid, 2019).

1.2.2. Type 2 Diabetes Mellitus (T2DM): it results from insulin resistance (IR), a condition in which cells fail to use insulin properly, and sometimes combined with an absolute insulin deficiency. This form was previously referred to as non-insulin dependent diabetes mellitus (NIDDM) or "adult-onset diabetes" (Obaid, 2019).

1.2.3. Hybrid Forms of Diabetes: attempts to distinguish T1DM from T2DM among adults have resulted in proposed new disease categories and nomenclatures, including slowly evolving immune-mediated diabetes and ketosis prone T2DM (WHO, 2019).

1.2.4. Other Specific Types of Diabetes: many factors could be a potential cause for diabetes such as monogenic diabetes, monogenic defects of β-cell function, monogenic defects of insulin action, diseases of the exocrine pancreas, endocrine disorders, drugs or chemical-induced diabetes, infection-related diabetes, uncommon specific forms of immune-mediated diabetes and Other genetic syndromes sometimes associated with diabetes (WHO, 2019).

1.2.5. Unclassified Diabetes: subtyping diabetes has become increasingly complex and it is not always possible to classify all newly diagnosed cases of diabetes as belonging to a specific category. Consequently, a category of “unclassified diabetes” has been introduced.

For most individuals given this label at diagnosis, it is a temporary category as they can be classified into an appropriate type at some point after diagnosis. The worldwide increase in the prevalence of obesity has resulted in T2DM being diagnosed in children and young adults and at the same time children and young adults with T1DM are more commonly overweight or obese than in the past. In addition, ketosis or frank ketoacidosis are not confined to T1DM. These issues make the classification of diabetes difficult, particularly at diagnosis (WHO, 2019).

1.2.6. Hyperglycaemia First Detected During Pregnancy: this type includes two categories of hyperglycaemia when first recognized in pregnancy. One is diabetes mellitus, defined by the same criteria as in non-pregnant persons. The other is gestational diabetes, defined by newly recommended glucose cut-off points that are lower than those for diabetes (WHO, 2019).

So, as well known, DM is characterized by recurrent or persistent hyperglycemia and is diagnosed by demonstrating any one of the following:

1- Fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL) (Adegbola and Ajayi, 2014).

2- Plasma glucose ≥ 11.1 mmol/L (200 mg/dL) two hours after a 75 g oral glucose load as in a glucose tolerance test (Adegbola and Ajayi, 2014).

3- Glycated hemoglobin (Hb A1c) ≥ 6.5 % (ADA, 2018).

A positive result, in the absence of unequivocal hyperglycemia, should be confirmed by a repeat of any of the above methods on a different day. It is preferable to measure a fasting glucose level because of the ease of measurement and the considerable time commitment of formal glucose tolerance testing, which takes two hours to complete and offers no prognostic advantage over the fasting test. According to the current definition, two fasting glucose measurements above 126 mg/dL (7.0

mmol/L) are considered diagnostic for diabetes mellitus. People with fasting glucose levels from 110 to 125 mg/dL (6.1 to 6.9 mmol/L) are considered to have impaired fasting glucose. Patients with plasma glucose at or above 140 mg/dL (7.8 mmol/L), but not over 200 mg/dL (11.1 mmol/L), two hours after a 75g oral glucose load are considered to have impaired glucose tolerance (Al-Fartosy and Mohammed, 2017b).

1.3. Insulin Resistance

Insulin resistance (IR) is the reducing of biological effects (glucose assimilation) in tissues and organs to the response for insulin action on the specific cell receptors. It can be divided into three levels:

1- Pre-receptor level: disorder of pancreas β-cell function.

2- Cell level: decreasing of tissue insulin sensitivity.

3- Liver level: increasing of glucose production.

As it is known, β-cells function is secreting insulin especially the clear reveals under the glucose-tolerant test. The sensitivity of cells to glucose is the most important quantitative parameter of their functional capacity. Under normal conditions, in response to an increase in blood glucose, insulin is secreted from the β-cells of pancreas and binds to insulin receptors on the membrane of insulin-responsive tissues. This result in a signaling transduction cascade which allows for the transport of glucose into the cell for utilization (Moisa, 2017). IR is considered the core factor in the pathogenesis of T2DM. It is often associated with a wide array of other pathophysiologic disorders including, obesity, dyslipidemia, hypertension and atherosclerosis. The coexistence of these diseases, termed the metabolic syndrome or syndrome X which was first described in 1988 (Després, 2018).

IR is also seen in obese individuals without diabetes mellitus. These individuals demonstrate high insulin levels to maintain euglycaemic status.

The syndrome X is also known as IR syndrome, Reaven's syndrome, Pluri-metabolic syndrome, deadly quartet, new world syndrome, civilization syndrome and metabolic syndrome or more appropriately as the dysmetabolic syndrome. It is characterized by the inability of insulin to produce its numerous actions in maintaining a normal blood glucose level, independent of the impaired secretion from the pancreatic β-cells. However, in an insulin-resistant state, potential defect in insulin receptors or in the insulin signaling pathway lead to the impaired insulin action and subsequently, less glucose is transported into the cell (Al-Fartosy and Mohammed, 2017c).

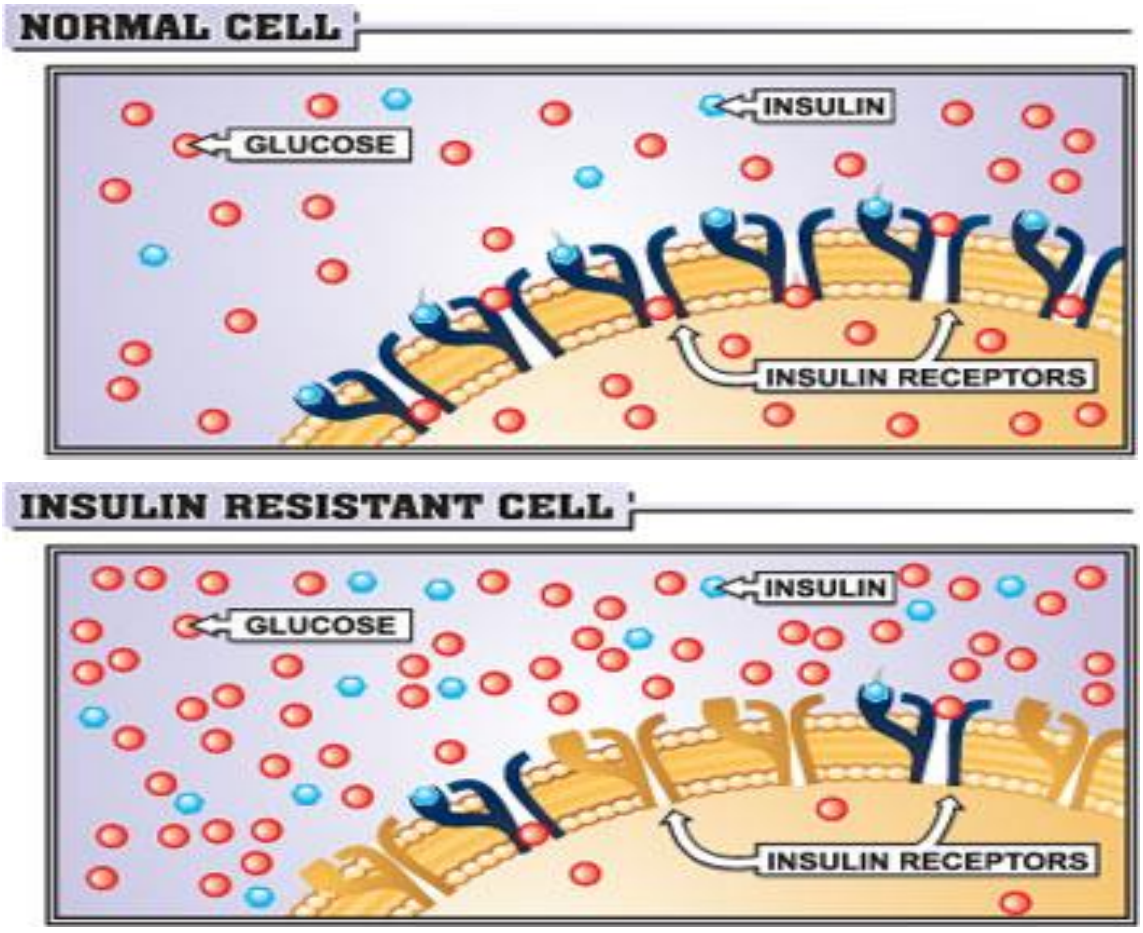


Figure (1.1): Normal cell and IR cell appearance of a typical IR (Al-Fartosy and Mohammed, 2017a).

IR occurs by various genetic and acquired conditions such as a high fat diet, reduced physical activity, and "glucose toxicity" due to

hyperglycemia (Angellotti *et al.,* 2018). Impairments in cellular events, distal to the interaction between insulin and its surface receptor, through alterations in the activities of signaling molecules, enzymes, and transcription factors are known to be the major cause. Antibodies against the insulin receptor or mutations in the insulin receptor gene have also been reported as possible causes (Fujita *et al.,* 2017).

There are two hypotheses which relate the intrauterine and infantile environment to the development of IR in future life. They are the 'thrifty phenotype' hypothesis and the 'thrifty genotype' hypothesis. The thrifty phenotype hypothesis states that the poor nutrition in fetal and infant life is detrimental to the development and function of the beta cells and insulin sensitive tissues, leading to IR under the stress of obesity (Smith and Ryckman, 2015). Maternal diabetes, resulting in the higher birth weights, may have effects similar to fetal malnutrition. The thrifty genotype hypothesis proposes that the defective insulin action in-utero results in a decreased fetal growth as a conservation mechanism, but at the cost of obesity-induced diabetes in later childhood or adulthood. The vast majority of diabetes in adults is polygenic and associated with obesity (Chan *et al.*, 2016).

With respect to the factors that cause the IR, studies have shown many of them including the following:

(1) Increased oxidative stress appears to be a deleterious factor leading to IR, β-cell dysfunction, impaired glucose tolerance, and ultimately type 2 diabetes mellitus (Park *et al.,* 2018).

(2) Obesity is a major risk factor for IR and type 2 diabetes mellitus. Adipocytes secrete numerous substances that might contribute to peripheral insulin sensitivity. These include leptin, tumor necrosis factor α, and interleukin-6 (Boughton *et al.,* 2017).

(3) A number of rare disorders such as genetic abnormalities of the insulin receptor or antibodies against the insulin receptor can cause IR, which is often very severe (Wang *et al.,* 2016).

(4) Genes: there is a genetic, or hereditary, contribution to IR which may be partly due to a genetic determination of body fatness in addition to an equally strong genetic determination of fat distribution in the body. So, some people are genetically prone to accumulate abdominal fat, which is important in generating IR.

(5) Gender: although women generally have 30 to 40 percent more fat on their body than men, women who have not reached menopause have about half as much intra-abdominal fat as men – and have a much better response to insulin. Unfortunately, after menopause, women lose this advantage which suggests that the pre-menopause benefit is related to estrogen (Rad, *et al.,* 2018).

(6) Diet: excess food intake, whether it is carbohydrate, protein or fat, leads to fat accumulation and IR. There are some evidences that the quantity and type (saturated fat) of fat in the diet also contribute to IR, but human research studies suggest that the total calorie or energy intake is more important than the fat content. Of course, it is easier to maintain a lower calorie intake and avoid obesity if the diet is low in fat (Budiyani *et al.*, 2017).

(7) Exercise: regular physical activity is extremely important in preventing IR and works in several ways. Firstly, it causes muscle to produce more glucose transport proteins which are stimulated by insulin to help the flow of glucose from the blood into muscle cells. Secondly, exercise increases blood flow through muscle and the biochemical machinery (enzymes) which convert glucose into energy. However, perhaps the most important effect of exercise is to reduce accumulation of intra-abdominal fat (Al-Attaby and Al-Lami, 2019).

1.4. Measurements of Insulin Resistance

The gold standard for investigating and quantifying IR is the "hyperinsulinemic euglycemic clamp". It is so-called because it measures the amount of glucose which is necessary to compensate for an increased insulin level without causing hypoglycemia. It is a type of glucose clamp technique. The test is rarely performed in clinical care, but it is used in medical research, for example, to assess the effects of different medications (Cicero *et al.,* 2015). Some studies found that insulin to be an effector although the accurate measurement of IR is challenging, the homeostasis model assessment (HOMA) score and area under curve (AUC) insulin during an oral glucose tolerance test (OGTT) appear to be comparable to those with the hyperinsulinemic-euglycemic clamp technique (Xin *et al.,* 2019).

1.5. Diabetic Complications

DM is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes as a chronic condition requires careful control. Without proper control and follow-up management, it can lead to various complications. These complications may be divided to short- and long-term complications.

1.5.1. Short-Term Complications:

It is acute metabolic complications manifested by increased lipolysis with fatty acid release and accumulation of fat in parenchymal organs further aggravates the metabolic disturbance such as Diabetic Keto Acidosis (DKA), Hyperosmolar Nonketotic States (HNS), Hypoglycemia and Infections (Kiprono *et al.,* 2015).

1.5.2. Long-Term Complications:

Many patients with T2DM are asymptomatic, and their disease is undiagnosed for many years. The kidney plays a pivotal role in myocardial failure. Therefore, interfering with the cardio-renal axis is an important therapeutic objective. The duration and intensity of high blood glucose level play an important role in glycosylation of proteins and lead to changes in the shape of the endothelial cells. Lining of the blood vessels, glycoprotein formation and basement membrane become thickening and weak (Devarapalli *et al.,* 2019).

The long-term complications of DM can be divided into micro-vascular (damage to small blood vessels) and macro-vascular (damage the arteries) diseases:

1.5.2.1. Microvascular Complications:

These are diabetic complications involving small vessels such as the capillaries. Prolonged and chronic high serum glucose plays the central role in the initiation of this condition. There are three major kinds of these Complications: Diabetic Nephropathy, Diabetic Neuropathy and Diabetic Retinopathy (Klemis *et al.,* 2017).

1.5.2.2. Macrovascular Complications:

These are diabetic complications peculiar to large vessels such as arteries and vein. Arteriosclerosis is the main pathological mechanism for developing complications in these vessels. There are three major kinds of these Complications: Cardiovascular Diseases (CVD), Cerebrovascular Diseases (Stroke) and Peripheral Artery Diseases (PAD) (Devarapalli *et al.,* 2019).

1.6. Diabetic Nephropathy (DN)

1.6.1. Definition and Epidemiology

Diabetic Nephropathy (DN) is kidney disease associated with long-standing hyperglycemia, first described in 1936 by Kimmelstiel and Wilson, as inter-capillary glomerulonephritis. The main features of DN include the nephrotic syndrome with excessive filtration of protein into the urine (proteinuria), high blood pressure (hypertension), and progressive impairment of kidney function. In severe cases DN leads to kidney failure, end-stage renal disease (ESRD) with the need for chronic dialysis or kidney transplantation. Clinically, DN is characterized as the presence of proteinuria > 0.5 g/24h, referred to as overt nephropathy or clinical proteinuria. Several studies revealed that small amounts of albumin in the urine, usually undetectable by conventional methods, were predictive of the later development of proteinuria in both T1DM and T2DM patients This stage of renal involvement was termed microalbuminuria or incipient nephropathy (Kopel *et al.*, 2019; Zanetti *et al.*, 2020).

Proteinuria occurs in 15–40% of T1DM patients, with a peak of incidence at around 15 years of diabetes duration. The cumulative incidence of proteinuria in T2DM patients is more variable, ranging from 5 to 20%. There is, however, evident racial/ethnic variability in the prevalence of nephropathy. It is much higher among African Americans, Asians, Hispanics, and Native Americans (especially Mexican-Americans) than Caucasians. In Pima Indians diabetic nephropathy has been observed in about 50% of all diabetic patients (Afkarian *et al.*, 2013).

Increased prevalence of T2DM and prolonged life expectancy of these patients in the last decade has led the number of diabetic patients that had progressed to the end staged renal disease (ESRD) to renal replacement therapy. In the United States, DN accounts for about 40% of new cases of

ESRD. About 20–30% of patients with T1DM or T2DM develop symptoms of nephropathy, but in T2DM, a considerably smaller fraction of them progress to ESRD. However, because of the much greater prevalence of type 2 diabetes, such patients represent over half of all the patients currently starting the dialysis program (Mise *et al.*, 2017).

1.6.2. Pathophysiology of DN (Renal Structural Damage)

Diabetes causes typical histopathological changes in kidney structure, presented by increase in mesangial matrix and GBM thickening, followed by tubular and interstitial changes. Macroscopically, an early feature of nephropathy in both experimental and human diabetes, is enlargement of whole kidney due to combination of tubular hypertrophy and interstitial expansion probably because of hyperglycemia and fluid reabsorbing. Furthermore, renal scarring and papillary necrosis may occur, mainly in women, due to urinary tract infections. Atherosclerosis of renal arterioles and renal artery sclerosis may follow. Diffuse mesangial expansion is a distinctive mark of DN. Kimmelstiel-Wilson nodules, which are areas of extreme mesangial accumulation observed in 40– 50% of patients developing proteinuria may be also present (Afkarian *et al.*, 2013).

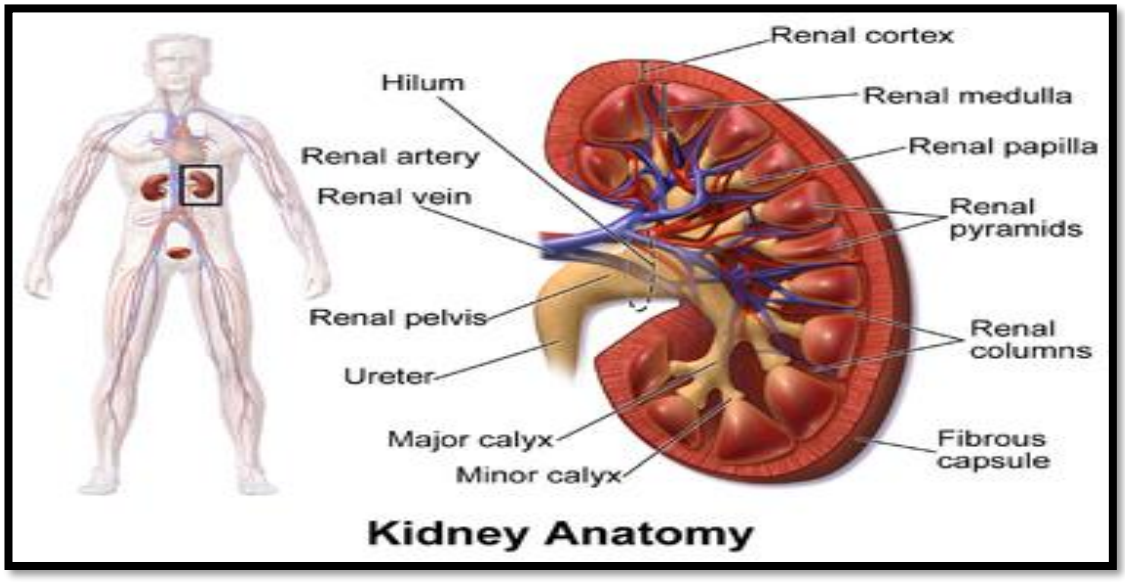


Figure (1.2): Location and structure of the kidney (Irshad *et al.*, 2018).

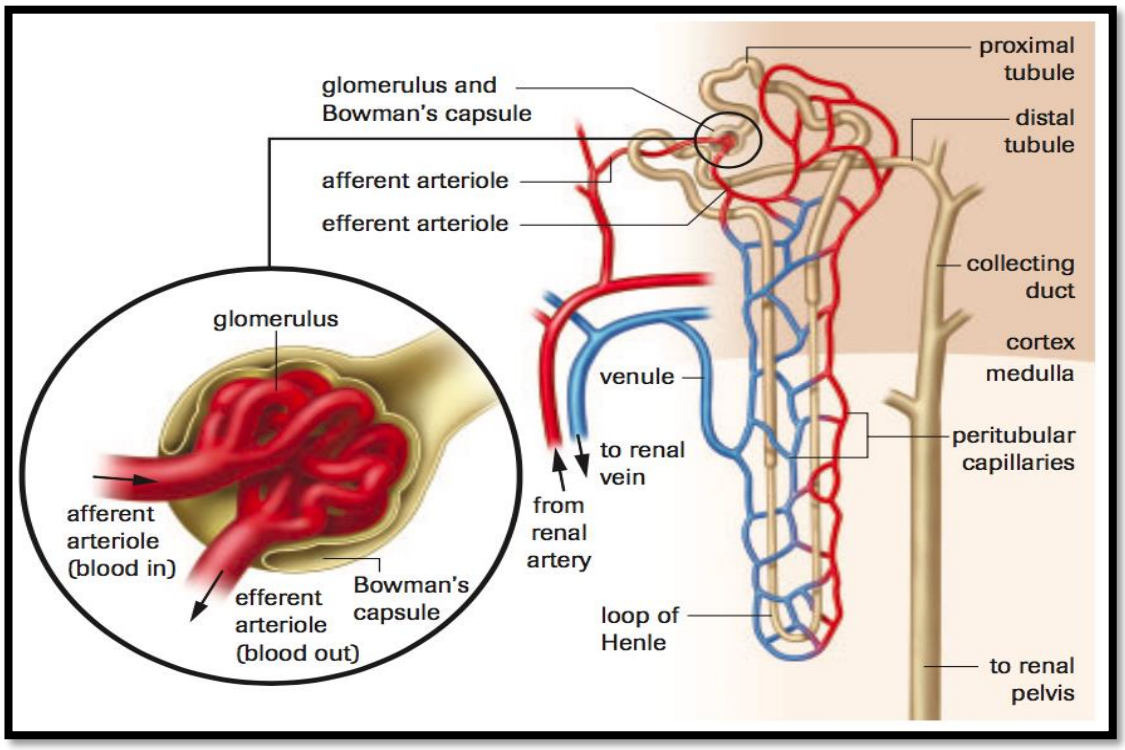


Figure (1.3): Structure of the nephron (Grujicic *et al.*, 2019).

Furthermore, mesangial matrix expansion correlates with both of proteinuria and deterioration of renal function. Accumulation of matrix in the mesangial area reduces the capillary surface area available for filtration, thus it contributes to the progressive loss of renal function. The result of advanced glomerulopathy is hyalinized glomerulus with no obvious capillary loops. Increase in matrix is secondary to a combination of its excess production and decreased degradation. Also, composition changes from normal to abnormal with elevated collagen IV and reduced proteoglycans (heparan sulfate). Another conspicuous feature of diabetic glomerulopathy, however not the specific one because it occurs irrespective of nephropathy status, is GBM thickening due to alterations in its architecture and composition. It would seem that thickened GBM participates in more effective barrier to the filtration of proteins, but it is in fact more porous to proteins (Sahay *et al.*, 2014).

In addition, the biochemical composition changes in several aspects. The increase in hydroxylation of amino acids (lysine, proline, glycine),

multiplication of disaccharide bounds and reduction in laminin and heparan sulphate content has been observed. The loss of charge selectivity in the GBM rises due to reduction in negatively charged proteoglycans allowing passage of positively charged proteins (albumin). This action partially explains the proteinuria (Irshad *et al.*, 2018).

On the other hand, the final barrier separating plasma proteins from the vasculature is the slit diaphragm of podocytes (glomerular visceral epithelial cell). The changes in podocytes are variable. There is certain reduction in number of podocytes and widening of pedicels which appears with onset of MAU, however, it does not fully correlate with degree of proteinuria. The remaining podocytes must compensate the lost by widening of pedicels. Taken together, all mentioned alterations of the GF barrier, composed of the glomerular endothelium, GBM, and podocytes result in the reduction of GFR and proteinuria. The harmful effect of proteinuria is observed also downstream, in tubules, presented by nephron atrophy and interstitial fibrosis (Norris *et al.*, 2018).

Moreover, tubular interstitial alterations reflect the degree of renal damage, although the structural and functional changes do not correlate. Persistent hyperglycemia impairs the tubular transport leading to haemodynamic unbalance and worsening of renal functions. Thickening of tubular basement membrane (TBM) is present, the most striking in proximal tubules. Interstitial fibrosis may be observed in later stages of DN, and it correlates with GFR. It may be accompanied by chronic inflammatory infiltration of T lymphocytes and macrophages. Furthermore, hyaline arteriosclerosis is early manifestation of DN, corresponding to glomerular impairment. Hypertension and hyperlipidemia worsen this condition by narrowing the lumen of arterioles resulting in the accelerated renal vascular hypertension (Klemis *et al.*, 2017).

Finally, in addition to typical features of nephropathy that induced by diabetic environment, nondiabetic renal lesions can be found in diabetic patients. They occur more often in T2DM patients. Possible reason for this is the presence of atherosclerosis, or the urinary tract infections typically present in these patients. The age may also play a role in the development of non-diabetic lesions. The most typical non-diabetic renal diseases found in diabetic patients are: membranous glomerulopathy, focal segmental glomerulosclerosis, or IgA nephritis (Grujicic *et al.,* 2019).

1.6.3. Stages and Clinical Course of DN

Approximately 25% to 40% of T1DM patients ultimately develop DN, which progresses through 5 clinically characterized stages. In T2DM, the clinical stages are less easily defined:

1.6.3.1. Stage 1 – Hyperfiltration and Hypertrophy:

Stage I is found in the patients with newly diagnosed diabetes and it is characterized by 20– 40% increase in glomerular filtration (GF) and increase in capillary permeability leading to microalbuminuria (MAU) and development of renal hypertrophy. Therapy with insulin leads to decline in MAU and GF (Bermejo *et al.,* 2017).

1.6.3.2. Stage 2 – Latent Diabetic Nephropathy:

It is a clinically silent phase with persistent hyperfiltration and hypertrophy. The GF remains elevated or returns to normal, but glomerular damage starts to manifest. The low or above-normal levels of albumin are present in the urine (30 mg/day, or 20μg/min). This is the earliest clinical evidence of nephropathy, referred to as MAU. Some authors consider this stage for stage III, and patients with MAU are defined as having incipient nephropathy. After 2 – 4 years the typical histopathological changes start to develop. Improved glycemic control decreases GF in this phase also (Gluhovschi *et al.,* 2016).

1.6.3.3. Stage 3 – Incipient Diabetic Nephropathy:

GBM thickening occurs in Albuminuria 30-300 mg/day (20-200 μg/min) and practically unaffected GF are run of the mill indications of this stage. About 20% of patients with MAU develop incipient nephropathy within 6-12 years on standard diabetic care, although up to 50% of diabetic patients may not develop MAU at all. Hypertension typically develops during stage 3. Except for being a predictor of manifestation and progression of nephropathy (mainly in T1DM), albuminuria is a marker of greatly increased cardiovascular morbidity and mortality, especially in patients with T2DM. Thus, screening for MAU on yearly basis in all diabetic patients is important for detection of possible vascular disease and for aggressive intervention in order to reduce all cardiovascular risk factors such as antihypertensive therapy, cessation of smoking, lowering of cholesterol levels, exercise, etc. (Murthy *et al.*, 2019).

1.6.3.4. Stage 4 – Overt Nephropathy:

Without specific interventions, 80% of subjects with T1DM who develop sustained MAU progress to the stage of overt nephropathy or clinical albuminuria. Over a period of 10–15 years the amounts of albumin in the urine exceed 300mg/day or 200μg/min and hypertension develops along the way in almost all patients. Glomerular damage continues, and the filtering ability of kidneys begins to decline steadily, with urinary albumin excretion (UAE) increased at a rate of 10–20% per year. The glomerular filtration rate (GFR) decreases about 10% annually. Glycemic compensation and tight blood pressure control slow down the progression, otherwise this stage leads to renal insufficiency, acceleration of hypertension and development of nephrotic syndrome with simultaneous progression of other diabetic complications, such are diabetic retinopathy, neuropathy or diabetic foot. A higher proportion of T2DM individuals are

found to have MAU and overt nephropathy shortly after the diagnosis because diabetes is actually present for many years before it is diagnosed. Without specific interventions, 20–40% of T2DM patients with MAU progress to overt nephropathy, but 20 years after onset of overt nephropathy, only 20% will progress to ESRD (Bermejo *et al.*, 2017).

1.6.3.5. Stage 5 – End Stage Renal Disease (ESRD):

Once overt nephropathy occurs, without specific interventions, GFR gradually falls over a period of several years at a rate that is highly variable from individual to individual (2–20 mL/min per 1 year) and renal replacement therapy is needed (i.e., hemodialysis or peritoneal dialysis or kidney transplantation). ESRD develops in 50% of T1DM individuals with overt nephropathy within 10 years and in 75% by 20 years. Survival of dialysis patients with diabetic ESRD comparing to non-diabetic patients is much lower due to cardiovascular risk and insufficient diabetes control (Mise *et al.*, 2017).

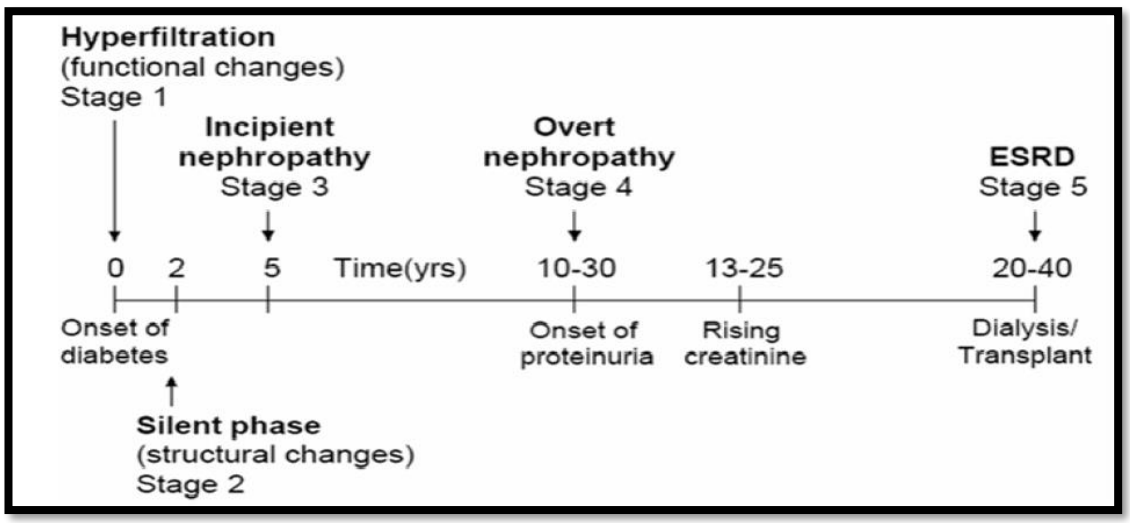


Figure (1.4): Different Stages of DN (Bermejo *et al.*, 2017).

1.6.4. Differential Diagnosis of DN

The physical examination, laboratory evaluation, personal history and imaging of the kidneys are basic tools of differential diagnosis in DN. It is aimed at proving the persistent albuminuria higher than 30 mg/day, exclusion of other renal disease and proving the presence of diabetes. Three methods can be used to confirm the presence of disease and its following up such as chemical and microscopic examination of urine and urine sediment, assessment of proteinuria and screening for determinants of renal functions (Afkarian *et al.*, 2013).

The first mentioned approach uses diagnostic strips to examine urine features: pH, presence of red blood cells, leucocytes, proteins, glucose, bilirubin, nitrites, and ketone bodies. The test for the presence of proteins is positive only in stage 4 of nephropathy. Test for the glucose in the urine is only for orientation and do not serve to follow the diabetes compensation (Kopel *et al.*, 2019).

The second tool demonstrates how it is very important for diagnosis of nephropathy through MAU assessment. MAU is present if urinary albumin excretion is > 30 mg/24h, equivalent to 20 μg/min in timed specimen, or 30 mg/g creatinine in random sample. In T2DM patients, a test for the presence of MAU should be performed at time of diagnosis because of the difficulty in precise dating of the onset of T2DM. If the absence of previously demonstrated MAU is observed, the test should be performed annually. In T1DM, screening of individuals begins in 5 years after disease diagnosis because MAU rarely occurs within shorter period of T1DM duration. Screening for MAU is preferably performed by measurement of the albumin-to-creatinine ratio in random spot collection, because it is easy to carry out in office settings and is accurate (Mise *et al.*, 2017). Morning collections are best because of the known diurnal variation

in albumin excretion. MAU measurement can also be done by 24h urine collection with creatinine, allowing the simultaneous measurement of creatinine clearance, or timed collection (e.g., 4-h or overnight). For such screening the standard hospital laboratory assays for urinary protein are not sufficiently sensitive, therefore specific assays are needed. MAU screening is important for the early diagnosis of DN, and institution of angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) therapy and blood pressure control as a prevention of DN (Gluhovschi *et al.,* 2016).

The third tool, which helps to determine right diagnosis of DN, is the measurement of specific markers of renal functions such as urea, creatinine GFR, etc., which will be discussed in this study. GF is basic examination in patients with nephropathy, because in initial phases it is elevated in 25-50% of T1DM patients till 5 years from diabetes diagnosis and more than 45% of T2DM patients at the time of diagnosis. It is a consequence of hyperfiltration and MAU, nevertheless, it stabilizes with progression to stage of persistent MAU. In addition, the progression of renal insufficiency to renal failure in late stages of nephropathy can be determined by concentrations of urea which are increased in diabetic nephropathy (Vanholder *et al.*, 2018).

In the special situations such as proteinuria over 1 g/24 h, renal impairment in the absence of retinopathy, or unexplained hematuria, renal biopsy is recommended to perform. Although the criteria for renal biopsy are not well established in type 1 diabetes the reason may be the presence of proteinuria in patients with short diabetes duration, especially in the absence of diabetic retinopathy (Parchwani *et al.*, 2015).

In patients with T2DM, the criteria are less clear. Non-diabetic renal complications are common in proteinuric T2DM patients, but the proportion varies according to the criteria used to perform the biopsy and to

the ethnic origin. The diagnosis of DN is easily established in long-term T1DM patients (more than 10 years of diabetes duration), especially if retinopathy is present. Typical, DN is also likely to be present in proteinuric T2DM patients with retinopathy. However, diagnostic uncertainty exists in some patients with T2DM since the onset of diabetes is unknown and retinopathy is absent in a significant proportion (28%) of these patients (Mise *et al.*, 2017).

The presence of symptoms during urination suggests urinary tract disorders such as obstruction, infection, or stones. Skin rash or arthritis may indicate systemic lupus erythematosus or cryoglobulinemia. Presence of risk factors for parenterally transmitted disease may raise the suspicion of kidney disease associated with HIV, hepatitis C, or hepatitis B. History of proteinuria and/or hypertension during childhood or pregnancy may suggest other glomerulonephritis. Also, family history of kidney disease may indicate the presence of polycystic kidney disease or other genetic diseases. Imaging of the kidneys, usually by ultrasonography, should be performed in patients with the family history of polycystic kidney disease or when symptoms of urinary tract obstruction, infection, kidney stones are present (Murthy *et al.*, 2019).

1.7. Specific Biomarkers of DN

1.7.1. Blood Biomarkers

1.7.1.1. Homocysteine (Hcy)

Homocysteine (Hcy) is a homologue of the amino acid cysteine, differing by an additional methylene (-CH2-) group. Hcy exists at neutral pH values as a zwitterion (Shin and Baik, 2016). Hcy is not obtained from the diet, it is biosynthesized from methionine via a multi-step process. First, methionine receives an adenosine group from ATP, a reaction catalyzed by S-adenosylmethionine synthetase, to give S-adenosyl methionine (SAM). SAM then transfers the methyl group to an acceptor molecule, (i.e., norepinephrine as an acceptor during epinephrine synthesis, DNA methyltransferase as an intermediate acceptor in the process of DNA methylation). The adenosine is then hydrolyzed to yield L-homocysteine. L-Homocysteine has two primary fates: conversion via tetrahydrofolate (THF) back into L-methionine or conversion to L-cysteine (Aouda and Hamza, 2017).

Furthermore, Several Hcy species have been identified in human plasma including albumin-(protein)-bound, free circulating disulfides and sulfhydryl forms. However, total Hcy plasma concentrations are commonly reported in the literature as current analytical methodology involves the reduction of Hcy disulfide bonds, quantifying all forms as free total Hcy (Platt *et al.*, 2017).

The metabolism of Hcy can be divided into three distinct pathways:

(1) The remethylation of Hcy to methionine by the vitamin B12 dependent methionine synthase.

(2) The transsulfuration pathway, converting Hcy to cystathionine and then cysteine via vitamin B6 dependent cystathionine β-synthase (CBS) enzyme.

(3) In the liver and kidneys, Hcy can be remethylated back to methionine by betaine homocysteine methyltransferase (Rujaswini *et al.*, 2018).

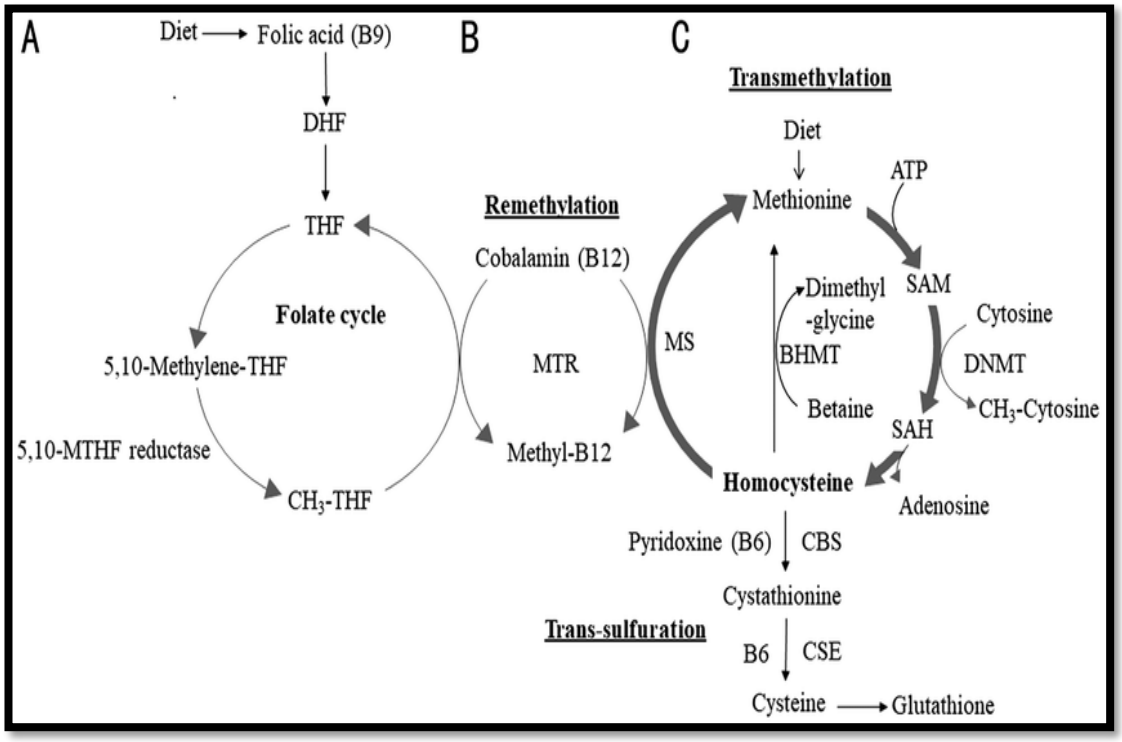


Figure (1.5): Schematic of Hcy metabolism (Kumawat *et al.*, 2016).

Moreover, extrarenal or intrarenal metabolism defects in Hcy can result in increased circulating Hcy concentrations in patients with diabetic kidney disease (DKD). Hcy remethylation, the main metabolized pathway in Hcy degradation, was diminished in patients with ESRD related to healthy controls (Ma *et al.*, 2018).

1.7.1.2. Fructosamine (FA)

Fructosamine (FA) is a compound that can be considered the result of a reaction between fructose and ammonia or an amine (with a molecule of water being released). FA is formed when carbonyl group of glucose reacts with an amino group of a protein, as the double bond to oxygen moves from the end carbon atom to the next carbon atom and water is released. FA also formed from blood proteins such as serum albumin, which known as Glycated Serum Protein (GSP) or Glycated Albumin, and

used to identify the plasma glucose concentration over time and so assess diabetic control over an intermediate period of time (Selvin *et al.*, 2018).

Glycation is a nonenzymatic mechanism resulting in the coupling of a sugar to a protein. In this process, glucose molecules are joined to protein molecules to form stable ketoamines, or fructosamines. In the case of fructosamines, the glycation involves a labile Schiff-base intermediate and Amadori rearrangement. The major component of the FA moiety is glycated albumin, with albumin constituting approximately 52-68% of total serum proteins. FA is the generally accepted name for 1-amino-l-deoxyfructosamine and is sometimes referred to as isoglucosamine. FA was first synthesized by Emil Fischer in 1886 (Soriano and Aquino, 2017).

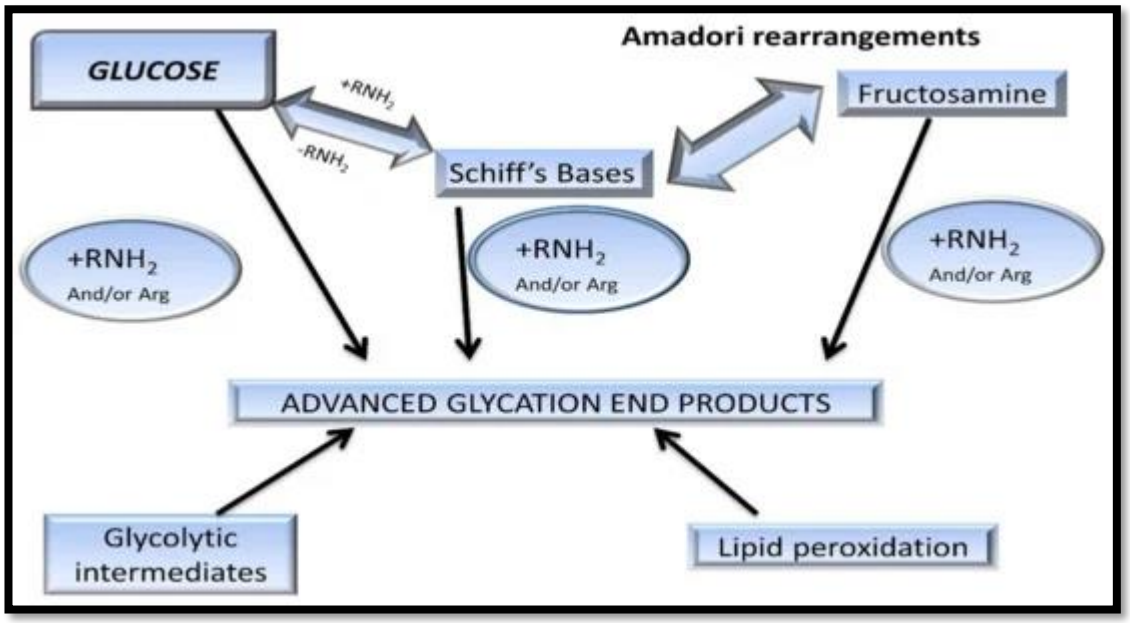


Figure (1.6): The reaction of glucose and protein to form FA and AGEs (Selvin *et al.*, 2015).

Glucose control is usually assessed in diabetes with the Glycosylated hemoglobin measurement that indicates average glucose levels over the preceding 6 weeks, as reflected by the permanent glycosylation of a small fraction of the hemoglobin molecules in their blood. However, this is not appropriate where there has been a recent change in diet or treatment within 6 weeks, or if there are abnormalities of red blood cell aging or mix

of haemoglobin subtypes. So, FA is used in these circumstances, as it also reflects an average of blood glucose levels, but over a shorter period of 2 to 3 weeks. On the other hand, FA measurement exploits the ability of serum proteins to undergo glycation (glycosylation) in conditions of increased ambient glycemia (Gupta and Khurana, 2019).

1.7.1.3. Cystatin C (Cys C)

Cystatin C (CysC) is a low molecular weight protein produced at a constant rate by all nucleated cells. Cys C or cystatin 3 (formerly gamma trace, post-gamma-globulin or neuroendocrine basic polypeptide), a protein encoded by the CST3 gene, is mainly used as a biomarker of kidney function. It also seems to play a role in brain disorder (Upadhyay and Sowdhamini, 2016). In addition to be an inhibitor of papain-like cysteine proteases, Cys C has recently been shown to inhibit another family of cysteine protease, called the peptidase family C13 with human legumain as a typical enzyme (Shelke and Tele, 2019). Determination of the structure of the human Cys C gene and its promoter has established that the gene is of the house-keeping type, which indicates a stable production rate of it by most nucleated cell types. The cystatin superfamily encompasses proteins that contain multiple cystatin-like sequences. Some of the members are active cysteine protease inhibitors, while others have lost or perhaps never acquired this inhibitory activity. There are three inhibitory families in the superfamily, including: Type 1 cystatins (stefins), Type 2 cystatins and the kininogens. The type 2 cystatin proteins are a class of cysteine proteinase inhibitors found in a variety of human fluids and secretions, where they appear to provide protective functions. Cys C is a non-glycosylated, basic protein and the crystal structure of it is characterized by a short alpha helix and a long alpha helix which lies across a large antiparallel, five-stranded

beta sheet. Like other type 2 cystatins, it has two disulfide bonds (Paramanick *et al.,* 2016).

Moreover, Cys C concentration is the highest of all known low-molecular-weight cystatins in most of the extracellular fluids in human. Cys C could play a pivotal role as a biomarker of kidney function, prediction of cardiovascular disease and neurological disorders. Cys C is mainly removed from bloodstream by the kidneys (Branda *et al.*, 2020). As Cys C is per se produced at a constant level, its concentration in the circulation remains nearly stable when kidney function is normal. Consequently, the rate at which Cys C is filtered at the glomerulus is the primary determinate of blood Cys C level. As a low molecular-weight globular protein with a high isoelectric point, Cys C is almost freely filtered through the glomerular membrane, and subsequently completely reabsorbed and catabolized by the proximal tubular cells (Boncheva *et al.*, 2016).

1.7.1.4. L-Carnitine (LC)

L-Carnitine (LC,), a quaternary ammonium compound, is a cofactor required for the transport of long-chain fatty acids into the mitochondria for energy production in peripheral tissues. LC inhibits free radical generation, helping to prevent impairment of fatty acid beta-oxidation in mitochondria and protecting tissues from damage by repairing oxidized membrane lipids. LC has also been reported to be a direct scavenger of O^{2-} and H_2O_2 (Giudetti *et al.,* 2016).

The synthesis of LC requires two essential amino acids (lysine and methionine), iron, and vitamin C, B6 and niacin in the form of nicotinamide dinucleotide (NAD). Carnitine is synthesized primarily in the liver and in the kidney and must be transported to other tissues. It is most concentrated in tissues that use fatty acids as their primary dietary fuel, such as skeletal and heart muscle (Shimizu *et al.,* 2019).

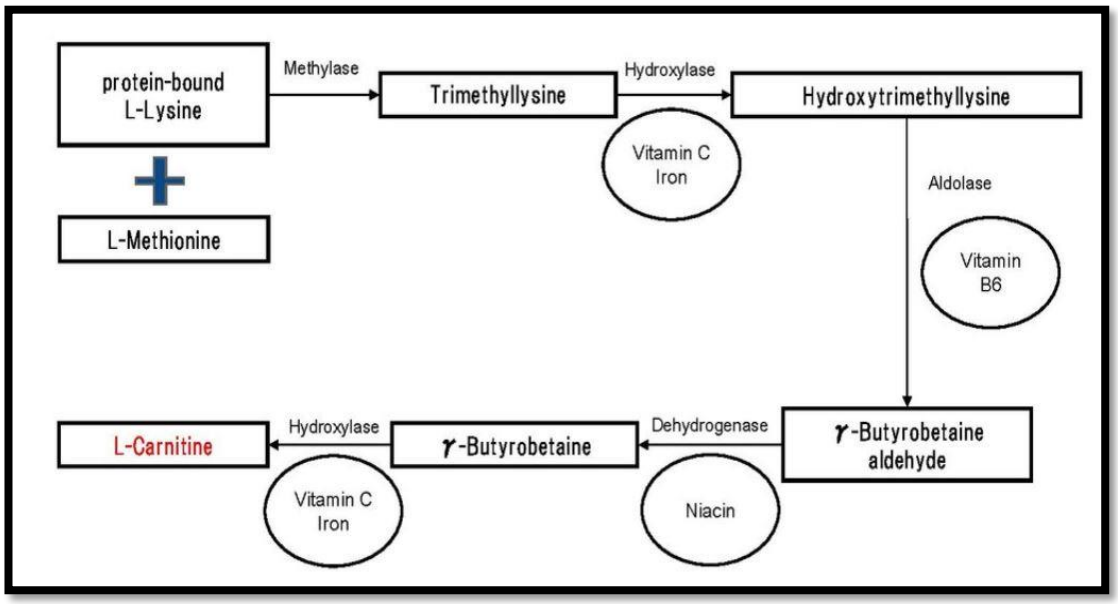


Figure (1.7): Biosynthesis of LC (Rehman *et al.*, 2017).

Furthermore, LC has been demonstrated to bear anti-inflammatory and antioxidant properties and improves insulin sensitivity, protein nutrition, dyslipidemia, and membrane stability. Due to its pivotal role in intermediary metabolism it is not surprising that plasma and tissue levels of LC are maintained within a relatively narrow homeostatic range which is controlled by carrier mediated gastrointestinal absorption from dietary sources, endogenous biosynthesis, extensive renal tubular reabsorption and compartmentalization through carrier-mediated transport between plasma and tissue (Bene *et al.*, 2018).

Moreover, Since the fundamental biological function of carnitine is its ester-forming capability with organic acids of both exogenous and endogenous origin, it can reduce the accumulated acyl CoA derivatives and/or their metabolites via transporting them out from the mitochondria. Therefore, carnitine could be a potential adjuvant in the treatment or prevention of IR and T2DM (Bonomini *et al.*, 2019).

1.7.1.5. Osteoprotegerin (OPG)

Osteoprotegerin (OPG), also known as Osteoclastogenesis Inhibitory Factor (OCIF) or tumor necrosis factor receptor superfamily member 11b

(TNFRSF11B), is a secreted glycoprotein that regulates bone resorption. OPG is synthesized as a monomer (60 kDa) and assembled as a homodimer within the cell, and then secreted mainly as a disulphide linked homodimer into the circulation. OPG was identified as a cytokine and member of the TNF receptor superfamily, and binds to two ligands, RANKL (receptor activator of nuclear factor κB ligand), a critical cytokine for osteoclast differentiation, and TRAIL (TNF-related apoptosis-inducing ligand), involved in immune surveillance. Thus, acting as a decoy receptor for RANKL and TRAIL, OPG inhibits the nuclear factor-κB's regulatory effects on inflammation, skeletal, and vascular systems and prevents TRAIL-induced apoptosis (Xia *et al.,* 2015).

Furthermore, OPG consists of 401 amino acids; however, the cleaving of a 21-amino acid signal peptide leads to the formation of a mature 380 amino acid form. It consists of 4 amino-terminal cysteine rich domains that are structurally like the extracellular portions of other associates in the TNF receptor superfamily. The carboxyterminal incorporates portions 5 and 6 that are death domain homologous regions (Lim and Han, 2017). The OPG molecule consists of three structural domains influencing the biological function. The N-terminal part is a cysteine-rich domain important for dimerization and osteoclastgenesis whereas the C-terminal contains a death domain and a domain for heparin binding (Dufresne *et al.* 2018).

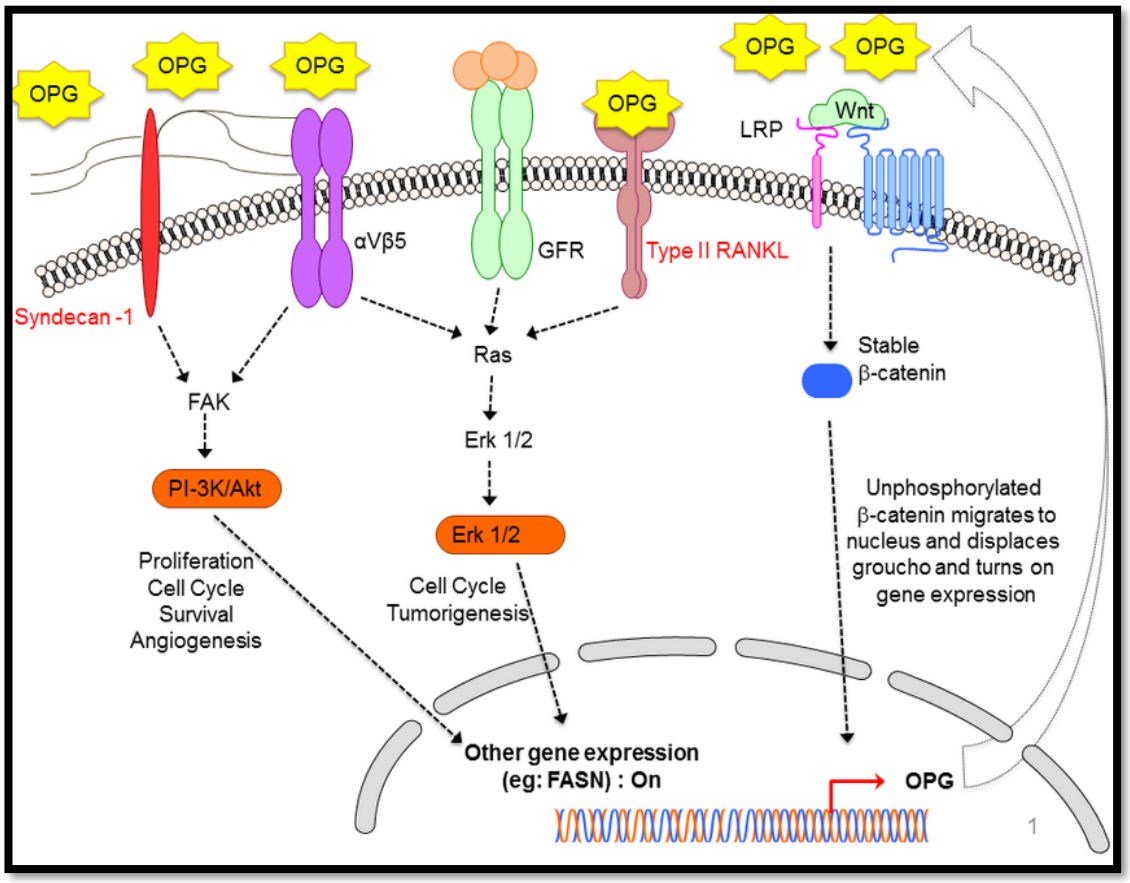


Figure (1.8): Schematic diagram depicting the diverse signalling pathways that are triggered or modulated by OPG (de Ciriza *et al.*, 2015).

On the other hand, OPG is highly expressed in heart, lung, kidney, liver and bone marrow among other tissues and produced by vascular endothelial and smooth muscle cells (SMC) and secreted into the circulation. OPG is found in the Weibel Palade bodies and in platelets where it is associated with von Willebrandt factor. Thus, TNF-α, interleukin-1 (IL-1), IL-4 and peroxisome proliferator activated receptor (PPAR) agonists upregulate the synthesis of OPG (Putri, 2019).

In addition to its presence in connective tissues, OPG also circulates in blood, although the concentrations here are considerably lower than in tissue. In some studies, the focus was on the relationship between markers of IR and OPG. IR is associated with increased coronary heart disease (CHD) and mortality, it is interesting that recent studies have suggested

plasma OPG as a predictor of cardiovascular disease (CVD). Many authors assume that this connection is due to an association between OPG and vascular calcifications (Wang *et al.,* 2019; Dufresne *et al.* 2018).

1.7.1.6. Sialic Acid (SA)

Sialic acid (SA), a generic term for a family of acetylated derivatives of neuraminic acid, is an essential component of glycoproteins and glycolipids. It acts as a co-factor of many cell receptors and is positively associated with most of the serum acute phase reactants. SA is a component of cell membranes and vascular permeability is regulated by SA moieties. The vascular endothelium carries a high concentration of SA, hence extensive microvascular damage accounts for its shedding into the circulation leading to increased vascular permeability and overall increased SA concentration. Thus, elevated levels indicate excessive damage of the vascular cells of retina of the eyes, kidneys, heart and brain. This leads to conditions like retinopathy, nephropathy and neuropathy (Zhu *et al.,* 2017).

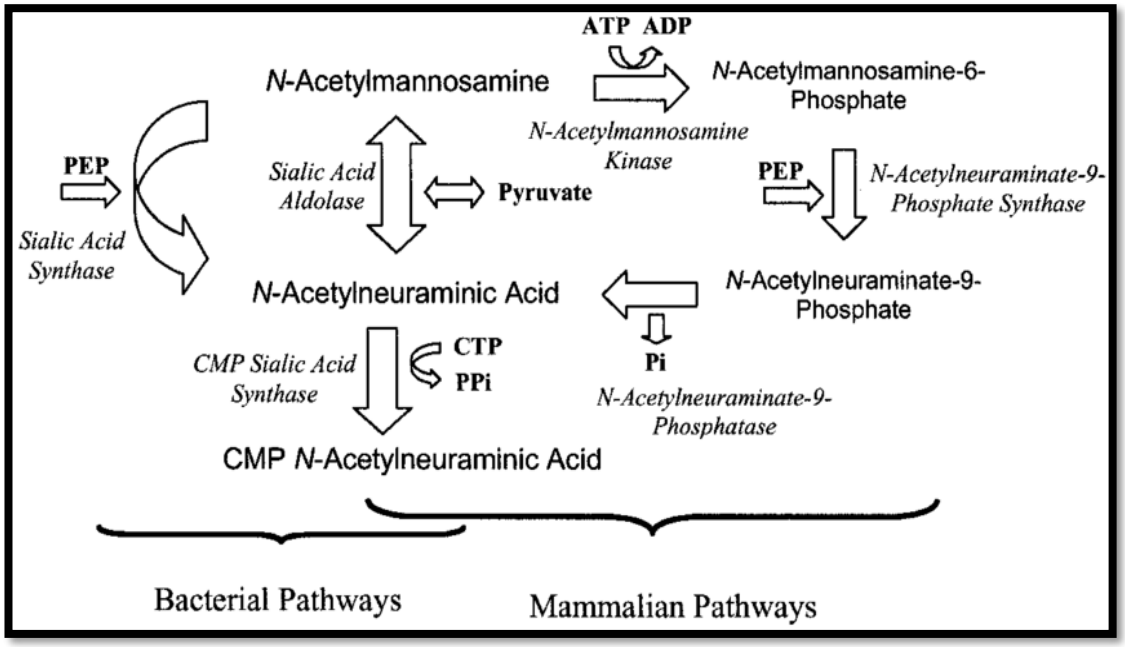


Figure (1.9): Bacterial and mammalian sialic acid metabolic pathways (Ghosh *et al.,* 2016).

Furthermore, sialic acids are found on the surfaces of all living cells in vertebrates, the negative charge on these sialic acids help to give the cells charge repulsion and preventing the cells from unwarranted interaction with itself that can lead to agglutination and destruction of these cells, a typical example is the erythrocytes. Because of the electronegative charge, SA is involved in capillary permeability, platelet aggregation, and activity of enzymes, as antigenic determinants and as essential components of receptors (El-Sayed *et al.*, 2018).

In addition, cells aggregation is prevented by sialic acids on cells surfaces. The presence of SA at the terminal end of glycolipids, glycoproteins and gangliosides prevent them from destruction and also the function depends on these. Alteration in SA level expression has been seen in several pathological diseases state. Due to their location and ubiquitous distribution and the pivotal role SA plays, any disturbances to its metabolism can lead to unhealthy medical condition. The level of SA is seen to be altered in several diseases such as CVD, cancer, diabetes and liver diseases. Also the level of SA can be used to predict disease risk and monitor prognosis during therapy (Jafri *et al.*, 2017).

Moreover, most of the SA in human serum is N-acetylneuraminic acid; other forms are found only in trace amounts. Total sialic acid in serum is the sum of protein-bound sialic acid, lipid-bound sialic acid, and free sialic acid, although free sialic acid represents only a very small fraction of total sialic acid. The molecular weights of sialic acids vary with their substitutions, but the average molecular weight is assumed to be 328.2 for sialic acid in human serum or plasma (Ghosh *et al.*, 2016).

1.7.2. Blood Trace Elements

Trace elements are defined as those elements occurring in human body but constituting < 0.01% of the body weight. Many trace elements are important for optimum human metabolic function. These micronutrients serve a variety of functions including catalytic, structural and regulatory activities. They interact with macromolecules such as enzymes, pro-hormones, and biological membrane receptors. Others play a crucial role in the immune system. Trace elements are uniquely required for maintenance of life and health. Lack or inadequate supply of such nutrients produces a functional impairment or can result in disease (Al-Fartosy *et al.,* 2017a).

The clinical significance and evaluation of trace elements such as, Zn and Se regarding different diseases including DM remain conflicting as well as controversial. Some of the important antioxidants contain trace elements. For example, Glutathione peroxidase has Se, Cu/Zn super-oxide dismutase has Cu and Zn in their structure, whereas, catalase has Iron as cofactor, and ciruloplasmin, the carrier of Cu. Decreasing of these trace elements causes the action of antioxidant systems to be lower and this leads to hyperactivity and inflammation in several diseases (Shazia *et al.,* 2012).

1.7.2.1. Selenium (Se)

1.7.2.1.1. Se in the Body

Selenium is an essential trace element for both humans and animals. It is involved in the complex system of defense against oxidative stress through selenium-dependent glutathione peroxidases and other selenoproteins. Selenoproteins including glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases and selenoprotein P (Sepp1) have important enzymatic functions. Through selenoproteins, selenium is involved in many biological functions, such as, protection against oxidative stress, immune function and thyroid function. The

concentration and activity of glutathione peroxidases and other selenoproteins increase with increasing intake of selenium until the dose response relationship reaches a plateau at plasma selenium levels 70 to 90 ng/mL. At greater levels, additional selenium intake further increases the plasma selenium level because of nonspecific incorporation of selenomethionine into albumin and other proteins rather than increased concentration or activity of glutathione peroxidases (Onah *et al.*, 2013).

Selenium is most widely recognized as a substance that speeds up the metabolism of fatty acids and works together with vitamin E (Tocopherol) as antioxidant. In addition to its antioxidant properties, selenium also appears to work as an anti-inflammatory agent in certain disorders. Selenium deficiency is relatively rare in healthy and well-nourished individuals. It is well recognized in several diseases like cardiovascular diseases, cataracts, cancer, rheumatoid arthritis, osteoarthritis, pancreatitis and asthma. Although selenium is an essential trace element, it is toxic if taken in excess. Exceeding the Tolerable Upper Intake Level (TUI) of (400µg/day) can lead to selenosis. Elemental selenium and most metallic selenides have relatively low toxicities because of their low bioavailability. By contrast, selenates and selenites are very toxic (Blessing *et al.*, 2019).

1.7.2.1.2. Se and DM

In addition to its pro-oxidant and antioxidant effects, selenium has been reported to have a strong antidiabetic and insulin-mimetic effects. In animal experiments, selenate decreased the activity of the enzyme tyrosine phosphatase, a negative regulator of insulin signal transmission, by 50% and effectively reduced IR. The anti-diabetic effect of selenium, however, is limited to selenate at very high doses and does not hold for any other form of selenium (Ahmed *et al.*, 2015). In a probability sample of the United States population, high serum selenium levels were positively associated with the prevalence of diabetes. The researchers suggested that,

selenium intake, including selenium supplementation, should not be recommended for primary or secondary diabetes prevention in populations with adequate selenium status such as United States population. In the same direction, some studies reported that selenium supplementation does not seem to prevent T2DM, and it may increase risk for the disease (Montali *et al.,* 2015).

1.7.2.2. Zinc (Zn)

1.7.2.2.1. Zn in the Body

Zinc is present in all body tissues. The total body zinc content has been estimated to be 30 mmol (2g). Skeletal muscle accounts for approximately 60% of the total body content and bone mass, with a zinc concentration of 1.5-3.0 μmol /g (100-200μg/g), for approximately 30%. The concentration of zinc in lean body mass is approximately 0.46μmol/g (30μg/g). Plasma zinc represents only about 0.1% of total body zinc content. This level appears to be under close homeostatic control. High concentrations of zinc are found in the choroid of the eye 4.2μmol/g (274μg/g) and in prostatic fluid 4.6-7.7mmol/L (300-500mg/L) (Alwan and Hamood, 2017).

Zinc is an essential component of a large number (>300) of metalloenzymes participating in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids as well as in the metabolism of other micronutrients. Zinc stabilizes the molecular structure of cellular components and membranes and in this way contributes to the maintenance of cell and organ integrity. Furthermore, zinc has an essential role in the polynucleotide transcription and thus in the process of genetic expression (Mohammed *et al.,* 2018).

Zinc plays a central role in the immune system, affecting several aspects of cellular and humoral immunity. Its involvement in such fundamental activities probably accounts for essentiality of zinc for all life

forms. The clinical features of sever zinc deficiency in humans are growth retardation, delay sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired appetite, increased susceptibility to infections mediated via defects in the immune system, and the appearance of behavioral changes (Farooq, 2019).

1.7.2.2.2. Zn and DM

Zinc is essential for the formation of both stored and active form of insulin. It may also be responsible for the conformational changes that allow insulin to bind to its receptors for activity. In addition, it has been suggested that zinc may be involved in the development or progression of both T1DM and T2DM diabetes. On the other hand, it was reported that zinc metabolism is also adversely affected by diabetes. High levels of zinc are eliminated in urine and absorption is impaired in both T1DM and T2DM and serum levels of zinc are (20-30) % lower as a result. Zinc protects cell from oxidative damage. Low levels of zinc may lead to damage the retinas (retinopathy), the nervous system (neuropathy), the kidneys (nephropathy) or cardiovascular system (Altoum *et al.*, 2019).

1.7.2.3. Magnesium (Mg)

1.7.2.3.1. Mg in the Body

The main biological role of Mg^{+2} in mammalian cells is involved with anion charge neutralization. Magnesium is particularly found in association with organic polyphosphates such as nucleotide triphosphate and nucleotide diphosphate (e.g. $ATP^{4-}.Mg^{2+}$ and $ADP^{3-}.Mg^{2+}$). Magnesium is also found associated with other highly anionic species including multisubstituted phosphates of sugar such as inositol triphosphates, nucleic acids (RNA and DNA) and some carboxylates (as a substrate for them such as isocitrate lyase and carboxylate group on proteins). Magnesium is normally bound between the Beta and Gama –

phosphates of nucleotide triphosphates such as ATP and between the α and β phosphate of nucleotide diphosphates (Kunutsor *et al.*, 2017).

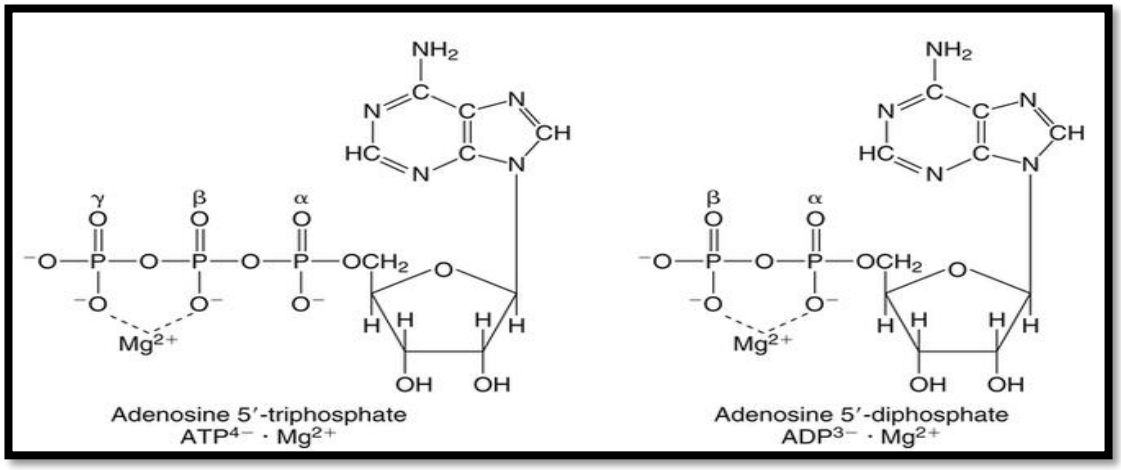


Figure (1.10): Physiological forms of Mg-ATP and Mg-ADP.

Magnesium has a fundamental role as a cofactor in more than 320 enzymatic reactions involving energy metabolism and nucleic acid synthesis. Magnesium involved numerous steps in central pathways of carbohydrate, lipid and protein metabolism and in mitochondrial ATP synthesis. For example, many steps in the glycolytic pathway require Mg^{2+}, either in the form of complex with ATP or ADP substrate or as a part of the metal enzyme itself. The steps catalyzed by hexokinase and phosphofructokinase require Mg-ATP as substrate whereas the steps catalyzed by phosphoglycerate kinase and pyruvate kinase require Mg–ADP. The transcription, translation, and replication of nucleic acid (RNA and DNA) require enzymes that catalyze the hydrolysis and formation of phosphodiester bonds. Almost all these enzymes require Mg^{2+} for optimal activity. Protein synthesis has been reported to be highly sensitive to magnesium depletion (Al-Jameil *et al.*, 2014).

Magnesium require for virtually every step of protein biosynthesis. Magnesium helps maintain normal muscle and nerve function keeps heart rhythm steady, supports a healthy immune system, and keeps bones strong. Magnesium also helps regulate blood sugar levels, promotes normal blood

pressure and is known to be involved in energy metabolism. There is an increased interest in the role of magnesium in preventing and managing disorders such as hypertension, cardiovascular disease, and diabetes. Magnesium plays an important role in carbohydrate metabolism it may influence the release and activity of insulin. Magnesium increases the body's ability to utilize Calcium, Phosphorus, Sodium, Potassium, Vitamin C, E and B-Complex (Sharma *et al.*, 2017).

Magnesium ion has a fundamental role in carbohydrate metabolism in general, and in the action of insulin in particular. Magnesium is a cofactor in the glucose–transporting mechanism of the cell membrane and the various enzymes in carbohydrate oxidation. Cellular magnesium seems to play an important role in glucose metabolism as it is a critical cofactor for the activities of various enzymes involving in glucose oxidation and may play role in the release of insulin. Magnesium involving at multiple levels in insulin secretion, binding, and activity. Magnesium deficiency has recently been proposed as a novel factor implicated in the pathogenesis of diabetic complication (Kumar *et al.*, 2018).

1.7.2.3.2. Mg and DM

Hypomagnesaemia a cause of diabetic complication. Linkage between magnesium deficiency and IR, carbohydrate intolerance, accelerated atherosclerosis, dyslipidemia, hypertension and adverse outcome in pregnancies complicating diabetes have been observed or postulated. DM has been suggested to be the most common metabolic disorder association with magnesium deficiency which had (25-39%) prevalence. Hypomagnesima in diabetes represents secondary magnesium depletion which requires more or less specific correction of the different perturbations of the control mechanism of magnesium deficiency that are involved with diabetes. The mechanism responsible for magnesium

deficiency in patients with DM is not completely known (Sakaguchi *et al.*, 2018).

Osmotic dieresis clearly accounts for a portion of the magnesium loss. It is believed that glycosuria which accompanies the diabetic status, impairs renal tubular reabsorption of magnesium for glomeular filtrate. A rise in the urinary magnesium excretion rates in diabetic patients with increasing insulin dosage has been reported despite maintenance of serum levels, suggesting the effect of insulin on renal magnesium handling. Dietary magnesium intake may also be a factor in deficiency, as the individuals do not consume the fully recommended daily allowance for magnesium. Glucose itself is a crucial part of cellular ion homeostasis, increasing intracellular calcium and decreasing intracellular magnesium (Al-Fartosy *et al.*, 2017b).

Recent evidence suggests that insulin can increase free magnesium entry into the cell. Furthermore, in the state of IR, insulin induced entry of magnesium is also impaired. Glycemic control in patient with T2DM, however, may not correct low magnesium concentration, suggesting that other factors may regulate magnesium levels in diabetic patients. Magnesium deficiency is a common characteristic of T2DM. In the United States about 40 % of outpatient diabetics have low levels of serum magnesium, also have been reported in several European countries, like Austria, Germany, Italy, France and Sweden (Khan *et al.*, 2013).

1.7.3. Urine Biomarkers

1.7.3.1. Albumin (Alb)

Albumin (Alb), a 65-kDa protein produced in the liver, is the most abundant plasma protein in the body. The main functions of Alb are to regulate the oncotic pressure, to act as an acid/base buffer, and to mediate the transportation of metabolites, hormones, vitamins, and drugs. In normal subjects, a small amount of Alb is filtered in the glomerulus, but almost all of it is reabsorbed by the tubules. Elevated urine Alb excretion (UAE) is considered a well-established marker of glomerular damage. In addition, it is known that tubular dysfunction by itself may cause albuminuria owing to decreased reabsorption of filtered Alb (Sekulic and Sekulic, 2015).

The UAE is considered normal when it is less than 30 mg/day or 20 μg/min or 2–19 μg/mL (normoalbuminuria). This threshold was determined because the UAE of 95% of "normal" patients falls below this value. However, it has been recognized that the risk of cardiovascular events and renal morbidity is elevated also in subjects in the "high normal" range. Based upon the ability of dipstick to measure urine Alb, the UAE has been classified as microalbuminuria, when the UAE is between 30 and 300 mg/day or 20 and 200 μg/min; macroalbuminuria, when the UAE is above 300 mg/day or 200 μg/min. The rate of progression from micro to macroalbuminuria in T2DM patients is 2- 3% annually (Kim *et al.,* 2014).

MAU is not only a risk factor for chronic kidney disease (CKD) and ESRD, but it is also a strong predictor of total and cardiovascular mortality and cardiovascular morbidity in diabetic patients. In patients with T2DM and nephropathy, albuminuria is the strongest risk marker for cardiovascular events (Debbarma *et al.,* 2015).

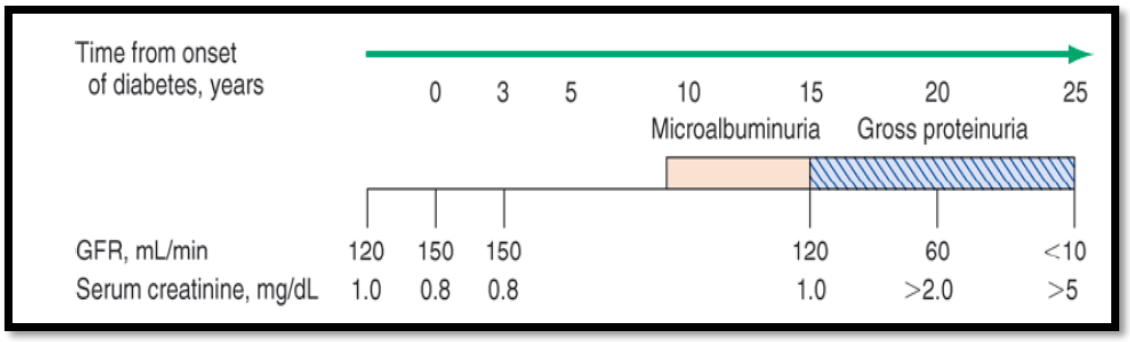


Figure (1.11): Relation between MAU, GFR, Creatinine and DM Duration (Kim *et al.*, 2014).

1.7.3.2. Neutrophil Gelatinase Associated Lipocalin (NGAL)

Neutrophil gelatinase–associated lipocalin (NGAL; also known as human neutrophil lipocalin, lipocalin-2, siderocalin, 24p3, or LCN2) is a small molecule of almost 25kD that belongs to the well-defined superfamily of proteins called lipocalins which is a family from small proteins that cells send out to bind things and carry them back and specialized in binding and transporting small hydrophobic molecules. The lipocalins share a molecular organization comprising 8β-strands arranged in a complex β-barrel structure delineating a calyx shape, which represents their binding site. The main features of NGAL were described because of some studies showed that the most important ligands of lipocalins are siderophores. Siderophores are small nonpeptidic iron containing molecules produced in bacteria, plants, and probably also mammals that, through iron transport and supply, are involved in cellular growth and survival. Interaction with iron-binding siderophores gives NGAL its characteristic bright red color and modulates most of its biological effects (AL-Mosawy and Hatroosh, 2019).

Furthermore, NGAL is released from neutrophils and many epithelial cell types including kidney tubular cells. NGAL covalently associated with human matrix metalloproteinase 9 (MMP-9) from human neutrophils. It is stored mainly in the specific granules of neutrophils, but also expresses at

very low-levels in several human tissues, including kidney, trachea, lungs, stomach, and colon. NGAL is found to possess diverse functions such as transporting, activating MMP-9, inducing apoptosis and regulating immune response. Some researchers have proved that NGAL can trigger nephrogenesis by stimulating the conversion of mesenchymal cells into kidney epithelia. NGAL also plays a reno-protective role through enhancing tubule cell proliferation in kidney injury, especially in ischemia-reperfusion injury (Ning *et al.*, 2018).

On the other hand, NGAL was found in secondary granules of human neutrophils. Also, NGAL is an iron-trafficking protein which is secreted from the ureteric bud in the embryonic kidney and regulate the primordial mesenchymal cell – the renal tubular epithelial progenitor. This is achieved by forming the NGAL:siderophore:Fe^{3+} complex, and this iron carrier is essential for cell differentiation and nephron formation. Moreover, the release of NGAL possesses kidney-protective activities as well. Its induction is one of the noticeable preservations of kidney function, reduced apoptosis, and an enhanced proliferative response (Tang *et al.*, 2019).

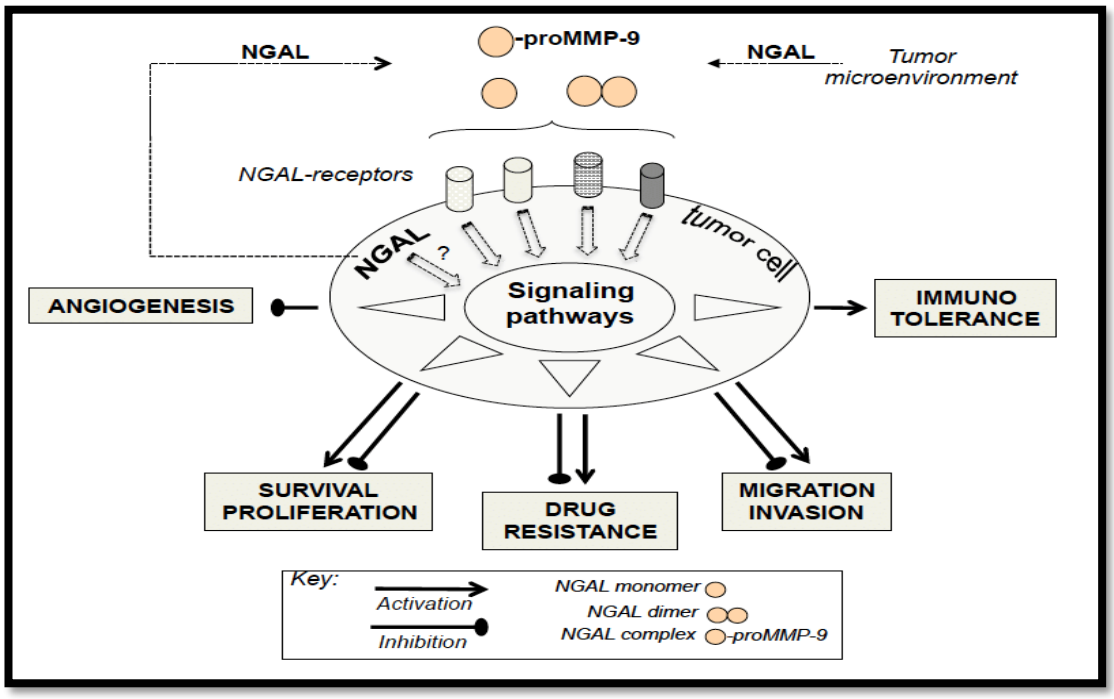


Figure (1.12): Schematic Diagram Illustrating the Putative Roles of NGAL in Modulating Major Cellular Processes (Gombert *et al.*, 2018).

1.7.3.3. 8-Hydroxy-2'-deoxyguanosine (8-OHdG)

8-Hydroxydeoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after reactive oxygen species (ROS) induced 8-hydroxylation of the guanine base in mitochondria and nuclear DNA. When damaged DNA is repaired, 8-OHdG is produced and excreted in urine without metabolism (Hojs *et al.*, 2015).

Furthermore, the most important oxygen-free radical causing damage to basic biomolecules (proteins, membrane lipids, and DNA) is the hydroxyl radical (HO•). The hydroxyl radical can be produced by various mechanisms, especially by the Fenton reaction of hydrogen peroxide (which diffuses into the nucleus) and metals and other endogenous and exogenous reactive oxygen species (ROS). The (HO•) attacks DNA strands when it is produced adjacent to cellular and mitochondrial DNA causing

the addition of DNA bases new radicals, which lead to the generation of a variety of oxidation products (Zhang *et al.*, 2018).

The interaction of (HO•) with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanosine). Initially, the reaction of the (HO•) addition leads to the generation of radical adducts, then by one electron abstraction, the 8-hydroxy-2'-deoxyguanosine (8-OHdG) is formed. The 8-OHdG undergoes keto-enol tautomerism, which favors the oxidized product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). In the scientific literature 8-OHdG and 8-oxodG are used for the same compound (Ye *et al.*, 2016).

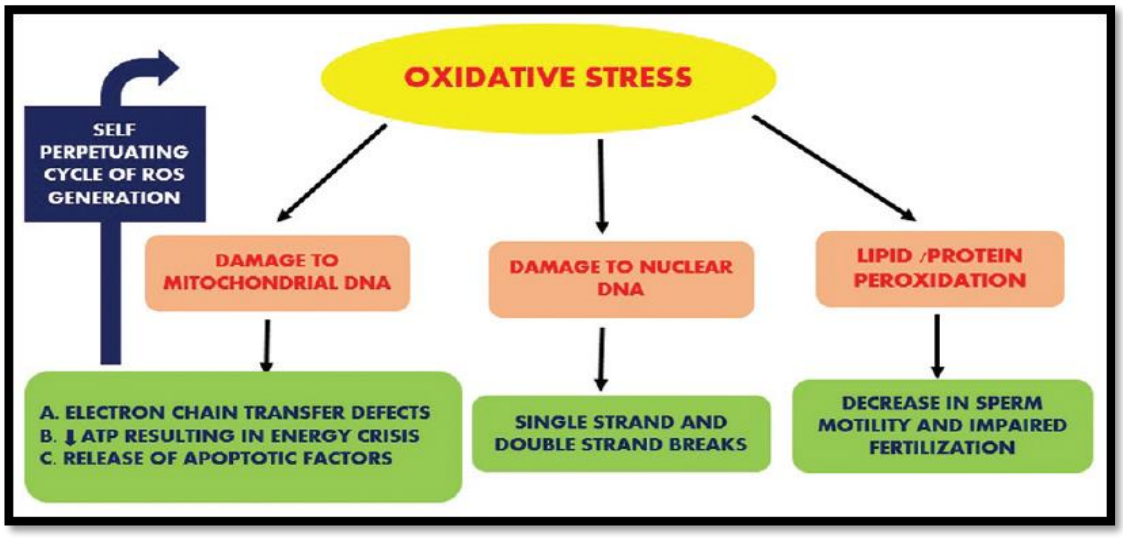


Figure (1.13): Effect of oxidative stress at cellular level (Korkmaz *et al.*, 2018).

1.7.3.4. Fibronectin (FN)

Fibronectin (FN), a high-molecular-weight glycoprotein (440kDa), is an intrinsic component of the glomerular extracellular matrix (ECM). It is produced in the liver, vascular endothelia and platelets. In diabetes, it may reduce erythrocyte deformity and filterability. It presents in a fibrillar form on the cell surface and in a soluble form in plasma (Uwaezuoke, 2017a).

Furthermore, FN is secreted as a disulfide-bonded dimer with 230–270 kDa subunits. It is a mosaic protein composed of three types of repeating modular consensus amino acid sequences. Alternative pre-mRNA splicing gives rise to combinations of these three regions and to a variable region (V). The two major forms of FN represent alternatively spliced products of a single gene designed for either the cellular or the plasma environment. Functional domains of fibronectin mediate interactions with cell surface receptors and several other macromolecules including fibrin, fibrinogen, collagen, heparin, immune complexes and FN itself. Alternatively, spliced isoforms of FN were detected in association with tissue remodeling within the synovial membrane. FN is rapidly degraded by many different types of proteinases (Scott *et al.*, 2015).

Moreover, Experimental evidence suggests an involvement of FN fragments in the regulation of cartilage metabolism and in the promotion of catabolic gene expression and chondrolysis. These fragments were shown to promote synoviocyte chemotaxis and glycosaminoglycan binding in the synovial fluid of patients with rheumatoid arthritis (RA). FN has been implicated in a variety of cellular functions including adhesion, cytoskeletal organization, oncogenic transformation, cell migration, platelet aggregation, wound healing, phagocytosis, hemostasis and embryonic differentiation. FN is released from endothelial cells and extracellular matrix after endothelial injury (Caterinoa *et al.*, 2018). FN enhances the initial adherence of neutrophils and monocytes to endothelium. FN also has opsonic properties although compared with antibody or complement; it plays a minor role. It also may have a direct phagocytosis-enhancing effect on monocytes and stimulated neutrophils. Fragments of FN also are chemotactic for monocytes. FN may also activate mononuclear phagocytes and Dendritic Cells (DCs) through Toll-like receptors TLR-4 (Lopes *et al.*, 2019).

1.8. The Aim of the Study

Although the scientific community has started resolving the secrets of the close linkage between obesity, IR, kidney dysfunction biomarkers and trace elements and their physiological effects in diabetic patients, a lot is still remaining to be discovered. In the province of Basrah (southern of Iraq), to date, no study has investigated on obesity, IR, renal dysfunction biomarkers and trace elements and their effects on T2DM patients with and without DN. Also, there is no scientific report on diabetes complications and its effects on T2DM patients. Hence, this study embarks on to explore the following aims:

(1) To evaluate the levels of glucose and insulin for a further calculation of insulin resistance in blood of healthy controls and T2DM patients with and without DN.

(2) To estimate the levels of BMI in healthy subjects and T2DM patients with and without DN.

(3) To study the effect of insulin resistance on the levels of some clinical biomarkers in blood serum (such as: urea, creatinine, GFR, CrCl, Hcy, FA, Cys C, LC, OPG and SA) and in urine (such as: ALB, NGAL, 8-OHdG and FN) with relation to obesity and study their effect on the development of DN in T2DM patients with and without DN compared with healthy controls.

(4) To find out the effects of obesity on insulin resistance and kidney function in healthy controls and T2DM patients with and without DN.

(5) To investigate the effect of insulin resistance on levels of some antioxidant elements (Se, Zn and Mg) with relation to obesity in T2DM patients with and without DN and healthy controls.

Chapter Two

Materials & Methods

Chapter Two

2.1. Chemicals and Instruments

The chemicals, kits and instruments used in this work listed in Tables (2.1), (2.2) and (2.3).

Table (2.1): Chemical compounds with their percentages, manufactures and origins

Chemical Compound	Percentage%	Manufacture / Origin
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	98	Hopkin & Williams / UK
Acetylene Gas (C_2H_2)	99.97	Jazan Industrial Gases / KSA
Selenium Dioxide (SeO_2)	98	Sigma-Aldrich / Germany
Zinc Metal (Zn)	99.54	Sigma-Aldrich / Germany
Sulfuric Acid (H_2SO_4)	98	CDH / India
Perchloric Acid ($HCLO_4$)	70	Sigma-Aldrich / Germany
Nitric Acid (HNO_3)	65	Merck / Germany
Hydrochloric Acid (HCl)	38	Avantor / USA

Table (2.2): Diagnostic kits with their catalogue No., manufactures and origins.

Biomarkers	Catalogue No.	Manufacture/Origin
Glucose	GL364	Randox / UK
Urea	1156015	Linear / Spain
Creatinine	CR 511/S	Randox / UK
Insulin	IS130D	Calbiotech / USA
Hcy	E3292Hu	BT-Laboratory / China
FA	E3232Hu	BT-Laboratory / China
Cys C	E1104Hu	BT-Laboratory / China
LC	E3426Hu	BT-Laboratory / China

OPG	E1558Hu	BT-Laboratory / China
SA	E1620Hu	BT-Laboratory / China
Alb	DEIA2299	Creative Diagnostics / USA
NGAL	E1719Hu	BT-Laboratory / China
8-OHdG	EA0048Hu	BT-Laboratory / China
FN	E2002Hu	BT-Laboratory / China

Table (2.3): Instruments with their models, manufactures and origins.

Instrument	Model - Manufacturer / Origin
Flame Atomic Absorption Spectrometer with (homemade) Hydride Generation System (HG-FAAS)	AA-630-12 Shimadzu / Japan
Flame Atomic Absorption Spectrometer	933 Plus GBC / Australia
UV-Vis Spectrophotometer (Double Beam)	V - 1200 / UV- EMC - LAB / Germany
ELISA	Human / Germany
Electronic Meter Balance	ACJ 220 - 4M KERN & Sohn GmbH / UK
Centrifuge	Biofuge B, Heraeus – Christ / Germany
Incubator	Memmert / Germany
Water Bath	Biofuge B - GFL / Germany
Oil Bath	F3 – HAKE / Germany
Stop Watch	Fisher Scientific Company / USA
Micropipette (10-100) µL	Gilson / France
Micropipette (100-1000) µL	Gilson / France
Vortex Mixer	Gallenkamp / Germany

2.2. Place of Work

This study was conducted in Department of Chemistry - College of Science and Department of Basic Medical Sciences - College of Nursing in Basrah University and the diabetes and endocrine glands center in Al-Mawany teaching hospital in province of Basrah–Iraq.

2.3. Study Design

In this section, the details of the study design including the subjects, target population, samples collection, the inclusion and exclusion criteria of cases and controls are extensively discussed.

2.3.1. Subjects

The present study is a cross-sectional clinical experiment. Samples were collected from the "diabetes and endocrine glands center" in Al-Mawany teaching hospital in Basrah province-Iraq, during the period from August 2019 until February 2020. In this study, 186 volunteers of patients and healthy controls (men and women) were participated in the present study aged between 36-65 years old. Ninety subjects (50 patients and 40 healthy controls) were excluded from the study due to enable to follow up study. Final 63 subjects (men and women) who suffering from T2DM were selected to share in this study and they divided into two groups: the first group was consisted of 31 patients who suffering from DN (15 men and 16 women) while the second group was consisted of 32 patients who free from DN (15 men and 17 women). These subjects were compared with 33 healthy controls (16 men and 17 women). Diagnosis of T2DM patients was based on the recommendation of American Diabetes Association (ADA, 2018). Duration of diabetes was considered as the time from which the patient was diagnosed with diabetes. Nephropathy was diagnosed on the basis of GFR level (<60 mL/min/1.73m^2) or persistent microalbuminuria

(>19 µg/mL) in the morning urine of patients (Jerums *et al.*, 2012; Zanetti *et al.*, 2020). All the volunteers were from Basrah province. All volunteers were given an informed written consent before participating in the study. Consent for the study was obtained from all enrolled patients. Demographical data were collected via a structural interview that was conducted during the visit. Standard self-administered questionnaire paper is used to define the age, duration of DM and DN, health habits (smoking, alcohol consumption and exercise), medical history and current medications as shown in Table (2.4).

2.3.1.1. Inclusion Criteria

The T2DM patients diagnosed by clinicians, with or without nephropathy, were selected in the respective group. The control group was health individuals; not suffering from type-2 diabetes nor having any family history of type-2 diabetes mellitus; not suffering from any acute or chronic cardiovascular diseases; not taking any drug believed to effect on plasma glucose level. All the volunteers had a stable clinical course for at least 3 months.

2.3.1.2. Exclusion Criteria

Patients, who were pregnant (women), had angina or heart failure, renal failure, hypertension, alcoholics, T1DM, urinary tract infection, uncontrolled thyroid disorders, severe liver dysfunction, human immunodeficiency viruses (HIV) infections, pancreatic diseases, hormonal abnormalities, genetic syndromes and severe concurrent illness were excluded from the study.

Table (2.4): Questionnaire paper form used in this study.

No.		Tel.			
The patient's name			Age		
Gender	Men		Height		
	Women		Weight		
Date of Birth					
Address					
Familial history of DM		Familial history of DN			
Familial history of CKD			Genetic History		
Age at diagnosis	T2DM				
	DN				
Duration of Disease	T2DM				
	DN				
Smoking Habits	Yes		Drinking alcohol	Yes	
	No			No	
Food Habits	Vegetarian		Non-Vegetarian		
Demographic Area	Urban		Rural		
Educational Status	Learned		Illiterate		
Employment Status	Employed		Not Employed		
Medicines	Regular				
	Irregular				
Any other remarks					

2.3.2. Samples

All samples were collected in the morning between 09:00 and 10:00 am after 12 hours fasting time and 30 minutes of rest in the supine position.

2.3.2.1. Blood Samples

Fresh venous blood (10 mL) were collected from patients and healthy volunteers by vein punch then divided into two parts, the first part was 1 mL and it was added into EDTA containing polypropylene tubes and shook gently to be utilized for the determination of the level of Selenium (Se). The second part (9 mL) was moved to plain tube (without anticoagulant) which admitted clotting for 20 minutes at room temperature. After the blood had clotted, it was moved into a centrifuge at 402 x g for 20 minutes to get the serum. The collected serum immediately utilized in the estimation of variables in this study, while the rest were stored in deep freezing at (-80ºC) until using.

2.3.2.1. Urine Samples

Fresh morning urine samples (20 mL) were collected from T2DM and healthy volunteers. The urine samples were collected in pre-sterile vials, after first voids of urine, and were brought to our laboratory in cool condition. The samples were centrifuged at 402 x g for 20 minutes to remove all suspended particles and cell derbies aliquoted. Then, the clear supernatants were collected carefully and utilized immediately in detection of variables in this study, while the rest were stored in deep freezing at (-80ºC) until using.

2.4. Methods

2.4.1. Body Mass Index (BMI)

Body mass index was calculated as weight in kilograms divided by height in meters squared using the following formula:

BMI (kg/m^2) = Weight (Kg) / Height (m^2)

Height and weight were measured by using a regularly calibrated stadiometer and balance-beam scale with participants wearing light clothing and no shoes (Al-Fartosy *et al.,* 2020).

2.4.2. Biochemical Estimation

To detect some of the levels of the biochemical parameters and trace elements in blood and urine of healthy controls and T2DM with or without DN, a number of biochemical measurements have been carried out, adopting standard procedures, as follows:

2.4.2.1. Estimation of Blood Biomarkers

2.4.2.1.1. Level of Glucose

Principle:

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone (Paraaminophenazone "PAP") to form a red – violent quinoneimine dye as indicator (Sirivole and Eturi, 2017):

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

$$2\,H_2O_2 + \text{Phenol} + \text{4-aminophenazone} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

Reagents:

1- R1a Vial: Buffer Solution (Phosphate Buffer 0.1mol/L with PH=7 & Phenol 11mmol/L).

2- R1b Vial: GOD-PAP Solution (4-aminophenazone 0.77mmol/L, Glucose oxidase 1.5kU/L & Peroxidase 1.5kU/L).

3- Standard Concentration Solution of Glucose (100.19 mg/dL).

Reagent Preparation:

Working Reagent Solution was prepared by mixing the content of R1a Vial with the content of R1b Vial together.

Procedure:

1- Three sets of tubes were prepared as below:

Table (2.5): Sets of tubes with their solutions and additives in glucose estimation

Tubes / Solutions	Blank	Standard	Serum
Working Reagent	1 mL	1 mL	1 mL
Standard	-	10 µL	-
Sample (Serum)	-	-	10 µL

2- All tubes were mixed well by vortex and incubated them for 10 minutes at 37ºC.

3- The absorbance (A) of the serum samples and standard were measured against the blank at wave length 500nm by using cuvette of 1 cm light path of Sepctrophotometer.

Calculations:

$$\text{Level of glucose (mg/dL)} = \frac{\text{A (Sample)}}{\text{A (Standard)}} \times \text{n}$$

Where: n = Concentration of glucose in standard solution = 100.19 mg/dL.

2.4.2.1.2. Assay of Insulin

Principle:

The insulin level is measured by the enzyme linked immunosorbent assay (ELISA). A solid phase ELISA is based on the sandwich principle. The microtiter wells are coated with monoclonal antibody directed towards a unique antigenic site on the insulin molecule. An aliquot of patient sample containing endogenous insulin is incubated in the coated well with enzyme conjugate, which is an anti-insulin antibody conjugated with biotin. After incubation, the unbound conjugate is washed off. During the second incubation step, streptavidin peroxidase enzyme complex binds to

the biotin- anti-insulin antibody. The amount of the bound Horseradish peroxidase (HRP) complex is proportional to the concentration of the insulin in the sample. Having added the substrate solution, the intensity of the developed colour is proportional to the concentration of insulin in the patient sample (Moisa, 2017).

Reagents:

1- Insulin Enzyme Conjugate.

2- Assay Diluent.

3- TMB Substrate.

4- Stop Solution.

5- 20X Wash Concentrate.

6- Insulin Standards Solutions with the Concentrations 0, 6.25μIU/mL, 12.5 μIU/mL, 25 μIU/mL, 50 μIU/mL and 100 μIU/mL.

Reagent Preparation:

1- Enzyme Conjugate: 1X working dilution was prepared at 1:20 with assay diluent.

2- Wash Buffer Concentrate: 1X wash buffer was prepared by adding the contents of the bottle to 475 mL of distilled water.

Procedure:

1- Reagents were allowed to stand at room temperature (20-25ºC) and mixed gently before use:

2- The coated strips were placed into the holder.

3- 25 μL of Insulin standards, control and patient's serum were pipetted into appropriate wells.

4- 100μL of working Insulin Enzyme Conjugate was added to all wells and thoroughly mixed completely for 10 seconds.

5- All wells were incubated for 60 minutes at room temperature (20-25ºC).

6- Liquid was removed from all wells. Wells were washed three times with 300 μL of 1X wash buffer and blotted on absorbent paper towels.

7- 100 µL of TMB substrate was added to all wells.

8- All wells were incubated for 15 minutes at room temperature.

9- 50µL of stop solution was added to all wells and shook the plate gently to mix the solution.

10- Absorbance on ELISA Reader was read at 450 nm within 15 minutes after adding the stopping solution for all wells.

Calculations:

The standard curve was constructed as follows:

1- Insulin standard value on each standard vial was checked.

2- The Standard Curve was constructed by plotting the absorbance for the insulin standards (vertical axis) versus the insulin standard concentrations in µIU/mL (horizontal axis) on a linear graph paper.

3- The absorbance for standards and each unknown sample from the curve was read.

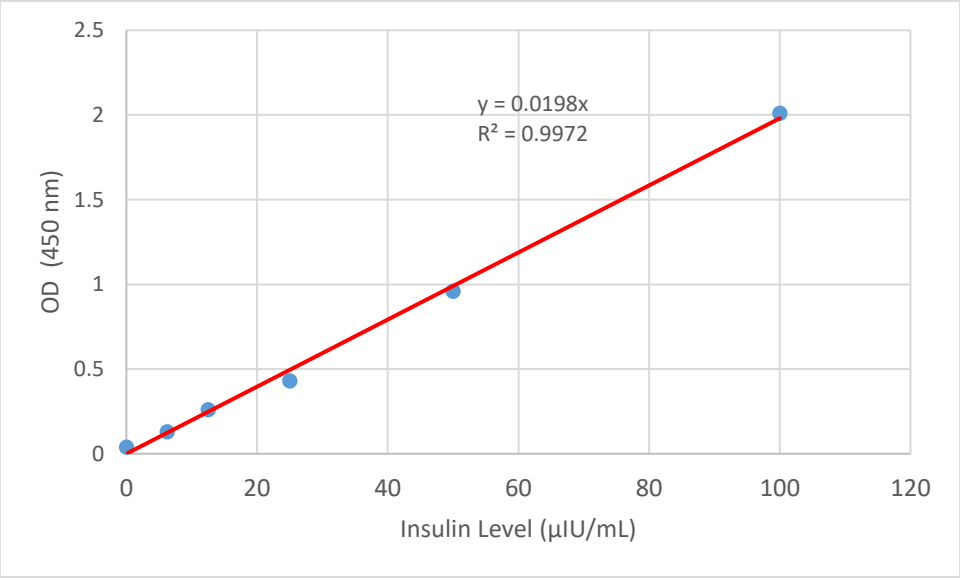


Figure (2.1): Standard Curve of Insulin Assay

2.4.2.1.3. Insulin Resistance (IR) Calculation

Insulin resistance (IR) was calculated by the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) equation (Al-Fartosy *et al.*, 2020):

HOMA-IR = Fasting insulin (μIU/mL) x Fasting glucose (mg/dL) / 405

2.4.2.1.4. Level of Urea

Principle:

Urea is hydrolyzed by urease into ammonia and carbon dioxide. The ammonia generated reacts with alkaline hypochlorite and sodium salycilate in presence of sodium nitroprusside as coupling agent to yield a green cromophore. The intensity of the color formed is proportional to the concentration of urea in the sample (Sirivole and Eturi, 2017).

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

$$NH_4^+ + \text{Salicylate} + NaClO \xrightarrow[OH^-]{\text{Nitroprusside}} \text{Indophenol} + NaCl$$

Reagents:

1- R1 Vial: Enzyme Reagent (Urease > 500 U/mL).

2- R2 Vial: Buffered Chromogen (Phosphate Buffer 20mmol/L with PH=6.9, EDTA 2mmol/L, Sodium salicylate 60mmol/L & Sodium nitroprusside 3.4mmol/L).

3- R3 Vial: Alkaline Hypochlorite (Sodium hypochlorite 10 mmol/L & Sodium hydroxide 150mmol/L).

4- Standard Concentration Solution of Urea (50 mg/dL).

Reagent Preparation:

Working Reagent Solution was prepared by mixing the content of R1 vial with the content of R2 vial together.

Procedure:

1- Three sets of tubes were prepared as below:

Table (2.6): Sets of tubes with their solutions and additives in urea estimation

Tubes / Solutions	Blank	Standard	Serum
Working Reagent	1 mL	1 mL	1 mL
Standard	-	10 µL	-
Sample (Serum)	-	-	10 µL
All tubes were mixed well by vortex, incubated them for 5 minutes at 37ºC. Then, 1mL of R3 vial solution was added to all tubes.			
R3 Vial	1 mL	1 mL	1 mL

2- All tubes were mixed well by vortex and incubated them for 5 minutes at 37ºC.

3- The absorbance (A) of the serum, urine and standard were measured against the blank at wave length 600nm by using cuvette of 1 cm light path of Sepctrophotometer.

Calculations:

$$\text{Level of urea (mg/dL)} = \frac{\text{A (Sample)}}{\text{A (Standard)}} \times \text{n}$$

Where: n = Concentration of urea in standard solution = 50 mg/dL.

2.4.2.1.5. Level of Creatinine

Principle:

Creatinine in alkaline solution reacts with picrate to form a colored complex (Sirivole and Eturi, 2017).

Reagents:

1- R1a Vial: Picric acid 35mmol/L.

2- R1b Vial: Sodium hydroxide 0.32mol/L.

3- R2 Vial: Trichloroacetic acid (TCA) 1.2mol/L.

4- Standard Concentration Solution of Creatinine (1.91 mg/dL).

Reagent Preparation:

Working Reagent Solution was prepared by mixing the content of R1a vial with the content of R1b vial together.

Procedure:

1- 1mL of serum was added to 1mL of TCA in a centrifuge tube and mixed well by using glass rod to evenly disperse the precipitate. The tube was placed in a centrifuge and rotated for 10min at 2500 RPM. Then, the resulted supernatant was collected in a small vial.

2- Three sets of tubes were prepared as below:

Table (2.7): Sets of tubes with their solutions and additives in creatinine estimation

Tubes / Solutions	Blank	Standard	Serum
Distilled Water	0.5 mL	-	-
Standard	-	0.5 mL	-
TCA	0.5 mL	0.5 mL	-
Supernatant	-	-	1 mL
Working Reagent	1 mL	1 mL	1 mL

3- All tubes were mixed well by vortex and incubated for 20 minutes at 25ºC.

4- The absorbance (A) of the serum, urine and standard were measured against the blank at wave length 520nm by using cuvette of 1 cm light path of Spectrophotometer.

Calculations:

$$\text{Level of creatinine (mg/dL)} = \frac{\text{A (Sample)}}{\text{A (Standard)}} \times n$$

Where: n = Concentration of creatinine in standard solution=1.91 mg/dL.

2.4.2.1.6. GFR Calculation

GFR was calculated by the Modification of Diet in Renal Disease Study (MDRD) equation (Chen *et al.*, 2016):

$$GFR\ (mL/min/1.73\ m^2) = 186 \times Serum\ Cr^{-1.154} \times age^{-0.203} \times 1.212\ (if\ subject\ is\ black) \times 0.742\ (if\ subject\ is\ woman)$$

2.4.2.1.7. CrCl Calculation

CrCl was calculated by the Cockcroft-Gault Equation (Chen *et al.*, 2016):

$$CrCl\ (mL/min) = (140 - age) \times Weight\ (Kg) \times 0.85\ (if\ subject\ is\ woman) / (72 \times Serum\ Cr)$$

2.4.2.1.8. Assay of Homocysteine (Hcy)

Principle:

Hcy level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human Hcy in serum. The plate has been pre-coated with Human Hcy antibody. Hcy present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human Hcy Antibody is added and binds to Hcy in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated Hcy antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of Human Hcy. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Rujaswini *et al.*, 2018).

Reagents:

1- Standard Solution (64nmol/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

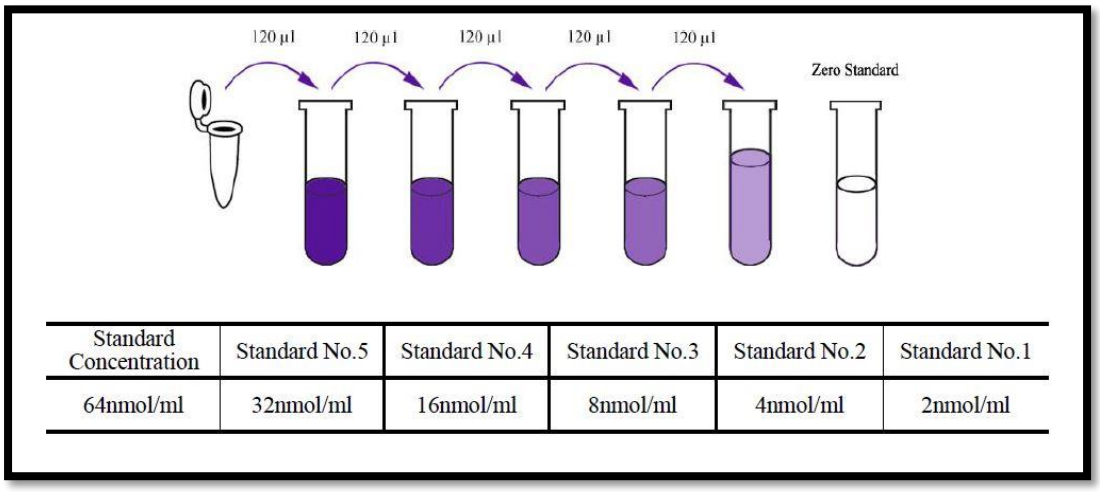
7- Wash Buffer Concentrate (25x).

8- Biotinylated Human Hcy Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (64nmol/mL) was reconstituted with 120µL of standard diluent to generate a 32nmol/mL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (32nmol/mL) 1:2 with standard diluent to produce 16nmol/mL, 8nmol/mL, 4nmol/mL and 2nmol/mL solutions. Standard diluent was served as the zero standard (0 nmol/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
64nmol/ml	32nmol/ml	16nmol/ml	8nmol/ml	4nmol/ml	2nmol/ml

Figure (2.2): Standards Solutions Preparation of Hcy.

2- Wash Buffer: 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standards wells.

4- 40µL of samples was added to samples wells and then 10µL of anti-Hcy antibody was added to these wells.

5- 50µL of streptavidin-HRP was added to samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubate for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL of substrate solution A was added to each well and then 50µL of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

9- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

10- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

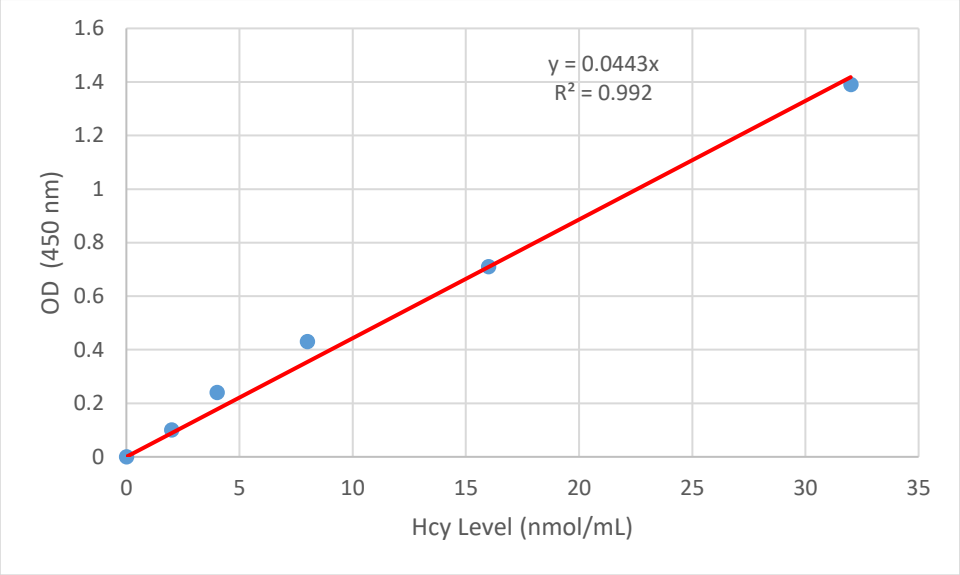


Figure (2.3): Standard Curve of Hcy Assay

2.4.2.1.9. Assay of Fructosamine (FA)

Principle:

FA level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human FA in serum. The plate has been pre-coated with Human FA antibody. FA present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human FA Antibody is added and binds to FA in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated FA antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of Human FA. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Danese *et al.*, 2015).

Reagents:

1- Standard Solution (16mmol/L).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

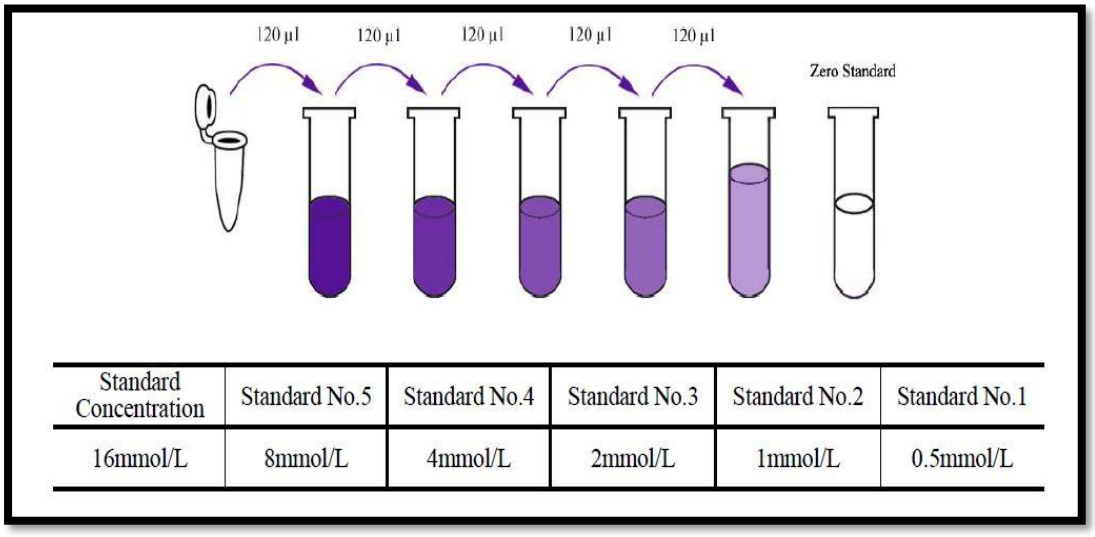
7- Wash Buffer Concentrate (25x).

8- Biotinylated Human FA Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (16mmol/L) was reconstituted with 120µL of standard diluent to generate a 8mmol/L standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (8mmol/L) 1:2 with standard diluent to produce 4mmol/L, 2mmol/L, 1mmol/L and 0.5mmol/L solutions. Standard diluent was served as the zero standard (0 mmol/L).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
16mmol/L	8mmol/L	4mmol/L	2mmol/L	1mmol/L	0.5mmol/L

Figure (2.4): Standards Solutions Preparation of FA.

2- Wash Buffer: 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standards wells.

4- 40µL of samples was added to samples wells and then 10µL of anti-FA antibody was added to these wells.

5- 50µL of streptavidin-HRP was added to samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubated for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1ml of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL substrate solution A was added to each well. Then, 50µL of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

9- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

10- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

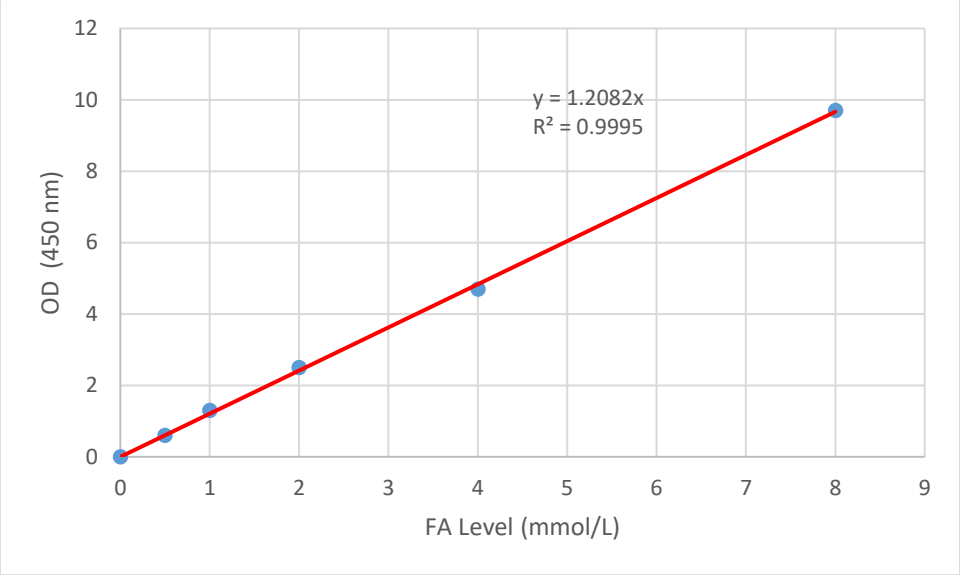


Figure (2.5): Standard Curve of FA Assay

2.4.2.1.10. Assay of Cystatin C (Cys C)

Principle:

Cys C level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human Cys C in serum. The plate has been pre-coated with human Cys-C antibody. Cys-C present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human Cys-C Antibody is added and binds to Cys-C in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated Cys-C antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of human Cys-C. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Miliku *et al.*, 2017).

Reagents:

1- Standard Solution (24µg/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

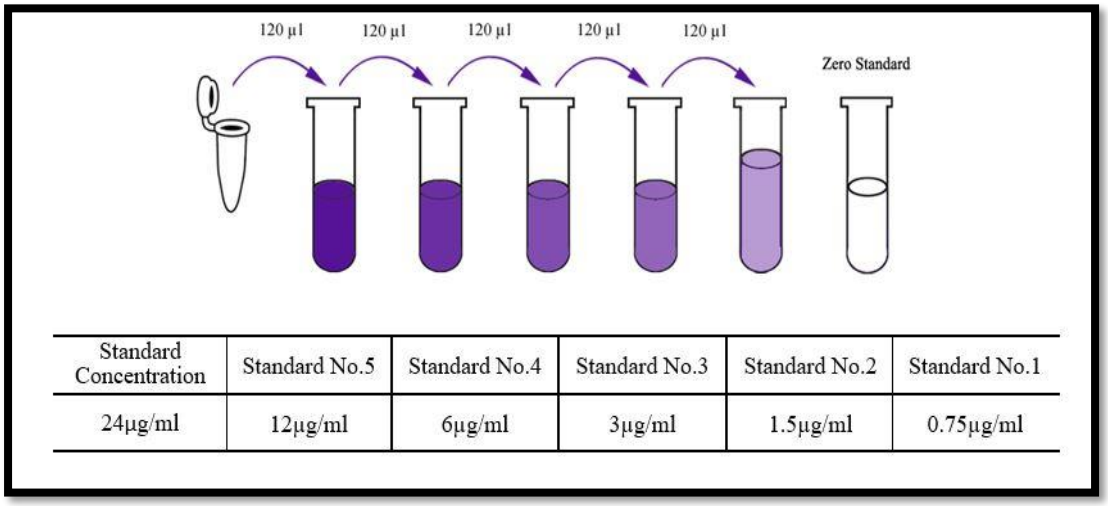
7- Wash Buffer Concentrate (25x).

8- Biotinylated human Cys-C Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (24µg/mL) was reconstituted with 120µL of standard diluent to generate a 12µg/mL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (12µg/mL) 1:2 with standard diluent to produce 6µg/mL, 3µg/mL, 1.5µg/mL and 0.75µg/mL solutions. Standard diluent was served as the zero standard (0 µg/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
24µg/ml	12µg/ml	6µg/ml	3µg/ml	1.5µg/ml	0.75µg/ml

Figure (2.6): Standards Solutions Preparation of Cys C.

2- Wash Buffer: 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standard wells.

4- 40µL of samples was added to sample wells and then 10µL of anti-Cys-C antibody was added to these wells.

5- 50µL streptavidin-HRP was added to all samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubated for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL of substrate solution A was added to each well. Then, 50µL of substrate solution B was added to each well.

9- The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

10- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

11- The optical density (OD value) was determined of each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

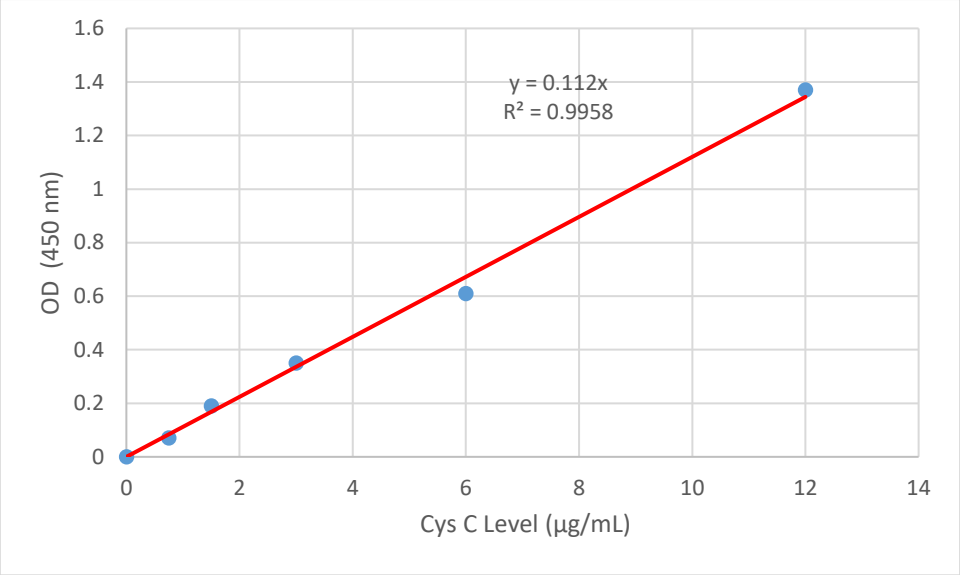


Figure (2.7): Standard Curve of Cys C Assay.

2.4.2.1.11. Assay of L-Carnitine (LC)

Principle:

LC level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human LC in serum. The plate has been pre-coated with Human L-carnitinel antibody. L-carnitinel present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human L-carnitinel Antibody is added and binds to L-carnitinel in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated L-carnitinel antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of Human L-carnitinel. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Rehman *et al.*, 2017).

Reagents:

1- Standard Solution (240nmol/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

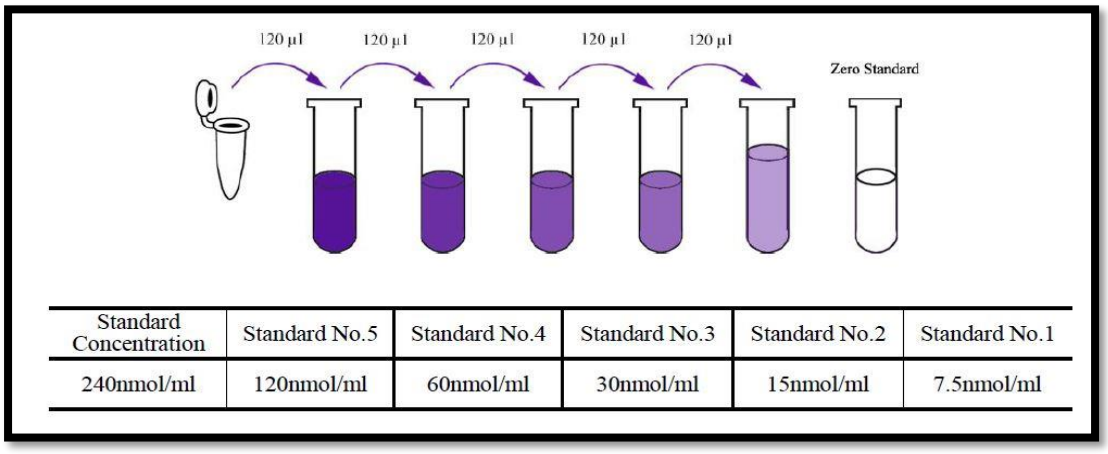
7- Wash Buffer Concentrate (25x).

8- Biotinylated Human L-carnitinel Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard**:** 120µL of the standard (240nmol/mL) was reconstituted with 120µL of standard diluent to generate a 120nmol/ml standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (120nmol/mL) 1:2 with standard diluent to produce 60nmol/mL, 30nmol/ml, 15nmol/mL and 7.5nmol/mL solutions. Standard diluent was served as the zero standard (0 nmol/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
240nmol/ml	120nmol/ml	60nmol/ml	30nmol/ml	15nmol/ml	7.5nmol/ml

Figure (2.8): Standards Solutions Preparation of LC.

2- Wash Buffer: 20mLof Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standards wells.

4- 40µL of samples was added to samples wells and then10µL of anti-L-carnitinel antibody was added to these wells.

5- 50µL of streptavidin-HRP was added to samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubated for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1 mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL of substrate solution A was added to each well and then 50µl of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

9- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

10- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

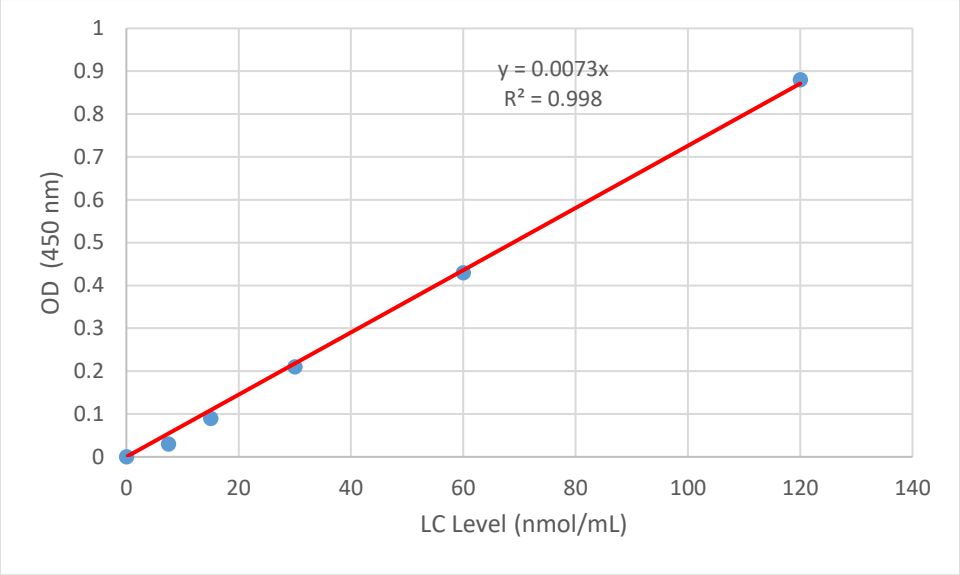


Figure (2.9): Standard Curve of LC Assay

2.4.2.1.12. Assay of Osteoprotegerin (OPG)

Principle:

OPG level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human OPG in serum. The plate has been pre-coated with Human OPG antibody. OPG present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human OPG Antibody is added and binds to OPG in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated OPG antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of Human OPG. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Bernardi *et al.*, 2016).

Reagents:

1- Standard Solution (16ng/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

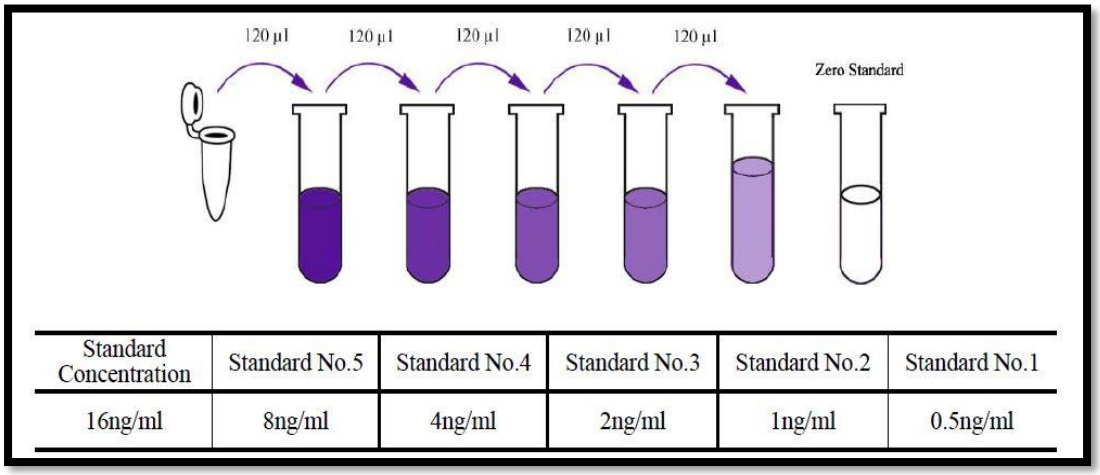
7- Wash Buffer Concentrate (25x).

8- Biotinylated Human OPG Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (16ng/mL) was reconstituted with 120µL of standard diluent to generate an 8ng/mL standard stock solution. The standard was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (8ng/mL) 1:2 with standard diluent to produce 4ng/mL, 2ng/mL, 1ng/mL and 0.5ng/mL solutions. Standard diluent was served as the zero standard (0 ng/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
16ng/ml	8ng/ml	4ng/ml	2ng/ml	1ng/ml	0.5ng/ml

Figure (2.10): Standards Solutions Preparation of OPG.

2- Wash Buffer: 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standards wells.

4- 40µL of samples was added to samples wells and then 10µL of anti-OPG antibody was added to these wells.

5- 50µL of streptavidin-HRP was added to samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubated for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1 mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL of substrate solution A was added to each well and then 50µL of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

9- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

10- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

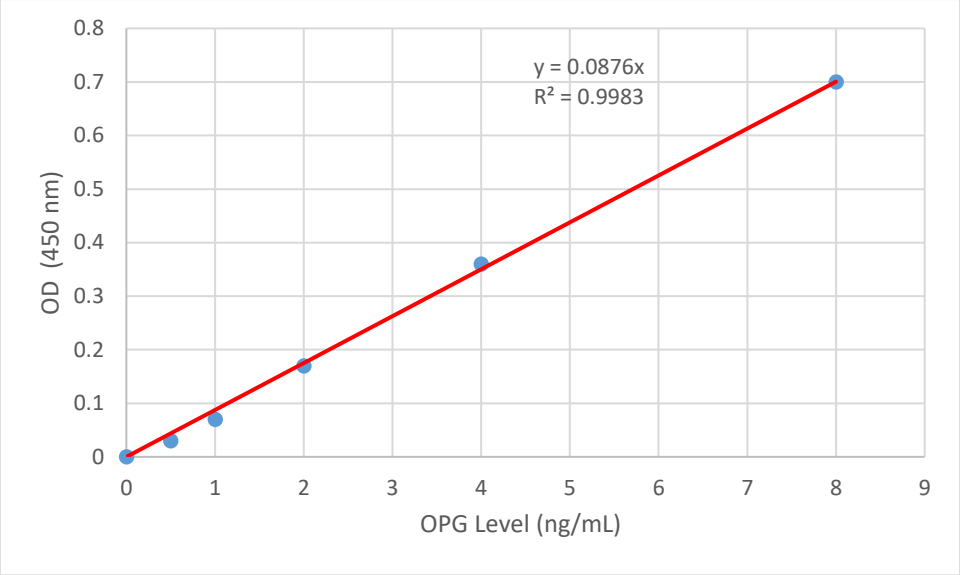


Figure (2.11): Standard Curve of OPG Assay

2.4.2.1.13. Assay of Sialic Acid (SA)

Principle:

SA level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human SA in serum. The plate has been pre-coated with Human SA antibody. SA present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human SA Antibody is added and binds to SA in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated SA antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of Human SA. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Ismail *et al.*, 2015).

Reagents:

1- Standard Solution (400mg/dL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

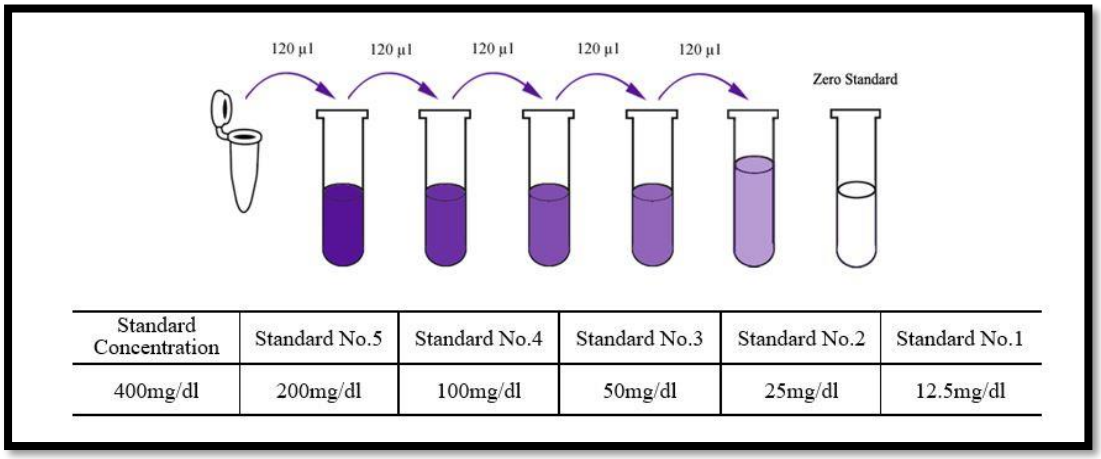
7- Wash Buffer Concentrate (25x).

8- Biotinylated Human SA Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (400mg/dL) was reconstituted with 120µL of standard diluent to generate a 200mg/dL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (200mg/dL) 1:2 with standard diluent to produce 100mg/dL, 50mg/dL, 25mg/dL and 12.5mg/dL solutions. Standard diluent was served as the zero standard (0 mg/dL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
400mg/dl	200mg/dl	100mg/dl	50mg/dl	25mg/dl	12.5mg/dl

Figure (2.12): Standards Solutions Preparation of SA.

2- Wash Buffer**:** 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standards wells.

4- 40µL of samples was added to samples wells and then 10µL of anti-SA antibody was added to these wells.

5- 50µL of streptavidin-HRP was added to samples and standards wells (Not blank control well) and mixed well.

6- The plate was covered with a sealer and incubated for 60 minutes at 37°C.

7- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1 mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

8- 50µL of substrate solution A was added to each well and then 50µL of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

9- 50µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

10- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

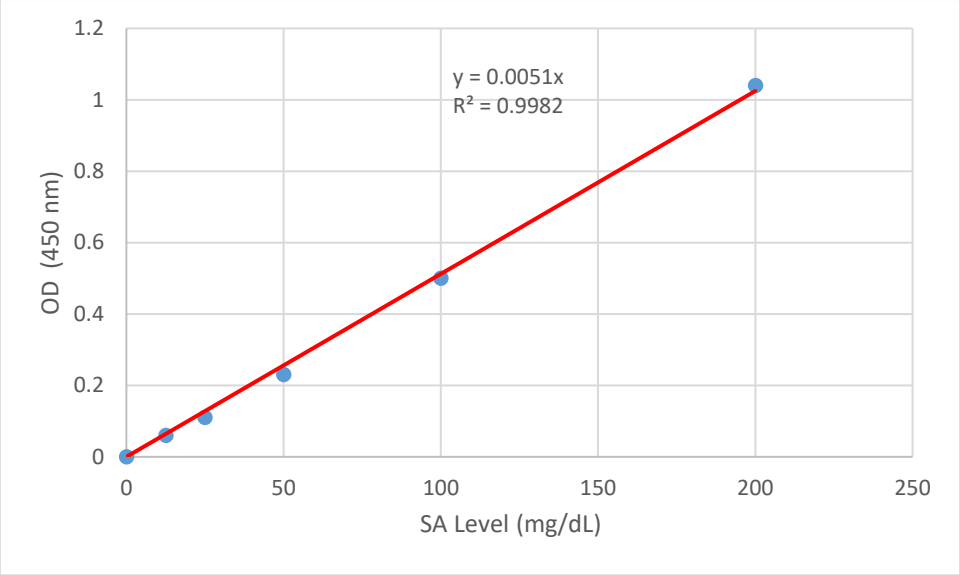


Figure (2.13): Standard Curve of SA Assay

2.4.2.2. Estimation of Blood Trace Elements

2.4.2.2.1. Digestion Procedures

2.4.2.2.1.1. Digestion Procedure of Selenium:

The blood must be digested in order to break out the organic materials associated with the selenium, the blood samples were digested by the following procedure (Al-Fartosy *et al.*, 2017a):

1- 1mL of the whole blood of each control individuals and T2DM with and without DN was transferred into Pyrex test tubes.

2- 1mL of Conc. Nitric acid (HNO_3) and 1mL of Conc. Sulfuric acid (H_2SO_4) were added to each tube.

3- The tubes were placed in an oil bath at 130°C until nitric acid (brown fumes) boiled away.

4- The tubes were removed from the oil bath and cooled to room temperature.

5- 1 mL of 5M Perchloric acid ($HClO_4$) was added to each tube.

6- The tubes were placed again in an oil bath at 130°C for 45 min.

7- The tubes were removed from the oil bath and cooled to room temperature.

8- 1 mL of 5M Hydrochloric acid (HCl) was added to each tube.

9- The tubes were placed again in an oil bath at 95°C for 30 min.

10- The tubes were removed from the oil bath and cooled to room temperature.

11- The tubes were diluted to 10ml by adding 5M HCl.

2.4.2.2.1.2. Digestion Procedure of Zinc and Magnesium:

The blood must be digested in order to break out the organic materials associated with the zinc and magnesium, the blood samples were digested by the following procedure (Al-Fartosy *et al.*, 2019):

1- 0.5mL of the blood serum of each control individuals and T2DM patients with and without DN was transferred into Pyrex test tubes.

2- 2mL of Conc. Nitric acid (HNO_3) and 1mL of Conc. Perchloric acid ($HClO_4$) were added to each tube.

3- The tubes were placed in an oil path at 160°C for 1hr (until the solutions in the tubes became clear).

4- The tubes were removed from the oil bath and cooled to room temperature.

5- The tubes were diluted to 10mL by adding 0.3M HCl.

2.4.2.2.2. Preparation of Standard Solutions

2.4.2.2.2.1. Preparation of Selenium Standard Solutions (1000 ng/mL):

Stock standard solutions of selenium were prepared by dissolving (1.405 g) of selenium dioxide (SeO_2) in (5 mL) of HNO_3 by warming and diluting to exactly (1000 mL) with distilled deionized water. Then take (1mL) of stock solution and diluting to (100 mL) by 1.5 M HCl, (10 µg/mL) standard was formed. Further dilution by 1.5 M HCl to prepare standard calibration curve (15 – 250) ng/mL (Yahya *et al.*, 2014).

2.4.2.2.2.2. Preparation of Zinc Standard Solutions (1000 µg/mL):

Dissolving (0.1 gm) of zinc metal in (0.5 mL) of Conc. HNO_3, then complete the volume to 100 mL by distilled deionized water to preparing

standard solution (1000 µg/mL). After that taking (1mL) of standard solution and diluting it to (100 mL) by 6 M HCl. A (10 µg/mL) standard was formed then it used to make up (0.1–1.6) µg/mL stock standards. All stock standards were prepared by using deionized water as diluent (Yahya *et al.*, 2014).

2.4.2.2.2.3. Preparation of Magnesium Standard Solutions (1000 µg/mL):

Standard solutions of magnesium were prepared by dissolving (1.014gm) of $MgSO_4.7H_2O$ in water containing (0.5mL) of Conc. HNO_3 and diluting the solution with distilled deionized water to (100 mL). This solution then used to make up (2.5-40) µg/mL stock standards. All stock standards were prepared by deionized water as diluents (Yahya *et al.*, 2014).

2.4.2.2.3. Determination of Trace Elements

Table (2.8) shows the optimum conditions for the determination of Se concentration used in this study according to previous studies (Al-Fartosy et al., 2019), Table (2.8) shows the concentration of HCl, flow rate of argon gas, time of mixing in reaction vessel, concentration and volume of reducing agent ($NaBH_4$) and the volume of selenium standard solution. The concentrations of Zn and Mg in standards and samples solutions were measured by flame atomic absorption spectrometry (FAAS).

2.4.2.2.3.1. Selenium Determination

Table (2.8): Optimized Conditions of Selenium Determination.

Parameter	Optimized Condition
Flow Rate of Argon Gas	0.3 L/min
Time of Mixing	(20-30) sec
HCl concentration	1.5 M
$NaBH_4$ Concentration	2% w/v
$NaBH_4$ Volume	200 µL

Se standard solution volume	200 µL
Lamp Current	10 mA
Wave length	196 nm

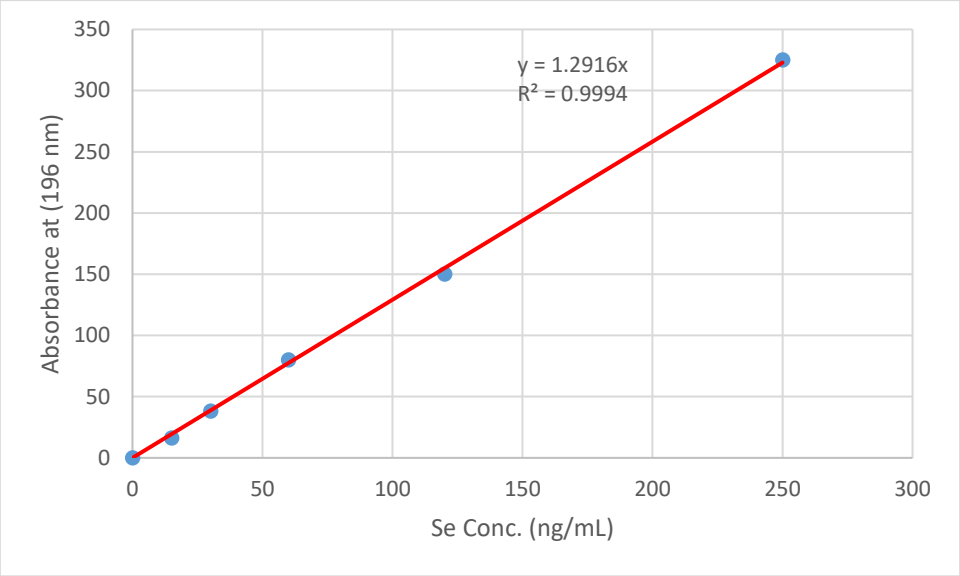


Figure (2.14): The Standard Curve of Se Determination

2.4.2.2.3.2. Zinc and Magnesium Determination

Table (2.9): Optimized conditions of Zn and Mg Determination

Parameter	Zn	Mg
Lamp Current (mA)	5	3
Wavelength (nm)	213.9	285.2
Slit Width (nm)	0.5	0.5
Slit Height	Normal	Normal
Read Time (sec)	3	3

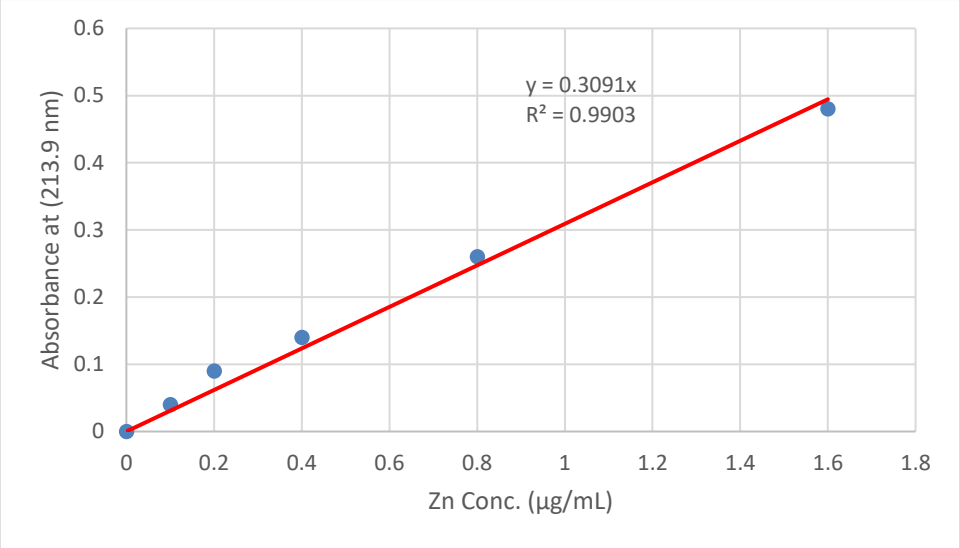


Figure (2.15): The Standard Curve of Zn Determination

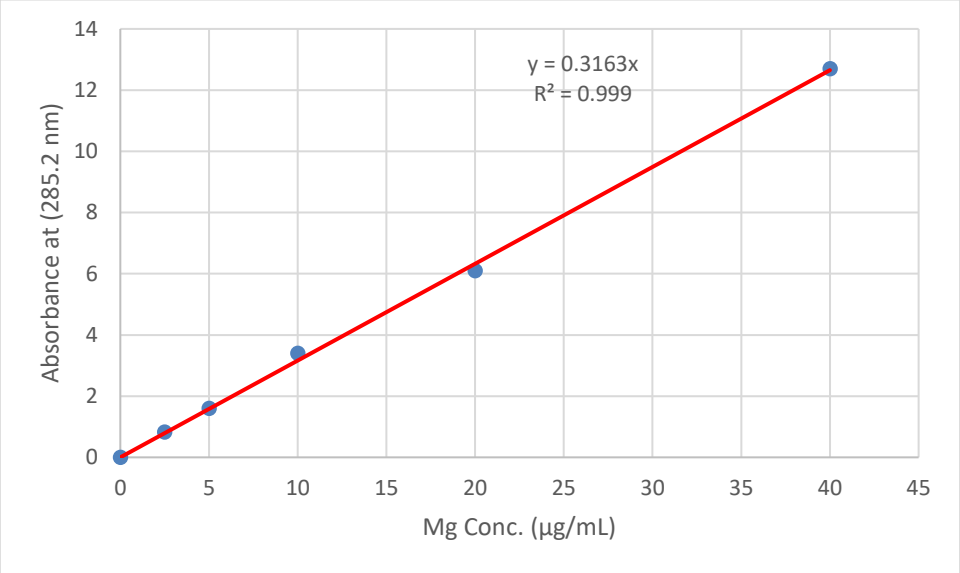


Figure (2.16): The Standard Curve of Mg Determination

2.4.2.3. Estimation of Urine Biomarkers:

2.4.2.3.1. Assay of Albumin (Alb)

Principle:

Alb level was measured by competitive enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human Alb in urine. Highly purified human Alb is bound to microwells. The reaction is based on a competitive ELISA method with these steps: Calibrators, controls and urine samples are incubated together with anti-

Alb peroxidase conjugate in the wells. Alb, if present, will compete with coated Alb for binding of the anti-Alb-conjugate. Washing of the microwells removes unspecific components. Bound enzyme conjugate will hydrolyze the enzyme substrate TMB. The addition of acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is inversely proportional to the concentration of albumin present in the original sample. (Norris *et al.*, 2018).

Reagents:

1- Calibrator A 0.15 µg/mL, containing serum/buffer matrix (PBS, BSA, detergent, NaN_3 0.09%), yellow.

2- Calibrator B 1.5 µg/mL, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

3- Calibrator C 6 µg/mL, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

4- Calibrator D 25 µg/mL, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

5- Calibrator E 100 µg/mL, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

6- Calibrator F 400 µg/mL, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

7- Control positive, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

8- Control negative, containing Alb in a serum/buffer matrix (PBS, detergent, NaN_3 0.09%), yellow.

9- Sample Buffer PA, containing PBS, detergent, preservative sodium azide 0.09%, yellow.

10- Enzyme Conjugate; containing anti-human Alb antibodies, HRP labelled; PBS, BSA, detergent, preservative Proclin 0.05%, light red.

11- TMB Substrate; containing 3,3', 5,5'-Tetramethylbenzidin, colorless.

12- Stop Solution; contains acid.

13- Wash Buffer, containing Tris, detergent, preservative sodium azide 0.09%; 50X conc.

Reagent Preparation:

All reagents were brought to room temperature before use: The contents of one vial of the buffered wash solution concentrate (50x) was diluted with distilled or deionised water to a final volume of 1000 mL prior to use.

Procedure:

1- 20 µL of calibrators, controls and patient samples was pipetted into the wells.

2- 100 µL of enzyme conjugate was dispensed into each well.

3- The wells were incubated for 30 minutes at room temperature (20°C - 28°C).

4- The contents of the microwells were discarded and washed 3 times with 300 µL of wash solution.

5- 100 µL of TMB substrate solution was dispensed into each well.

6- The wells were incubated for 15 minutes at room temperature

7- 100 µL of stop solution was added to each well of the modules

8- The wells were incubated for 5 minutes at room temperature.

9- The optical density was read at 450 nm and calculate the results. The developed colour is stable for at least 30 minutes. The reading was done during this time.

Calculations:

Normal and logarithmic calibration curve were plotted using the standard concentrations and their corresponding absorbance as presented in Figures (2.17 & 2.18). The concentration of each sample was calculated from the regression equation in the log-log plot.

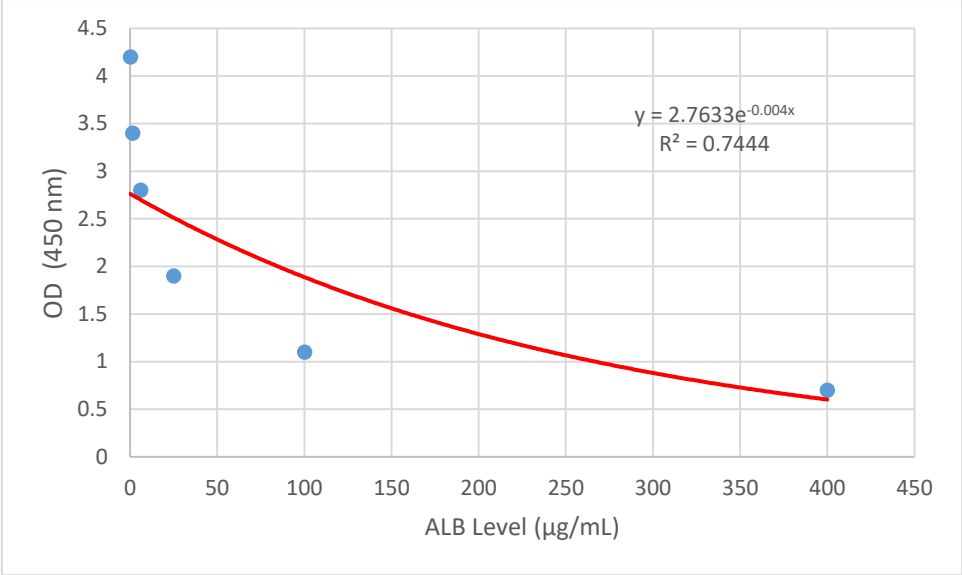


Figure (2.17): Normal Standard Curve of Alb Assay

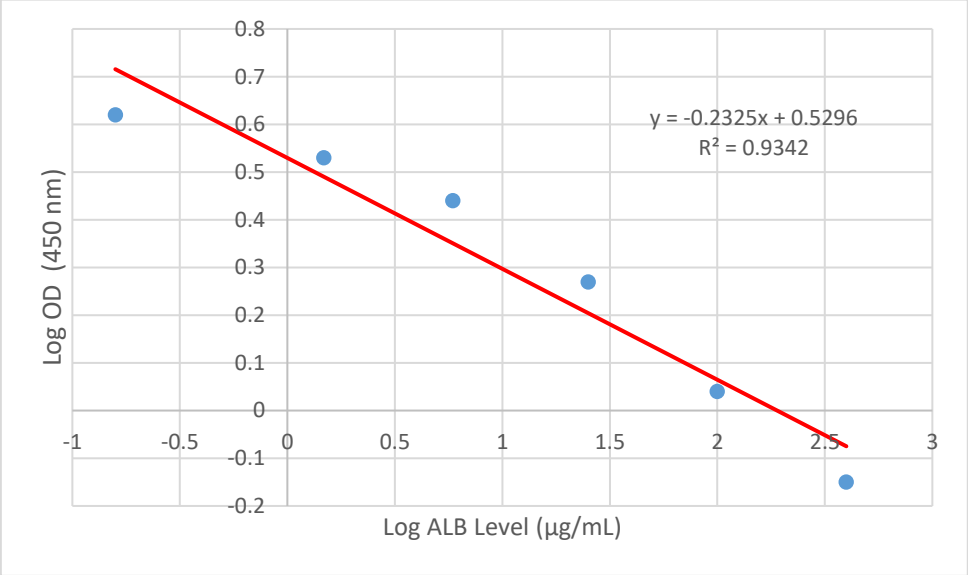


Figure (2.18): Logarithmic Standard Curve of Alb Assay

2.4.2.3.2. Assay of Neutrophil Gelatinase Associated Lipocalin (NGAL)

Principle:

NGAL level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human NGAL in urine. The plate has been pre-coated with human NGAL antibody. NGAL present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human NGAL Antibody is

added and binds to NGAL in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NGAL antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of human NGAL. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Ali *et al.*, 2018).

Reagents:

1- Standard Solution (640ng/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

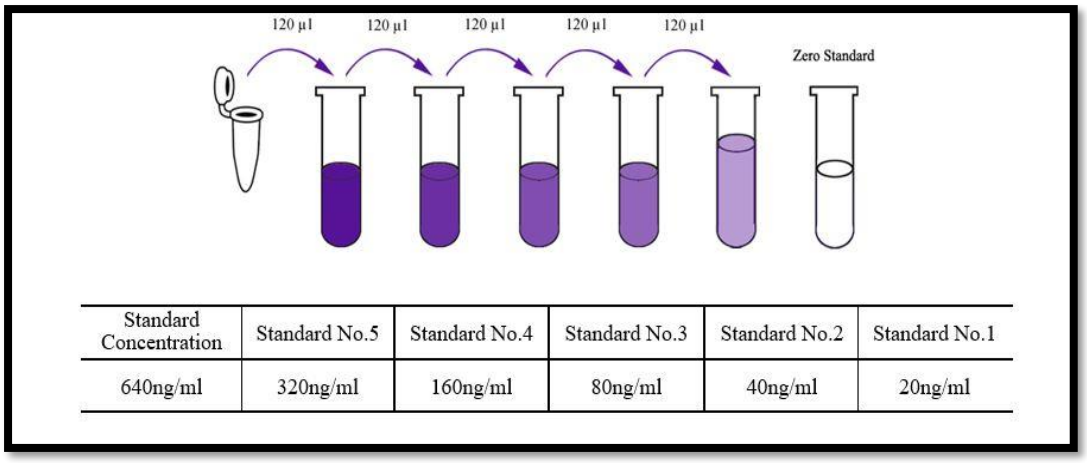
7- Wash Buffer Concentrate (25x).

8- Biotinylated human NGAL Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (640ng/mL) was reconstituted with 120µL of standard diluent to generate a 320ng/mL standard stock solution. The standard was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (320ng/mL) 1:2 with standard diluent to produce 160ng/mL, 80ng/mL, 40ng/mL and 20ng/mL solutions. The Standard diluent was served as the zero standard (0 ng/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
640ng/ml	320ng/ml	160ng/ml	80ng/ml	40ng/ml	20ng/ml

Figure (2.19): Standards Solutions Preparation of NGAL.

2- Wash Buffer: 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standard was added to standard well.

4- 40µL of sample was added to sample wells. Then, 10µL of anti-NGAL antibody was added to sample wells.

5- 50µL of streptavidin-HRP was added to sample and standard wells (Not blank control well) and mixed well. The plate was covered with a sealer and incubated 60 minutes at 37°C.

6- The sealer was removed, and the plate was washed 5 times with wash buffer. The wells were soaked with 1 mL of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

7- 50µL of substrate solution A was added to each well. Then 50µL of substrate solution B was added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

8- 50µL of Stop Solution was added to each well, the blue color changed into yellow immediately.

9- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

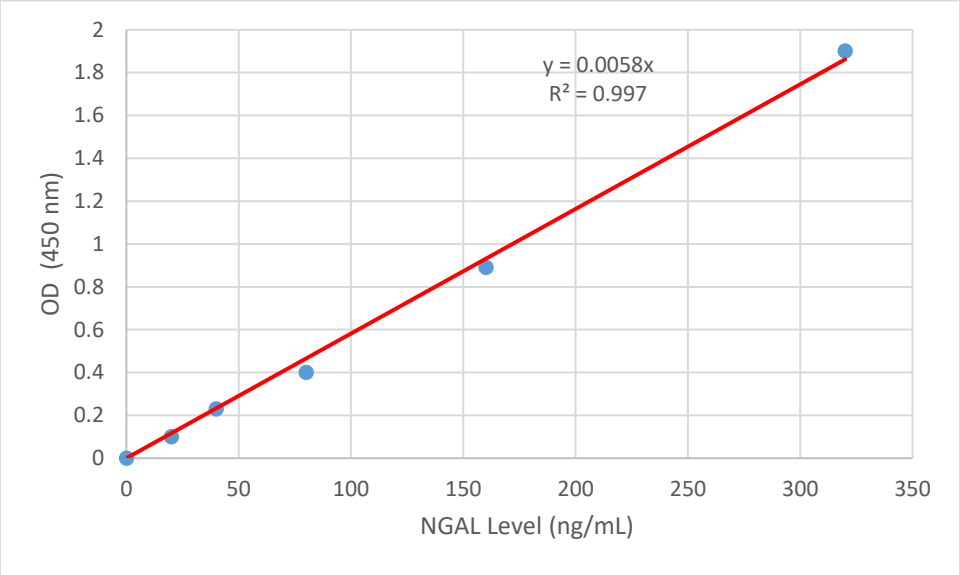


Figure (2.20): Standard Curve of NGAL Assay

2.4.2.3.3. Assay of 8-Hydroxy-2'-deoxyguanosine (8-OHdG):

Principle:

8-OHdG level was measured by competitive enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human 8-OHdG in urine. 8-OHdG standards or samples are added to the wells pre-coated with a monoclonal antibody. Then biotin-conjugated target antigen is added to the wells. The antigens in the standards or samples compete with the biotin-conjugated antigen to bind to the capture antibody and incubate. Unbound antigen is washed away during a washing step. An avidin-HRP is then added and then incubate.

Unbound avidin-HRP is washed away during a washing step. TMB Substrate is then added and color develops. The reaction is stopped by addition of acidic stop solution and color changes into yellow that can be measured at 450 nm. The intensity of the color developed is inversely proportional to the concentration of 8-OHdG in the sample. The concentration of 8-OHdG in the sample is then determined by comparing the O.D. of the samples to the standard curve (Korkmaz *et al.*, 2018).

Reagents:

1- Human 8-OHdG Standard, lyophilized: 150µL.

2- Standard/Sample Diluent.

3- Biotinylated Antigen, lyophilized.

4- Avidin-HRP Concentrate.

5- Biotinylated Antigen Diluent.

6- Avidin HRP Diluent.

7- Substrate Solution A.

8- Substrate Solution B.

9- Stop Solution.

10- Wash Buffer Concentrate (25x).

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: The standard was reconstituted with 150 µL of standard diluent to generate a 1600ng/mL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution 1:2 with diluent to produce 800ng/mL, 400ng/mL, 200ng/mL, 100ng/mL and 50ng/mL solutions. The sample diluent was served as the zero standard (0 ng/mL).

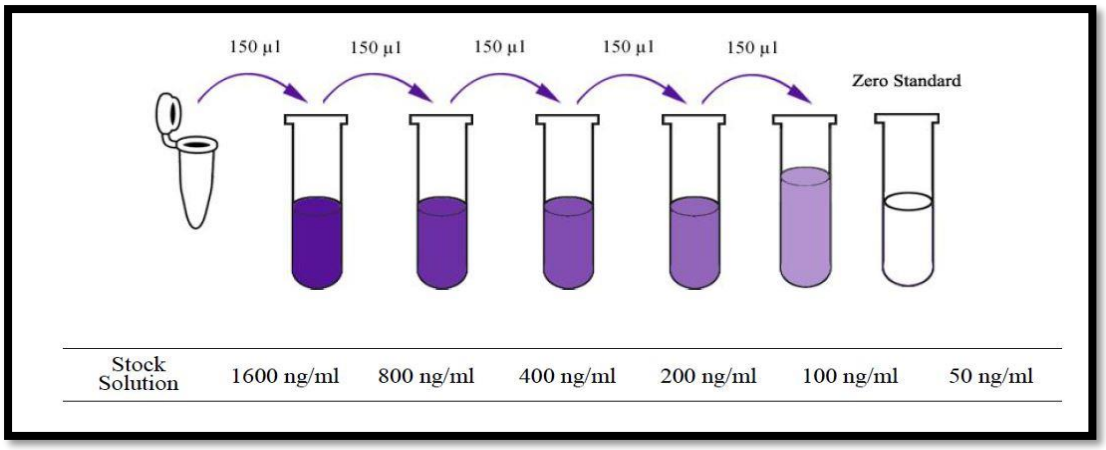


Figure (2.21): Standards Solutions Preparation of 8-OHdG.

2- Biotinylated Antigen: The biotinylated antigen vial was briefly centrifuged and reconstituted with diluent to produce a stock solution. This stock solution was pipetted into the diluent vial, mixed well and allowed to sit for 10 minutes with gentle agitation prior to making dilutions.

3- Avidin-HRP Concentrate: The avidin-HRP was centrifuge briefly by low- speed centrifugation. Then, it was pipetted into the diluent vial, mixed well and allowed to sit for 10 minutes with gentle agitation prior to making dilutions.

4- Wash Buffer Concentrate 25x: The concentrated wash buffer was diluted with distilled water to yield 500 mL of 1x wash buffer. The Wash Buffer Concentrate was diluted 1: 25 with reagent grade water.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50 µL of standards and samples was added to their wells. Then, 50 µL of biotinylated antigen was added to each well (Not blank control well) and mixed well. The plate was covered with a sealer and incubated for 30 minutes.

4. The plate was washed five times with 300 µL of wash buffer manually, inverted each time, decanted the contents and hit 5 times on absorbent

material to complete removing liquid. The plate was blotted on absorbent material.

5- 50 µL of concentrated avidin-HRP was added to standards and samples wells. The plate was covered with a sealer and incubated for 30minutes.

6- The plate was washed as described above after removing the sealer.

7- 50 µL of substrate solution A and 50 µL of substrate solution B were added to each well respectively. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

8- 50 µL of Stop Solution was added to each well, the blue color was changed into yellow immediately.

9- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 15 minutes after adding the stop solution.

Calculations:

Normal and logarithmic calibration curve were plotted using the standard concentrations and their corresponding absorbance as presented in Figures (2.22 & 2.23). The concentration of each sample was calculated from the regression equation in the log-log plot.

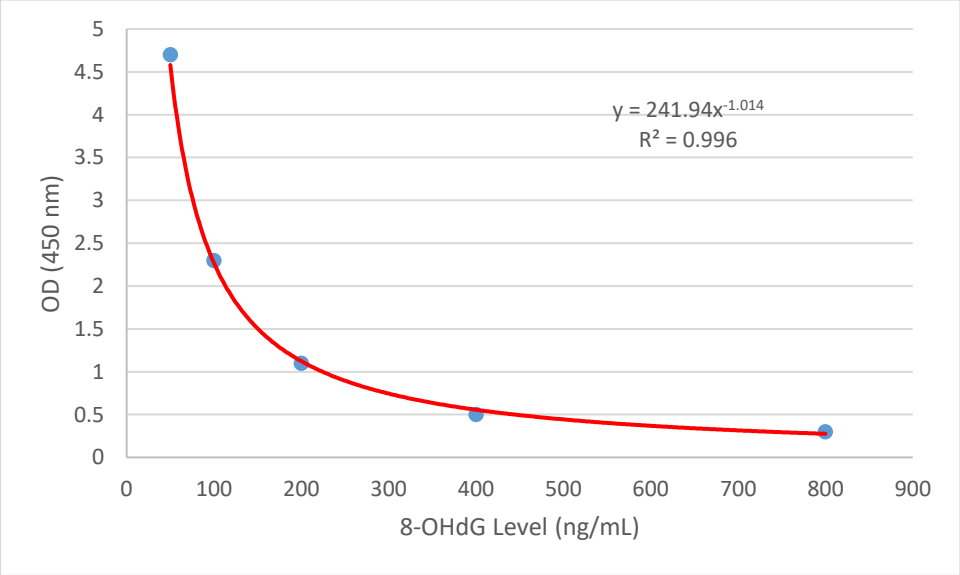


Figure (2.22): Normal Standard Curve of 8-OHdG Assay

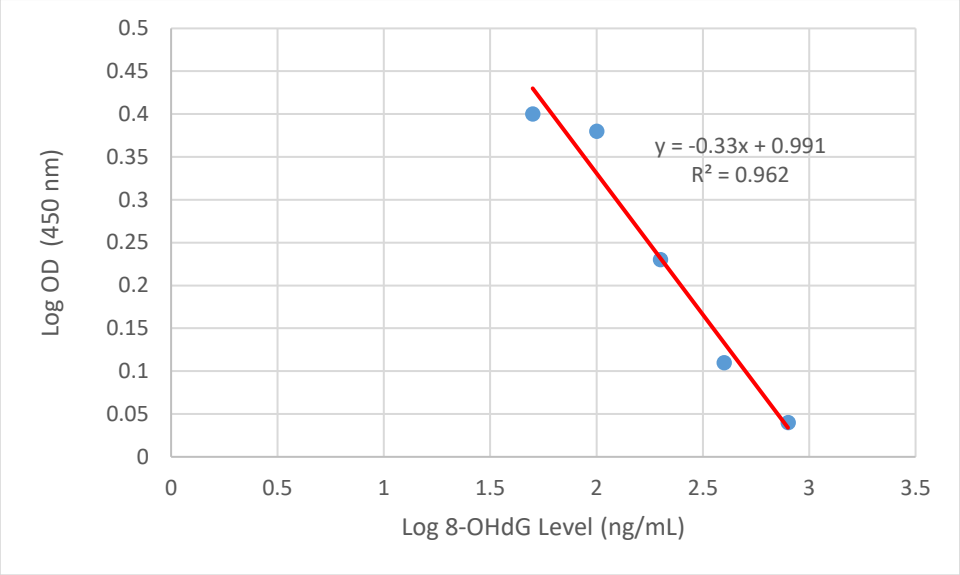


Figure (2.23): Logarithmic Standard Curve of 8-OHdG Assay

2.4.2.3.4. Assay of Fibronectin (FN)

Principle:

FN level was measured by sandwich enzyme-linked immune-sorbent assay (ELISA) technology for the quantitative measurement of human FN in urine. The plate has been pre-coated with human FN antibody. FN present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human FN Antibody is added and binds to FN in the sample. Then, Streptavidin-HRP is added and binds to the Biotinylated FN antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human FN. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm (Caterinoa *et al.*, 2018).

Reagents:

1- Standard Solution (800ng/mL).

2- Standard Diluent.

3- Streptavidin-HRP.

4- Stop Solution.

5- Substrate Solution A.

6- Substrate Solution B.

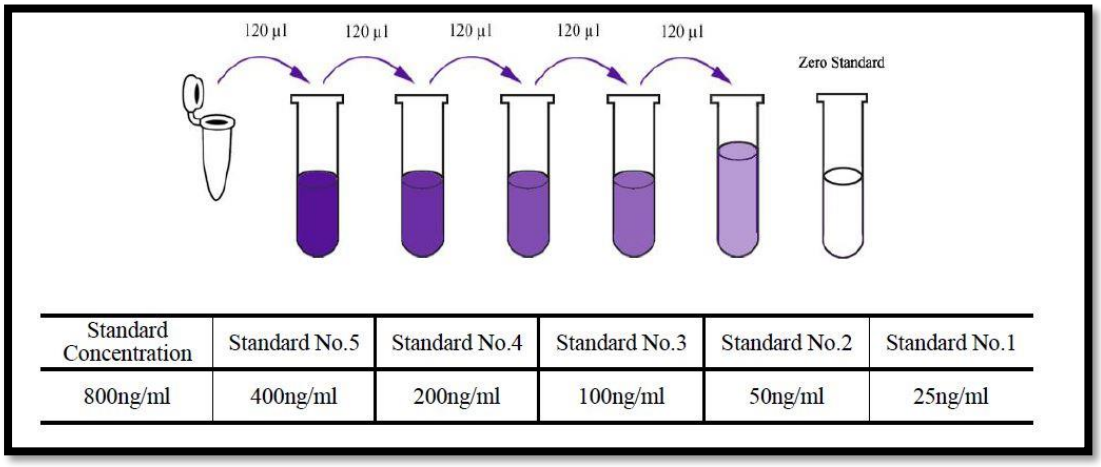
7- Wash Buffer Concentrate (25x).

8- Biotinylated human FN Antibody.

Reagent Preparation:

All reagents were brought to room temperature before use:

1- Standard: 120µL of the standard (800ng/mL) was reconstituted with 120µL of standard diluent to generate a 400ng/mL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (400ng/mL) 1:2 with standard diluent to produce 200ng/mL, 100ng/mL, 50ng/mL and 25ng/mL solutions. Standard diluent was served as the zero standard (0 ng/mL).



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
800ng/ml	400ng/ml	200ng/ml	100ng/ml	50ng/ml	25ng/ml

Figure (2.24): Standards Solutions Preparation of FN.

2- Wash Buffer**:** 20mL of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 mL of 1x Wash Buffer.

Procedure:

1- All reagents were brought to room temperature before use.

2- The strips were inserted in the frames for use.

3- 50µL of standards was added to standard wells.

4- 40µL of samples was added to sample wells. Then, 10µL of anti-FN antibody was added to sample wells.

5- 50µL of streptavidin-HRP was added to sample and standard wells (Not blank control well) and mixed well. The plate was covered with a sealer and incubated 60 minutes at 37°C.

5- The sealer was removed, and the plate was wash 5 times with wash buffer. The wells were soaked with 1 ml of wash buffer for 1 minute for each wash. The plate was blotted onto paper towels.

6- 50µL of substrate solution A was added to each well. Then, 50µL of substrate solution B was added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

7- 50µL of Stop Solution was added to each well, the blue color changed into yellow immediately.

8- The optical density (OD value) was determined for each well immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations:

The Standard Curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing the best fit line through the points on the graph.

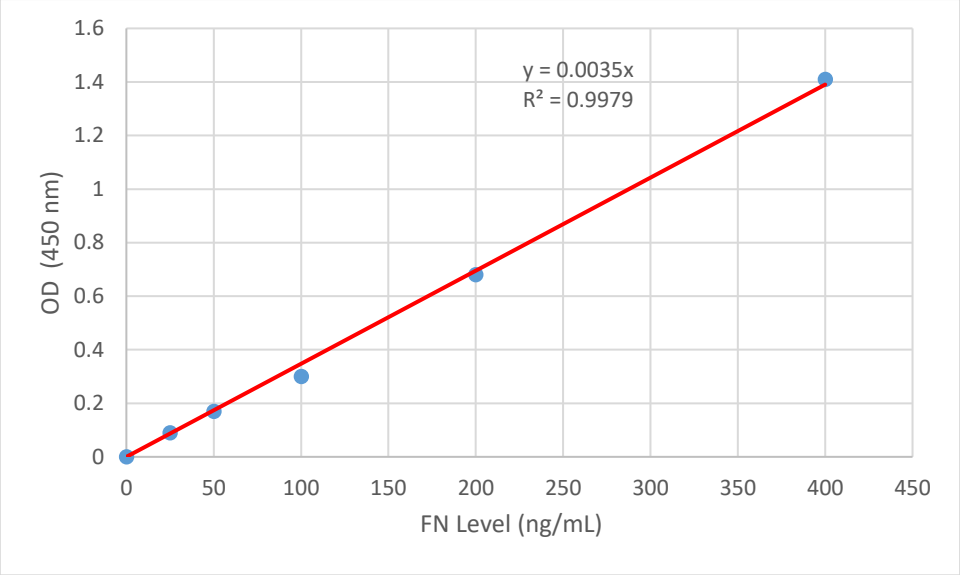


Figure (2.25): Standard Curve of FN Assay

2.5. Statistical Analysis

Results were analyzed using the SPSS software (Version 22) and the values were expressed as mean ± standard deviation (SD). All comparisons were 2-tailed and p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

Chapter Three

Results & Discussions

Chapter Three

3.1. Basic Characteristics of Individuals in the Present Study

Sixty-three subjects (men and women) who suffering from T2DM were selected to share in this study and they divided into two groups: the first group was consisted of 31 patients who suffering from DN (15men and 16women) while the second group was consisted of 32 patients who free from DN (15men and 17women). These subjects were matched with 33 healthy controls (16 men and 17 women). The general characteristics of the subjects participated in the present study are presented in Table (3.1).

Table (3.1): The demographic characteristics of the present study.

The Characteristics		T2DM Patients		Healthy Controls
		with DN	without DN	
Total Subjects No.		31	32	33
Age (Years) (Mean±SD)		57.26±4.47	53.94±4.74	51.70±5.07
DM Duration (Years) (Mean±SD)		13.57±2.53	5.23±1.66	-
DN Duration (Years) (Mean±SD)		6.80±0.63	-	-
Demographic Area	**Urban**	28	27	29
	Rural	3	5	4
Educational Background	**Learned**	25	24	26
	Illiterate	6	8	7
Smoking Habits	**Positive**	0	0	0
	Negative	31	32	33
Food Habits	**Vegetarian**	5	6	8
	Non-Vegetarian	26	26	25
Employment Status	**Employed**	19	14	28
	Not Employed	12	18	5
Subjects Gender	**Men**	15	15	16
	Women	16	17	17

In the existing study, data presented that all the patients and healthy control subjects were non-smokers. Furthermore, most of the volunteers from both patients and healthy controls were from urban, all of them acquired a well-educated and they had a good work place. The major variations between urban and rural regions are the differences in environments, pollution, social, psychic,

genetic, food habits and others which are increasing dramatically in urban areas (Al-Fartosy *et al.*, 2020; El-Zamar *et al.*, 2019). Moreover, same Table 3.1 showed that most of the volunteers from the diabetic patients with or without nephropathy and healthy controls were from the Basrah province. Therefore, our results cannot appear the actual status of the whole patients' groups in Iraq due to the low number of patients who attends in the diabetes and endocrine glands centre in Al-Mawany teaching hospital and counts on the cooperatively of patients and their ready to engage in the present study as well.

3.2. Determination of Total Parameters in this Study

Table (3.2) and Table (3.3) show the levels of all parameters were measured in this our work.

Table (3.2): Levels of total parameters measured in the present study for T2DM patients with DN and control group. The values are the Mean ± SD.

Biomarkers	T2DM Patients with DN n=31 (Men & Women)					Healthy Controls n=33 (Men & Women)
				95% CI		
	Mean ± SD	SE	Range	Lower	Upper	Mean ± SD
BMI (Kg/m²)	30.63±1.35	0.24	28.7-34.1	26.88	34.38	30.79±1.46
F. Glucose (mg/dL)	167.67±5.92**	1.06	159-180	151.23	184.1	101.9±4.8
F. Insulin (µU/mL)	28.99±2.06**	0.37	24.65-33.47	23.27	34.7	11.09±1.57
HOMA-IR	12.01±1.08**	0.19	9.9-14.9	9.01	15.01	2.80±0.53
F. Urea (mg/dL)	56.72±4.06**	0.73	51.01-65.75	45.45	67.99	27.27±3.98
F. Creatinine (mg/dL)	1.60±0.22**	0.04	1.35-1.96	0.99	2.21	0.87±0.07
GFR (mL/min/1.73m²)	41.70±5.25**	0.94	36.8-58	27.12	56.27	85.48±8.96
CrCl (mL/min)	64.47±7.45**	1.34	54-85	43.79	85.15	128.12±10.46
F. Hcy (nmol/mL)	21.30±3.07**	0.55	14.73-29.02	12.78	29.82	10.83±2.21
F. FA (mmol/L)	0.45±0.12**	0.02	0.32-0.97	0.12	0.78	0.26±0.04
F. CysC (ng/mL)	1.33±0.32**	0.06	0.89-2.39	0.44	2.22	0.76±0.13
F. LC (nmol/mL)	28.90±4.02**	0.72	20.15-36.35	17.74	40.06	46.15±4.91

F. OPG (ng/mL)	3.25±0.38**	0.07	2.33-4.01	2.20	4.30	1.87±0.27
F. SA (mg/dL)	86.89±3.86**	0.69	80.40-94.96	76.18	97.61	60.72±4.78
F. Se (ng/mL)	57.29±6.64**	1.19	49.27-67.65	38.86	75.72	86.29±6.80
F. Zn (µg/mL)	0.76±0.24**	0.04	0.42-1.31	0.09	1.43	1.29±0.43
F. Mg (µg/mL)	14.97±2.77**	0.50	8.71-18.95	7.28	22.66	21.74±3.65
F. Alb (µg/mL)	29.35±4.98**	0.89	19.99-37.09	15.52	43.17	6.03±2.11
F. NGAL (ng/mL)	66.64±11.89**	2.14	53.32-86.42	33.63	99.65	25.57±11.03
F. 8-OHdG (ng/mL)	26.01±5.31**	0.95	15.09-34.13	11.26	40.75	13.13±3.36
F. FN (ng/mL)	15.94±4.28**	0.77	9.27-24.17	4.06	27.82	8.51±2.28

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, n: No. of subjects, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

Table (3.3): Levels of total parameters measured in the present study for T2DM patients without DN and control group. The values are the Mean ± SD.

Biomarkers	T2DM Patients without DN n=32 (Men & Women)					Healthy Controls n=33 (Men & Women)
				95% CI		
	Mean ± SD	SE	Range	Lower	Upper	Mean ± SD
BMI (Kg/m²)	30.60±1.79	0.32	26.3-34.6	25.63	35.57	30.79±1.46
F. Glucose (mg/dL)	163.38±5.57**	0.98	151-174	147.91	178.84	101.9±4.8
F. Insulin (µU/mL)	28.08±2.12**	0.37	24.32-32.02	22.19	33.96	11.09±1.57
HOMA-IR	11.33±1.04**	0.18	9.2-13.8	8.45	14.22	2.80±0.53
F. Urea (mg/dL)	33.20±4.03*	0.71	24.9-40.9	22.02	44.39	27.27±3.98
F. Creatinine (mg/dL)	1.05±0.11*	0.02	0.89-1.29	0.75	1.36	0.87±0.07
GFR (mL/min/1.73m²)	67.35±6.97*	1.23	56.6-83.8	48	86.7	85.48±8.96
CrCl (mL/min)	98.03±10.45*	1.85	81-116	69.02	127.04	128.12±10.46
F. Hcy (nmol/mL)	17.92±3.93**	0.69	11.30-24.71	7.02	28.83	10.83±2.21
F. FA (mmol/L)	0.35±0.08**	0.01	0.22-0.50	0.13	0.57	0.26±0.04

F. Cys C (ng/mL)	1.23±0.24*	0.04	0.95-1.75	0.56	1.89	0.76±0.13
F. LC (nmol/mL)	36.28±3.57*	0.63	27.71-44.57	26.37	46.19	46.15±4.91
F. OPG (ng/mL)	2.65±0.21*	0.04	1.93-3.37	2.07	3.24	1.87±0.27
F. SA (mg/dL)	78.32±3.94**	0.70	69.28-87.23	67.38	89.26	60.72±4.78
F. Se (ng/mL)	64.87±5.91**	1.04	58.47-73.64	48.46	81.27	86.29±6.80
F. Zn (µg/mL)	0.86±0.19**	0.03	0.53-1.25	0.33	1.39	1.29±0.43
F. Mg (µg/mL)	17.28±2.81**	0.50	12.43-21.75	9.48	25.08	21.74±3.65
F. Alb (µg/mL)	10.91±2.86*	0.51	6.14-16.22	2.98	18.85	6.03±2.11
F. NGAL (ng/mL)	60.04±13.63**	2.41	39.35-80.04	22.20	97.88	25.57±11.03
F. 8-OHdG (ng/mL)	21.25±5.79**	1.02	10.05-32.13	5.18	37.32	13.13±3.36
F. FN (ng/mL)	11.76±3.47*	0.61	5.29-17.87	2.13	21.40	8.51±2.28

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, n: No. of subjects, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

3.3. Level of Body Mass Index (BMI)

The degree of obesity is usually estimated using a simple anthropometric parameter called body mass index (BMI) which is calculated by weight in (Kg) divided to height in squared meter (m^2) and can be utilized to determine the extent of glycemic control (Al-Fartosy *et al.*, 2020).

The present study showed a non-significant change (p>0.05) in BMI levels in T2DM patients with and without DN as compared to control subjects (Men: 31.27±1.67, 30.30±2.56 vs. 31.56±0.25 kg/m^2; Women: 30.12±0.51, 30.86±0.38 vs. 30.08±1.74 kg/m^2), Table (3.4) and Figure (3.1).

Table (3.4): Levels of BMI in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		BMI (kg/m²)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		31.27±1.67	30.12±0.51	30.30±2.56	30.86±0.38	31.56±0.25	30.08±1.74
SE		0.43	0.13	0.66	0.09	0.06	0.42
Range		28.7-34.1	29.8-32.1	26.3-34.6	29.6-31	30.6-31.9	27.4-33.1
95% CI	Lower	26.64	28.7	23.19	29.81	30.86	25.25
	Upper	35.91	31.53	37.41	31.92	32.25	34.91

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

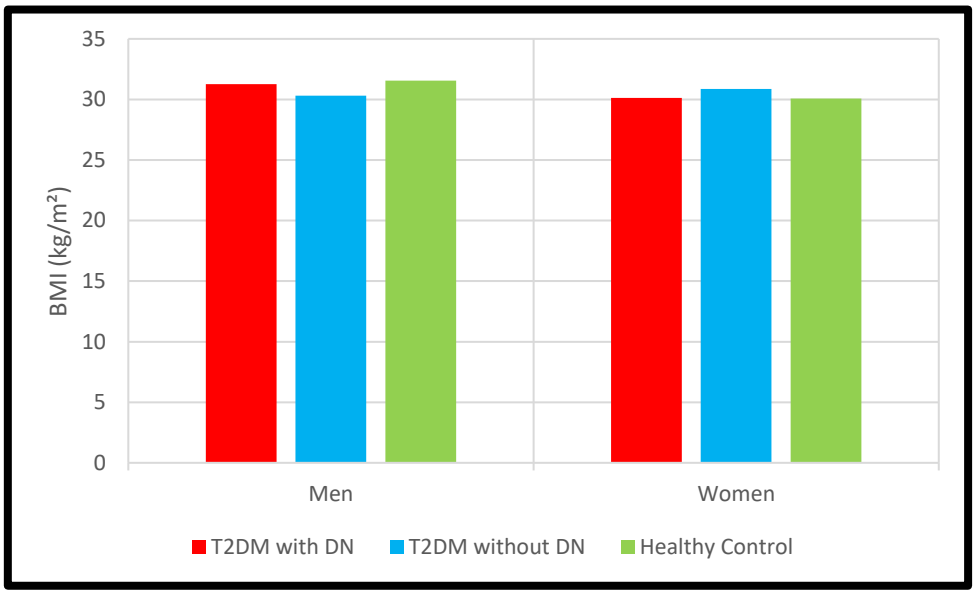


Figure (3.1): Levels of BMI in T2DM patients with or without DN and control group.

Obesity perhaps acts as a diabetogenic factor via elevating resistance to the action of insulin in those genetically predisposed to enhance T2DM. IR leads to higher plasma levels of insulin which bring about an increase in appetite. Consequently, people eat more and gain more weight (Al-Goblan *et al.*, 2014). So, obesity could be one of the etiological factors in the development of T2DM, and mostly because of loss of early phase insulin secretion in response to glucose which happens relatively earlier in the development of T2DM (Park *et al.*, 2018). This loss is critically crucial as the early blast of insulin secretion plays a

substantial role in priming target tissues of insulin, especially the liver responsible for normal glucose homeostasis after food uptake and mealtime glucose deflection take place when this process was deteriorated (Boughton *et al.*, 2017).

Obesity is considered one of the modifiable cardiovascular risk factors that is far more predominant in those people with T2DM than in the general population. Moreover, obesity and physical inactivity are important independent risk factors for type 2 middle aged men (Wang *et al.*, 2016).

3.4. Biochemical Estimation

3.4.1 Estimation of Blood Biomarkers

3.4.1.1. Level of Glucose

This study showed a high significant increase ($p<0.01$) in serum glucose level in T2DM patients with and without DN as compared to control subjects (Men: 172.73±3.35, 168.60±3.09 vs. 104.26±3.82 mg/dL; Women: 162.38±2.34, 158.76±2.15 vs. 99.68±4.56 mg/dL), Table (3.5) and Figure (3.2).

Table (3.5): Levels of glucose in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Glucose (mg/dL)					
		T2DM Patients				Healthy Control	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		172.73±3.35**	162.38±2.34**	168.6±3.09**	158.76±2.15**	104.26±3.82	99.68±4.56
SE		0.86	0.59	0.80	0.52	0.96	1.11
Range		162-180	159-171	158-174	151-163	98.03-110.5	91.21-104.3
95% CI	Lower	163.43	155.88	160.02	152.8	93.65	87.02
	Upper	182.03	168.87	177.18	164.73	114.86	112.34

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

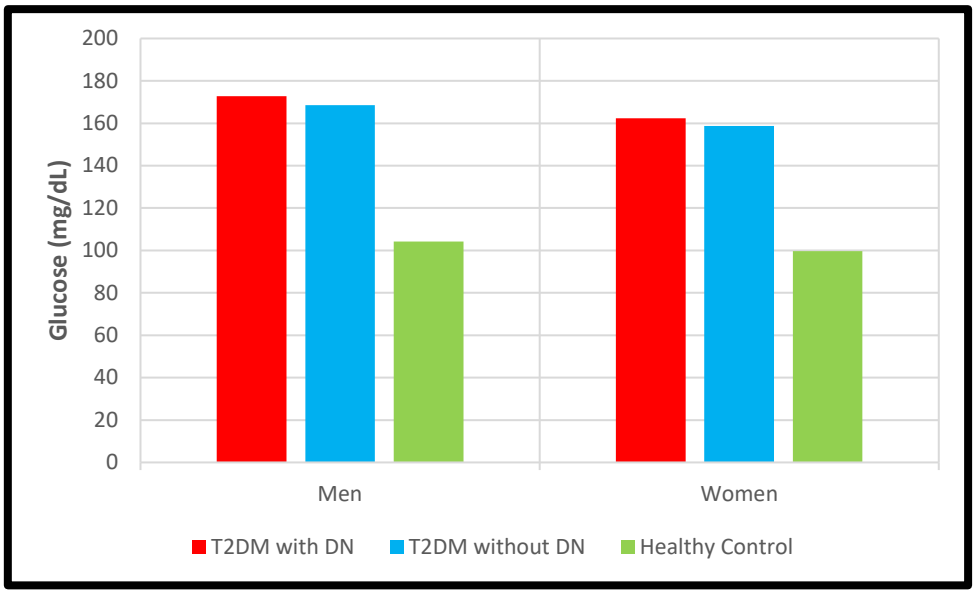


Figure (3.2): Levels of glucose in T2DM patients with or without DN and control group.

Any elevating in glycemia is the net result of glucose influx overriding glucose outflow from the plasma section. In the fasting state, hyperglycemia is directly associated to raise hepatic glucose production. In the postprandial state, further glucose deflection result from the set of insufficient repression of this glucose output and inglorious insulin energizing of glucose disposal in target tissues, fundamentally skeletal muscle (Angellotti *et al.*, 2018). Abnormal islet cell action is a key and necessary feature of type 2 diabetes. In early disease stages, insulin production is normal or elevated in absolute terms, but disproportionately low for the degree of insulin sensitivity, which is typically decreased. However, insulin kinetics such as the capability of the pancreatic β-cells to release sufficient hormone in phase with increasing glycemia are deeply compromised (Kelany *et al.*, 2016). This practical islet inability is the major quantitative determinant of hyperglycemia and progresses over time. In addition, in type 2 diabetes, pancreatic α-cells hyper-secrete glucagon, further encourage hepatic glucose production. Improving insulin action alleviate β-cells secretory burden, and any intervention that enhances glycaemia from energy limitation to, most strikingly, bariatric surgery can progress β-cells dysfunction to a range

(Levine *et al.,* 2016). Recently known abnormalities in the incretin system represented by the gut hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are found in T2DM whether these comprise primary or secondary disorders. In most patients with T2DM, especially the obese, IR in target tissues (liver, muscle, adipose tissue and myocardium) is an outstanding feature, these findings in both glucose overproduction and underutilization (Babiker and Al Dubayee, 2017). Furthermore, the increasing of delivery of fatty acids to the liver favours their oxidation, which participate to elevating gluconeogenesis, whereas the absolute overabundance of lipids boosts hepatosteatosin. The elevated hyperglycaemia-induced permeability of the glomeruli that is linked with hyperfiltration and microalbuminuria is not debated (Papadopoulou-Marketou *et al.,* 2017).

Moreover, the higher amounts of fasting blood sugar levels in men than women in our study may indicate poor glycemic control in men which is an indicator of DN. This can be clarified that rigorous glycemic control lowers the risk of nephropathy and other diabetic complications (Sirivole and Eturi, 2017).

3.4.1.2. Level of Insulin

Insulin is an anabolic hormone which acts on several target tissues including the liver, skeletal muscle and fat tissue. It is regulating the blood glucose level. The activity of enzymes that rule metabolic responses like glycogen synthesis, glycogenolysis, gluconeogenesis and lipogenesis is strictly controlled through intracellular signalling mechanisms downstream of the insulin receptor (Bogan, 2012). Insulin does its influence on the glucose uptake in a peripheral tissue by connecting to the insulin receptor which is a cell surface protein belongs to the family of tyrosine kinase receptors (Rad *et al.,* 2018). In T2DM, it is frequently associated with the basal hyperinsulinemia, decreased sensitivity to insulin, and disorders in insulin release. There was a late onset of insulin secretion in response to glucose, followed by an extended hyperinsulinemic phase. These disorders are

because of IR at the level of the β-cells might we expect an insulin secretory flaw in any condition connected with generalized hyperinsulinemia and IR in this section (Chan *et al.*, 2016).

This study showed a high significant increase ($p<0.01$) in serum insulin hormone in T2DM patients with or without DN as compared to control subjects (Men: 29.06±2.72, 28.17±2.37 vs. 11.50±1.81 µU/mL; Women: 28.78±1.14, 27.99±1.88 vs. 10.70±1.17 µU/mL), Table (3.6) and Figure (3.3).

Table (3.6): Levels of insulin in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Insulin (µU/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		29.06±2.72**	28.78±1.14**	28.17±2.37**	27.99±1.88**	11.5±1.81	10.7±1.17
SE		0.70	0.29	0.61	0.46	0.45	0.28
Range		24.65-33.47	26.84-30.62	24.32-32.02	24.69-31.13	8.23-14.93	8.69-12.75
95% CI	Lower	21.51	25.62	21.59	22.77	6.47	7.46
	Upper	36.61	31.95	34.75	33.21	16.52	13.95

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

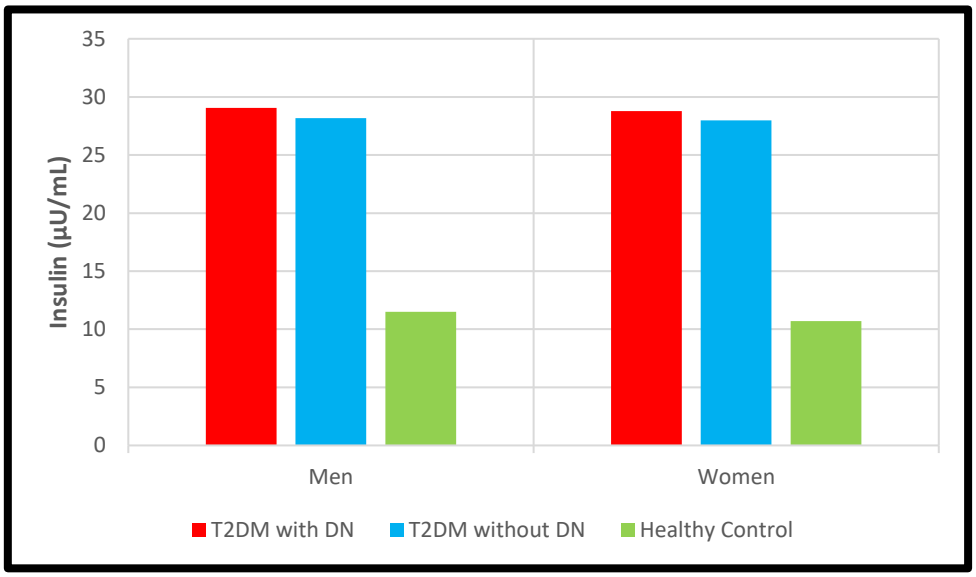


Figure (3.3): Levels of insulin in T2DM patients with or without DN and control group.

The elevation in insulin levels in T2DM patients with and without DN in our study could be due to decreased insulin receptor binding and significant hypertriglyceridemia which was correlated with the degree of hyperinsulinemia. We conclude that hyperinsulinemia in the basal state may lead to generalized IR linked with disturbances in glucose metabolism and insulin secretion. Our bodies need to produce the right quantity of insulin and respond to the insulin appropriately to be healthy. Confounding factors which are hyperglycemia and hyperinsulinemia in themselves (Al-Attaby and Al-Lami, 2019). The body begins to be more resistant to insulin with increasing duration of diabetes, so, insulin level is high or normal in the body but the accessible insulin is inadequate (Budiyani *et al.,* 2017). As recently pointed out in one study, it is practically impossible to improve diabetes due to severity of insulin resistance found in most T2DM patients unless the capacity to secrete external quantities of insulin to recompense for the IR is damaged because of the feedback between glucose concentration (the main stimulant for insulin release) and β-cells insulin secretion (Al-Fartosy and Mohammed, 2017a).

3.4.1.3. Level of Insulin Resistance

Insulin resistance (IR) can be known as a form of biological misinformation in the body in which the insulin hormone receptors on the cell membrane are not suitably responding to the insulin, thus the glucose in blood becomes unable to get into cells, which makes a hypoglycaemic reaction. This condition makes the pancreas produces high doses of insulin to attempt to get the glucose out of blood into cells, so decreased of the ability of insulin hormone to adjust and to signal changes the levels of glucose in the blood and possibly grows IR (Al-Fartosy *et al.,* 2020). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was utilized as a substitute measure of insulin resistance in our study. Although HOMA-IR was not the gold standard for assessment of insulin sensitivity, but it was a clinically useful index (Cicero *et al.*, 2015).

Data obtained in the present study showed a high significant increase ($p<0.01$) in HOMA-IR level in T2DM patients with and without DN (Men: 12.41±1.31, 11.74±1.11 vs. 2.98±0.58; Women: 11.54±0.55, 10.98±0.82 vs. 2.64±0.41) as compared to healthy group, Table (3.7) and Figure (3.4).

Table (3.7): Levels of HOMA-IR in T2DM patients with or without DN and control group. The values are the Mean ± SD.

	HOMA-IR					
	T2DM Patients				Healthy Controls	
	with DN		without DN			
	Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD	12.41±1.31**	11.54±0.55**	11.74±1.11**	10.98±0.82**	2.98±0.58	2.64±0.41
SE	0.34	0.14	0.29	0.20	0.15	0.10
Range	9.9-14.9	10.5-12.9	9.5-13.8	9.2-12.5	2-4.1	2-3.3
95% CI Lower	8.78	10.01	8.66	8.70	1.36	1.50
95% CI Upper	16.05	13.06	14.82	13.25	4.59	3.77

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

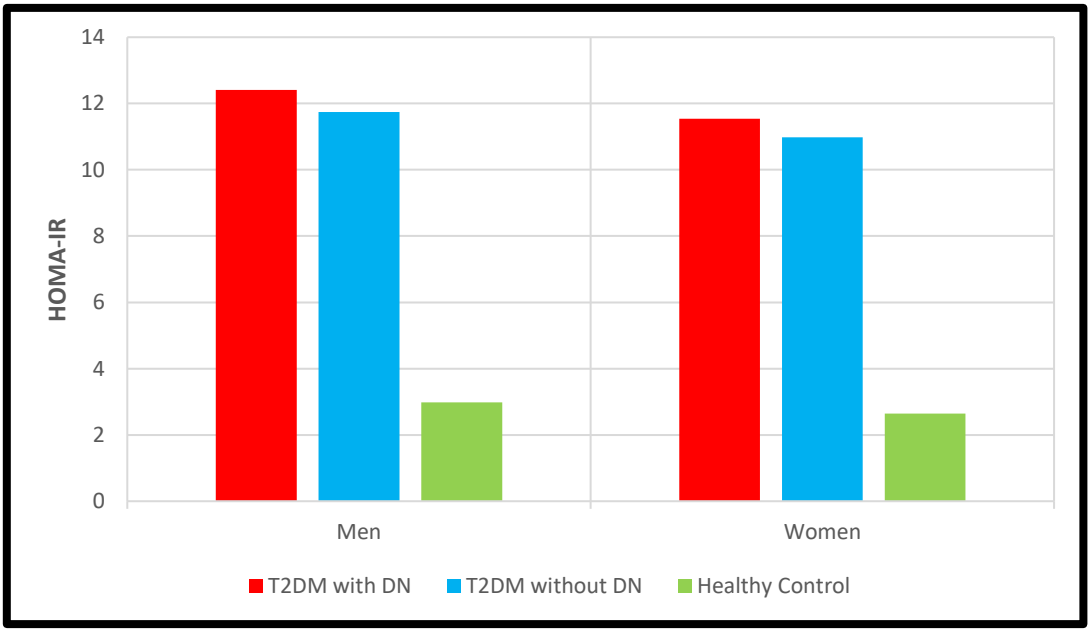


Figure (3.4): Levels of HOMA-IR in T2DM patients with or without DN and control group.

Pancreatic β-cells function may be damaged during adolescence or later. Its deterioration ranges from hyperinsulinemia, secondary to IR, with normal glucose tolerance to β-cells failure with T1DM. When IR is present, or when

insulin secretion is decreased in the later stages of the disease, free fatty acids (FFA's) are released in large amounts followed by an elevated production of glucose, triglycerides (TG's) and very low-density lipoprotein (VLDL) (Budiyani *et al.,* 2017).

In addition, FFA's also decreased insulin sensitivity in muscles by discouraging insulin mediated glucose uptake. On the other hand, elevated blood glucose level, and to some extent circulating FFA, raise insulin secretion and lead to increase hyperinsulinemia. It is obvious that IR causes blood glucose and FFA's levels to rise, thus, worsening the IR and hyperglycemia with released FFA's further rises insulin secretion forming a wicked circle (Xin *et al.,* 2019).

Furthermore, the pivotal role of the fat mass does not rule out the significance of heritability in the evolution of the metabolic syndrome. Environmental factors such as the obesity epidemic, due to the lack of physical exercise and increased caloric intake, is obviously responsible for the present elevation in the incidence of the metabolic syndrome worldwide (Febbraio, 2017**)**. So far, the predilection to get weight is highly individual and determined by genetic factors. It has been speculatd that TG's accumulation in skeletal muscles plays a lineal role in the etiology of IR. The results of several studies have demonstrated that the degree of IR is positively correlated with intramuscular TG's content. So, chronic hyperglycemia and dyslipidemia in T2DM can both produce hurtful effects on β-cell structure and function (Al-Fartosy and Mohammed, 2017c; Angellotti *et al.,* 2018). Although inter-relationships between glucotoxicity and lipotoxicity have not yet been elucidated, it is presumed that glucotoxicity leading to β-cell apoptosis occurs independently of dyslipidemia, whereas lipotoxicity additionally damaging β-cells occurs only in the presence of hyperglycemia. In the case of normoglycemia, elevated FFAs should be readily oxidized in the mitochondrion and should not harm the β-cell of pancreas (Tangvarasittichai, 2015).

Moreover, as DN progress, IR may be accelerating the decline in renal function toward end-stage renal disease. High triglyceride levels are a risk factor for proteinuria development and triglyceride-rich apolipoprotein B-containing lipoproteins clearly promote the progression of renal insufficiency (Mahfouz *et al.*, 2016).

3.4.1.4. Level of Urea

Urea is a small water-soluble molecule with a molecular weight of 60 g/mol which is the end-product of proteins and nitrogen metabolism in humans. It is the compound with the highest level found in the blood of renal failure patients. It is freely filtered by the glomerulus and not secreted by the tubules, but about (40–70%) is passively reabsorbed from the renal tubules (Vanholder *et al.*, 2018).

In the present study, there was a high significant increase ($p<0.01$) in the level of serum urea in T2DM patients with DN as compared to the healthy controls (Men: 59.31±3.97 vs. 29.63±4.05 mg/dL; Women: 53.94±1.80 vs. 25.04±2.28 mg/dL). While, there was a significant increasing ($p<0.05$) in levels of urea in T2DM patients without DN when compared with the healthy group (Men: 36.70±2.59 vs. 29.63±4.05 mg/dL; Women: 30.12±2.11 vs. 25.04±2.28 mg/dL), Table (3.8) and Figure (3.5).

Table (3.8): Levels of urea in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Urea (mg/dL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		59.31±3.97**	53.94±1.8**	36.7±2.59*	30.12±2.11*	29.63±4.05	25.04±2.28
SE		1.03	0.45	0.67	0.51	1.01	0.55
Range		52.87-65.75	51.01-56.86	32.5-40.9	24.9-37.09	23.03-36.23	20.82-31.18
95% CI	Lower	48.29	48.94	29.51	24.26	18.39	18.71
	Upper	70.33	58.93	43.89	35.97	40.87	31.37

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

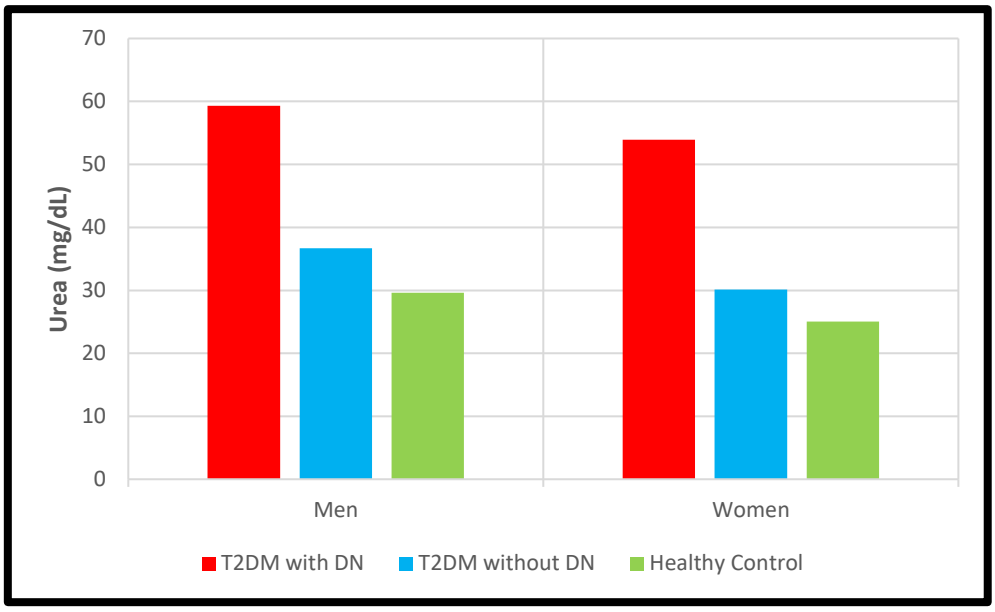


Figure (3.5): Levels of urea in T2DM patients with or without DN and control group.

The significant increasing in serum urea level in T2DM patients with and without DN in our study could be due to high intake of proteins, increase in serum creatinine level, elevated HbA1c, arterial hypertension, dyslipidemia, obesity, low hepatic function, burns, stress and myocardial infections (Sirivole and Eturi, 2017).

Furthermore, hyperglycemia may cause a damage to the kidney or the kidney is not functioning properly by causing irretrievable damage to millions of nephrons - tiny filtering units within each kidney. As a result, kidneys are unable to maintain the fluid and electrolyte homeostasis which lead to elevate urea levels in T2DM patients with and without DN (Tunçdemir *et al.*, 2018).

Moreover, measurement of serum urea is easily available test for this purpose which can assist in detection and prevention diabetic kidney disease at an early stage and can limit the progression to ESRD (Bamanikar *et al.*, 2016).

3.4.1.5. Level of Creatinine

Serum creatinine is primarily a metabolite of creatine, almost all of which is located in skeletal muscle. The amount of creatine per unit of skeletal muscle

mass is consistent and the breakdown rate of creatine is also consistent. Thus, serum creatinine concentration is very stable and a direct reflection of skeletal muscle mass. Creatinine is filtered by the glomerulus, and a small amount is also secreted into the glomerular filtrate by the proximal tubule (Mahmoud *et al.*, 2017).

In the present study, there was a high significant increase ($p<0.01$) in the level of serum creatinine in T2DM patients with DN (Men: 1.77±0.19 vs. 0.93±0.05 mg/dL; Women: 1.43±0.05 vs. 0.81±0.05 mg/dL) as compared to the healthy controls. While, there was a significant increasing ($p<0.05$) in levels of creatinine in T2DM patients without DN, when compared with the healthy group (Men: 1.15±0.08 vs. 0.93±0.05 mg/dL; Women: 0.97±0.05 vs. 0.81±0.05 mg/dL), Table (3.9) and Figure (3.6).

Table (3.9): Levels of creatinine in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Creatinine (mg/dL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		1.77±0.19**	1.43±0.05**	1.15±0.08*	0.97±0.05*	0.93±0.05	0.81±0.05
SE		0.05	0.01	0.02	0.01	0.01	0.01
Range		1.38-1.96	1.35-1.5	1.01-1.29	0.89-1.05	0.85-1	0.73-0.89
95% CI	Lower	1.24	1.29	0.93	0.83	0.79	0.67
	Upper	2.30	1.56	1.37	1.11	1.06	0.95

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

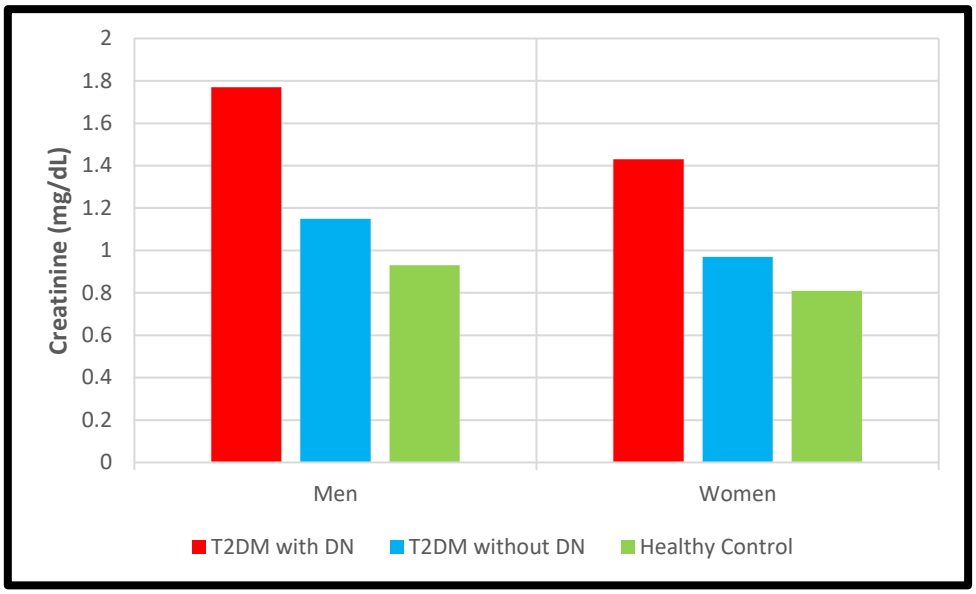


Figure (3.6): Levels of creatinine in T2DM patients with or without DN and control group.

The significant increasing in serum creatinine level in T2DM patients with and without DN in our study could be attributed to hyperglycemia in uncontrolled diabetics, high muscle mass, high protein meal intake, arterial hypertension, dyslipidemia, obesity and race, malnutrition, prescribed medication, elevated microalbuminuria, elevated HbA1c, and renal damage. Also, our results illustrated that creatinine level is higher in men than women and this may be due to high blood glucose levels and high muscle mass in men compared to women (Sirivole and Eturi, 2017).

Furthermore, serum creatinine is filtered by the glomerulus; therefore, serum creatinine level is used as an indirect measure of glomerular filtration. So, any decreasing in glomerular filtration rate (GFR) could lead to rise in serum levels of creatinine. Raised serum creatinine levels and reduced GFR has become fairly reliable indicators of kidney dysfunction (El-Zamar *et al.*, 2019).

From the other hand, serum creatinine is one of the simplest ways to assess the kidney functional in T2DM patients with and without DN because it accumulates in the body in cases of renal dysfunction thus raising their levels in the blood. Serum creatinine is a more sensitive index of kidney function

compared to serum urea level. This is because creatinine fulfils most of the requirements for a perfect filtration marker (Elnajjar *et al.,* 2016).

Moreover, estimation of renal function tests via creatinine is simple, reliable, economic and sensitive that can now be considered as an adjunct in the management and long duration treatment of T2DM disorder (Bamanikar *et al.,* 2016).

3.4.1.6. Level of GFR

Glomerular filtration rate (GFR) is an important indicator of the filtering capacity of kidneys and is considered the best overall index of renal function currently used. Estimated glomerular filtration rate (eGFR) is the most important variable in the assessment of patients with suspected or known kidney disease in clinical practice. Rigorous assessment of GFR requires the measurement of an ideal filtration marker, defined as a substance that is freely filtered by the kidney, not bound to plasma proteins, non-toxic and does not undergo metabolism, tubular secretion or absorption such as creatinine. GFR measures the rate at which the kidneys' two million glomeruli filter plasma in order to process it and remove waste products from it (Sur, 2016; Jayaraman *et al.,* 2017).

In the present study, there was a high significant decrease ($p<0.01$) in the level of GFR in T2DM patients with DN (Men: 43.24±6.92 vs. 91.19±6.86 mL/min/1.73m^2; Women: 40.40±2.17 vs. 80.11±7.22 mL/min/1.73m^2) as compared to the healthy controls. While, there was a significant decreasing ($p<0.05$) in levels of GFR in T2DM patients without DN, when compared with the healthy group (Men: 70.83±7.32 vs. 91.19±6.86 mL/min/1.73m^2; Women: 64.28±4.91 vs. 80.11±7.22 mL/min/1.73m^2), Table (3.10) and Figure (3.7).

Table (3.10): Levels of GFR in T2DM patients with and without DN and control group. The values are the Mean ± SD.

		GFR (mL/min/1.73m²)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		43.24±6.92**	40.4±2.17**	70.83±7.32*	64.28±4.91*	91.19±6.86	80.11±7.22
SE		1.79	0.54	1.89	1.19	1.72	1.75
Range		36.8-58	37-44.1	60-83.8	56.6-72.9	80.7-103.1	69.2-92.9
95% CI	Lower	24.03	34.38	50.51	50.65	72.14	60.07
	Upper	62.45	46.42	91.15	77.91	110.23	100.15

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

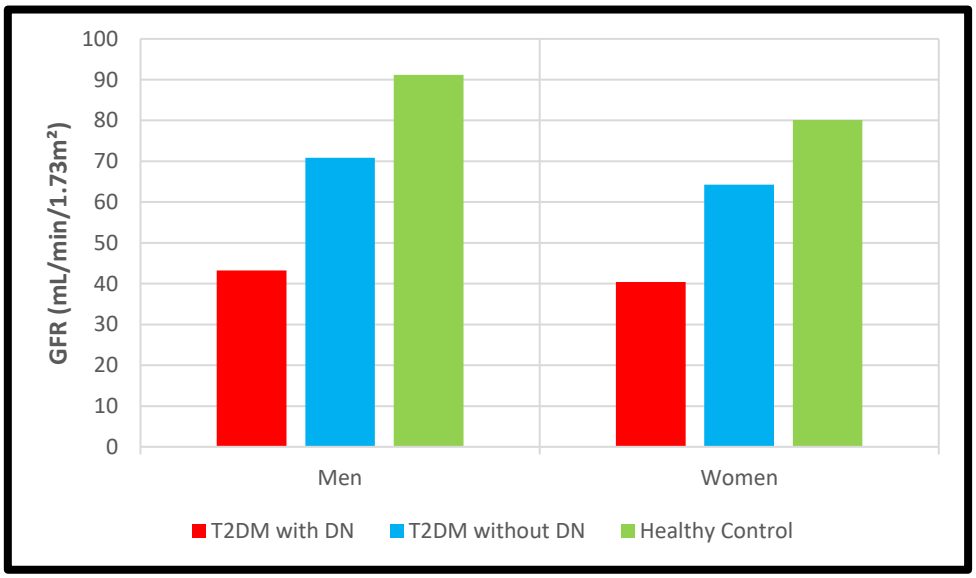


Figure (3.7): Levels of GFR in T2DM patients with or without DN and control group.

The declining in GFR level in T2DM patients with and without DN in our study could be due to renal injury, increased urinary Alb excretion, hypoalbuminemia and decreased haemoglobin levels (Mahmoud *et al.*, 2017).

Furthermore, guidelines recommend that clinical laboratories compute and report estimated GFR by using estimating equations, such as the Modification of Diet in Renal Disease (MDRD) corrected for Body Surface Area (BSA) equation. That's because it was re-expressed for use with a standardized serum creatinine assay, allowing GFR estimates to be reported in clinical practice by using

standardized serum creatinine. So, the decreasing in GFR level in T2DM patients with and without DN in our study could be attributed to the variables in MDRD equation such as elevated creatinine level, sex, older age and ethnicity (Lebherz-Eichinger *et al.,* 2015).

Moreover, serum creatinine level does not increase significantly until the GFR is reduced to less than 50% of its normal value because of increased tubular secretion of creatinine. Thus, GFR is usually accepted as the best overall estimate of kidney function and therefore is commonly used to evaluate onset and progression of chronic kidney disease (CKD) in various groups including diabetes (Drosos *et al.,* 2018).

3.4.1.7. Level of CrCl

Creatinine clearance (CrCl) is the volume of blood plasma cleared of creatinine per unit time. It is a rapid and cost-effective method for the measurement of renal function. Both CrCl and GFR can be measured using the comparative values of creatinine in blood and urine. The CrCl rate approximates the calculation of GFR since the glomerulus freely filters creatinine. However, it is also secreted by the peritubular capillaries, causing CrCl to overestimate the GFR by approximately 10% to 20%. However, because a small amount of creatinine is released by the filtering tubes in the kidneys, creatinine clearance is not exactly the same as the GFR. In fact, creatinine clearance usually overestimates the GFR, particularly in patients with advanced kidney failure (Seki *et al.,* 2019).

In the present study, there was a high significant decrease ($p<0.01$) in the level of CrCl in T2DM patients with DN (Men: 66.53±8.83 vs. 130.31±12.13 mL/min; Women: 62.88±5.31 vs. 126.06±8.06 mL/min) as compared to the healthy controls. While, there was a significant decreasing ($p<0.05$) in levels of CrCl in T2DM patients without DN, when compared with the healthy group

(Men: 96.07±10.91 vs. 130.31±12.13 mL/min; Women: 99.76±9.71 vs. 126.06±8.06 mL/min), Table (3.11) and Figure (3.8).

Table (3.11): Levels of CrCl in T2DM patients with and without DN and control group. The values are the Mean ± SD.

		CrCl (mL/min)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		66.53±8.83**	62.88±5.31**	96.07±10.91*	99.76±9.71*	130.31±12.13	126.06±8.06
SE		2.28	1.33	2.82	2.36	3.03	1.95
Range		58-85	54-71	81-113	85-116	112-150	113-139
95% CI	Lower	42.02	48.13	65.78	71.81	96.64	103.68
	Upper	91.05	77.62	126.35	126.72	163.99	148.43

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

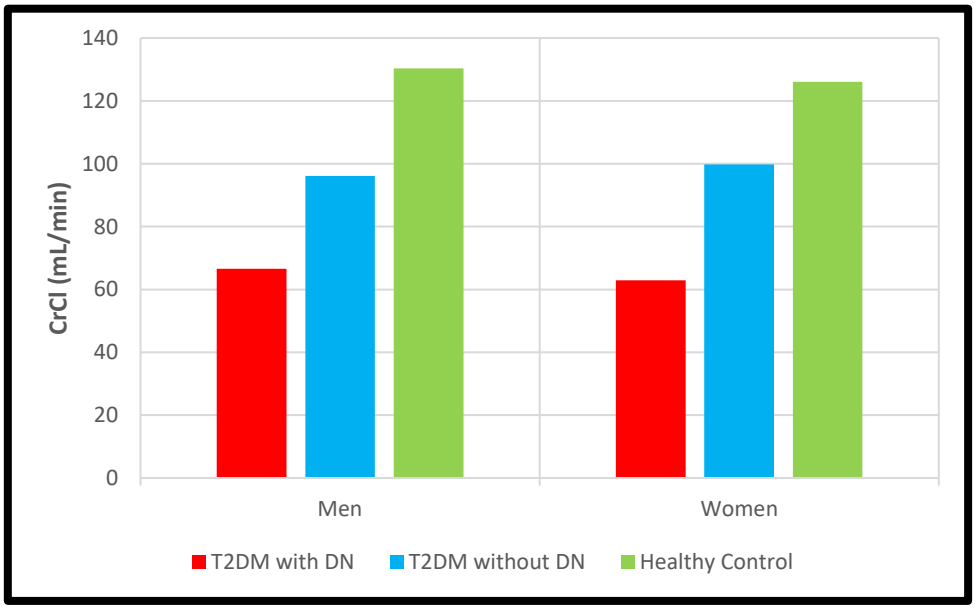


Figure (3.8): Levels of CrCl in T2DM patients with or without DN and control group.

The declining in CrCl level in T2DM patients with and without DN in our study might be due to renal injury, deteriorating kidney function, race, increased urinary Alb excretion and consuming some drugs such as cimetidine, trimethoprim and cephalosporins (Kim *et al.*, 2019).

Furthermore, CrCl was calculated by the Cockcroft-Gault equation. So, the declining in CrCl level in T2DM patients with and without DN in our study may

be because of the variables in this equation such as elevated creatinine level, sex, older age and weight (Chen *et al.,* 2016).

From the other hand, obstruction within the kidney or dysfunction from another disease such as congestive heart failure may play a pivotal role in decreasing CrCl level in T2DM patients with and without DN (Makris and Spanou, 2016).

Moreover, CrCl overestimates GFR due to the secretion of creatinine from the tubules in normal individuals. In patients with CKD, there is increased extra-renal and decreased urinary elimination of creatinine leading to overestimation of GFR from serum creatinine (Raman *et al.,* 2017).

3.4.1.8. Level of Hcy

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the metabolism of methionine. It manifests direct toxic effects on endothelial cells and indirect ones on normal methylation in endothelial cells. This contributes to enhanced production of free radicals, stimulates proliferation of smooth muscle cells and changes of platelet activity. Elevated levels of homocysteine are toxic to vascular endothelium, inducing endothelial dysfunction and contributing to development of atherosclerosis independent of standard CVD risk factors in diabetic and nondiabetic subjects. Hcy may be involved in the development of glomerular and interstitial renal disease proportional to plasma homocysteine levels which cause a progression of renal dysfunction is nitric oxide dependent endothelial dysfunction via reduced activity of the enzyme involved in asymmetric dimethylarginine metabolism (Cvetković *et al.*, 2015).

In the present study, there was a high significant increase ($p<0.01$) in the level of Hcy in T2DM patients with and without DN (Men: 23.26±2.77, 18.27±3.97 vs. 10.89±2.21 nmol/mL; Women: 19.05±1.61, 17.62±3.87 vs. 10.78±2.19 nmol/mL) as compared to the healthy controls, Table (3.12) and Figure (3.9).

Table (3.12): Levels of Hcy in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Hcy (nmol/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		23.26±2.77**	19.05±1.61**	18.27±3.97**	17.62±3.87**	10.89±2.21	10.78±2.19
SE		0.72	0.40	1.03	0.94	0.55	0.53
Range		20.76-29.02	14.73-23.81	11.83-24.71	11.3-23.94	8.27-15.16	8.71-15.24
95% CI	Lower	15.57	14.58	7.25	6.88	4.75	4.70
	Upper	30.95	23.52	29.29	28.36	17.02	16.86

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

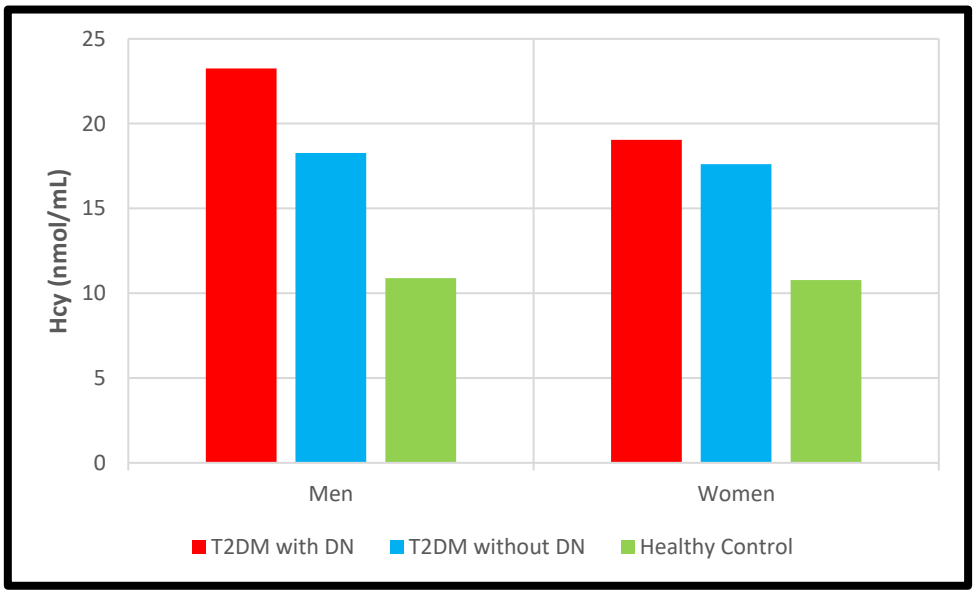


Figure (3.9): Levels of Hcy in T2DM patients with or without DN and control group.

The significant increasing in Hcy level in T2DM patients with and without DN in our study could be attributed to obesity, hyperuricemia, diabetic macroangiopathy, inflammation, elevated levels of CRP and IL-6, impaired adrenal function, oxidative stress, older age, elevated Cys C and creatinine levels, elevated UACR, coronary artery disease, heart attack, high blood pressure, increased cholesterol, hypertriglyceridaemia, malnourishment and malabsorption, elevated BMI and HbA1c levels, occlusive vascular disease, thrombosis, stroke, increased Alb excretion rate, methionine loading, medical

conditions such as cardiovascular disease, the rare hereditary disease homocystinuria, the methylenetetrahydrofolate reductase polymorphism genetic traits, increased glutathione peroxidase activity, intact pancreatic β-cell function, inhibition of methionine synthase activity by nitrous oxide (N_2O), myocardial infarction, cerebral and peripheral vascular diseases, neurodegenerative diseases, liver fibrosis and elevated apolipoprotein B (Kumawat *et al.,* 2016; Rujaswini *et al.,* 2018).

Furthermore, the significant elevation in Hcy level in T2DM patients with and without DN in our study might be due to insulin resistance. Insulin resistance has been hypothesized to play an important role in the development of atherosclerotic disease through hypertension, dyslipidemia and hyperglycemia. Insulin may affect activities of enzyme involved in homocysteine metabolism cystathione β-synthase (CBS) and 5, 10-methylenetetrahydrofolate reductase (MTHFR), which are key enzymes of homocysteine transsulfation and remethylation pathways, respectively. Hence, hyperhomocysteinemia promotes insulin resistance by inducing endoplasmic reticulum stress, elevating glucose output and upregulating phosphoenolpyruvate carboxykinase (PEPCK). This mechanism leads to raise Hcy levels in blood which may exert an atherothrombotic effect through increasing oxidative stress, which may induce endothelial dysfunction (Platt *et al.,* 2017). Also, the insufficiency or malabsorption of B vitamins may lead to elevate Hcy level because they drive Hcy metabolism, with 5-methyltetrahydrofolate serving as substrate for B12-dependent methionine (MS) in the remethylation of homocysteine back to methionine and B6 (as pyridoxal-5-phosphate) as a cofactor for cobalamin-dependent enzyme methionine synthase (CBS) in the transsulfuration pathway (Sheweita *et al.,* 2011).

From the other hand, the significant raising in Hcy level in T2DM patients with and without DN in our study could be due to impaired renal function. The

kidney is a major site for removal and metabolism of Hcy. The main source of Hcy is adenosylmethionine-dependent methylation of guanidoacetate to form creatine and its anhydride creatinine. Renal function plays a central role for clearance of both creatinine and Hcy. Serum Hcy concentrations in diabetic patients can be affected by both glomerular hyper- and hypofiltration, which can respectively decrease and increase the Hcy concentrations. Hcy is ultra-filtrated through the glomeruli, almost completely reabsorbed in the tubuli and degraded in kidney tissue by transmethylation and transsulfuration. Decreased renal clearance of homocysteine results in homocysteinemia. However, reduced glomerular filtration rate (GFR) accompanies microalbuminuria (MAU) in the late phase of diabetic nephropathy, and reduction of GFR causes elevation of plasma Hcy levels (Aouda and Hamza, 2017).

Moreover, treatment with some drugs such as anti-diabetic therapy like metformin and glitazones could cause the significant elevation in Hcy level in T2DM patients with and without DN. Additionally, other drugs may also elevate Hcy levels such synthetic hormonal contraceptives, anti-carcinogenic agents such as methotrexate and sulfasalazine which used for treatment of intestinal inflammatory disorders, anticonvulsants given for epilepsy such as carbamazepine and phenytoin and immunosuppressive agents such as cyclosporine. These drugs may lead to lowering of serum vitamin B12 and folate, which act as coenzyme and substrate for homocysteine remethylation to methionine, resulting in serum homocysteine accumulation (Shin and Baik, 2016).

3.4.1.9. Level of FA

Fructosamine (FA) is a ketoamine formed in a two-step process involving the reaction of the carbonyl group of glucose to the amino group of protein while the second step involves the transformation of the non-reversible Amadori rearrangement to the corresponding ketoamine. The ketoamine formed is FA and

is considered the most used alternative to HbA1c. Fructosamine is considered a reliable indicator of glycemic control (Soriano and Aquino, 2017).

In the present study, there was a high significant increase ($p<0.01$) in the level of FA in T2DM patients with and without DN (Men: 0.47±0.15, 0.36±0.08 vs. 0.26±0.03 mmol/L; Women: 0.42±0.07, 0.34±0.07 vs. 0.25±0.04 mmol/L) as compared to the healthy controls, Table (3.13) and Figure (3.10).

Table (3.13): Levels of FA in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. FA (mmol/L)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		0.47±0.15**	0.42±0.07**	0.36±0.08**	0.34±0.07**	0.26±0.03	0.25±0.04
SE		0.04	0.02	0.02	0.02	0.01	0.01
Range		0.33-0.97	0.32-0.55	0.22-0.50	0.22-0.46	0.21-0.31	0.21-0.42
95% CI	Lower	0.06	0.22	0.14	0.15	0.18	0.14
	Upper	0.89	0.61	0.58	0.54	0.35	0.36

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

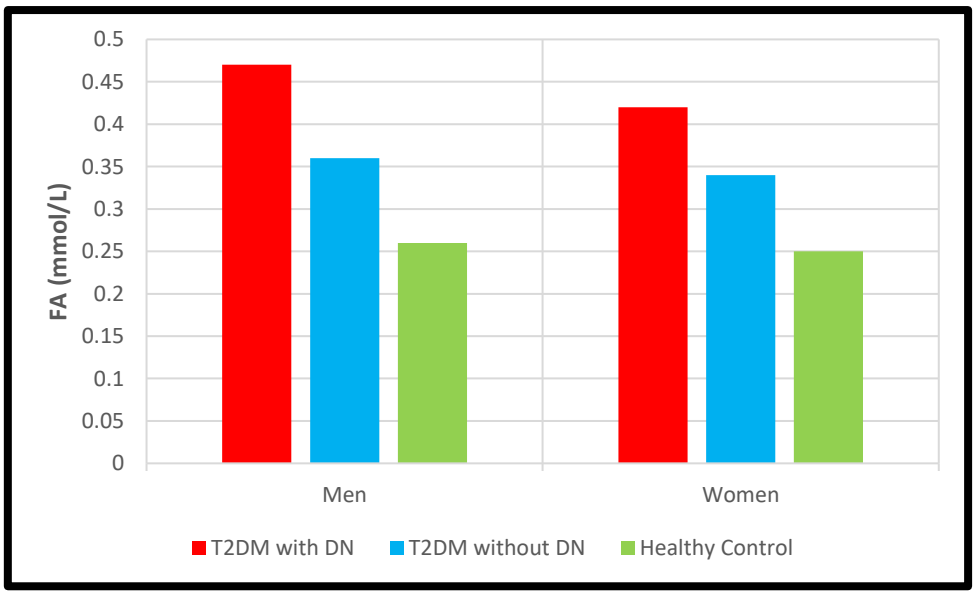


Figure (3.10): Levels of FA in T2DM patients with or without DN and control group.

The significant increasing in FA level in T2DM patients with and without DN in our study could be due to elevated HbA1c levels, older age, sex, black

race/ethnicity, hypertension, elevated liver markers (ALT, AST, or GGT), parental history of diabetes mellitus, low HDL, low kidney function (GFR declining), elevated urea level, high triglycerides and elevated cholesterol (Selvin *et al.*, 2015; Gupta and Khurana, 2019).

Furthermore, the elevation in FA level in T2DM patients with and without DN might be attributed to protein losing states such as nephrotic syndrome, diminished protein production (ie, hepatic cirrhosis), physiologic or pathologic conditions linked to hypoproteinemia (ie, pregnancy or malnutrition) and immunoglobulins (Danese *et al*., 2015). It is possible that protein metabolism is altered in obese adults and may be affected by inflammation. C-reactive protein is known to be highly associated with body mass index and is lowered by weight loss. In addition to alterations in protein metabolism, other conditions that may affect the interpretation of FA levels include liver disease, hyperuricemia, acute illness or infection (Selvin *et al.*, 2015).

In addition, hyperglycemia could be considered as another possible cause for the significant increasing in FA level in T2DM patients with and without DN. The risk of secondary complications in patients with T2DM is highly associated to the chronic level of blood glucose. Nonenzymatic glycosylation is accelerated in hyperglycemic conditions and chronic complications related to diabetes. The products of nonenzymatic glycation accumulate in kidney tissues and play an important role in diabetic nephropathy. So, to prevent the progression of diabetic complications, it is very necessary to reduce and maintain blood glucose levels under the physiological range. Hence, it is important to monitor the blood glucose level time to time and to prevent the further processing of diabetes complications, has led to the widespread use of a marker which can give the short time glycemic status of patients such as FA rather than HbA1c (Neelofar and Ahmad, 2017).

Moreover, FA can provide information on blood glucose levels over the forgoing 2-4 weeks therefore being a short-term marker. This results from a more

rapid turnover of glycated proteins, in contrast to HbA1c that is dependent on erythrocytes turnover taking about 120 days, therefore not being affected by erythrocyte or hemoglobin characteristics (Ribeiro *et al.*, 2016).

Finally, FA measurement had shown many advantages as compared with glucose and HbA1c: (a) FA can accurately reflect short-term changes in glycemia that corresponds to the half-life of Alb. (b) The patients in whom the HbA1c values are falsely low level ongoing haemolytic anaemia associated with Myelodysplastic syndrome. FA is an alternate indicator of glycemic control that has proven utility in situations when HbA1c is less accurate. (c) HbA1c levels must be interpreted with caution in patients with hematologic diseases that change RBC survival lifetime. Nevertheless, FA is unaffected by disorders of red blood cells or haemoglobin, and this test is useful in patients with blood loss or haemolytic anaemia. (d) FA may be reliably measured irrespective of fasting or non-fasting status. (e) FA provides a significantly better parameter for estimating glycemic control and is associated with morbidity (hospitalizations and infections) in diabetic patients on hemodialysis. (f) FA enables intervention, improves diabetes control, and provides a more accurate indicator for ongoing glycemic control. (g) FA measurement is rapid, inexpensive, precise and technically simple (Chen *et al.*, 2016).

3.4.1.10. Level of Cys C

Cystatin C, a 13 kD protein, is produced by a housekeeping gene in all nucleated cells. This cysteine protease inhibitor is freely filtered by the glomerulus without stearic restrictions and does not appear to be secreted by the renal tubules. Serum levels of Cystatin-C are a more precise test of kidney function (as represented by the GFR) than serum creatinine levels. It has been suggested to be closer to the “Ideal” endogenous marker (Gupta *et al.*, 2017).

In the present study, there was a high significant increase ($p<0.01$) in the level of CysC in T2DM patients with DN (Men: 1.36±0.35 Vs. 0.79±0.07 µg/mL;

Women: 1.28±0.28, vs. 0.74±0.16 µg/mL) as compared to the healthy controls. While, there was a significant increasing ($p<0.05$) in levels of Cys C in T2DM patients without DN, when compared with the healthy group (Men: 1.25±0.26 vs. 0.79±0.07 µg/mL; Women: 1.21±0.21 vs. 0.74±0.16 µg/mL), Table (3.14) and Figure (3.11).

Table (3.14): Levels of Cys C in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Cys C (µg/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		1.36±0.35**	1.28±0.28**	1.25±0.26*	1.21±0.21*	0.79±0.07	0.74±0.16
SE		0.09	0.07	0.07	0.05	0.02	0.04
Range		0.89-2.39	0.91-1.81	0.95-1.75	1.01-1.67	0.77-1.05	0.59-1.01
95% CI	Lower	0.39	0.50	0.53	0.62	0.60	0.29
	Upper	2.33	2.05	1.97	1.79	0.99	1.18

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

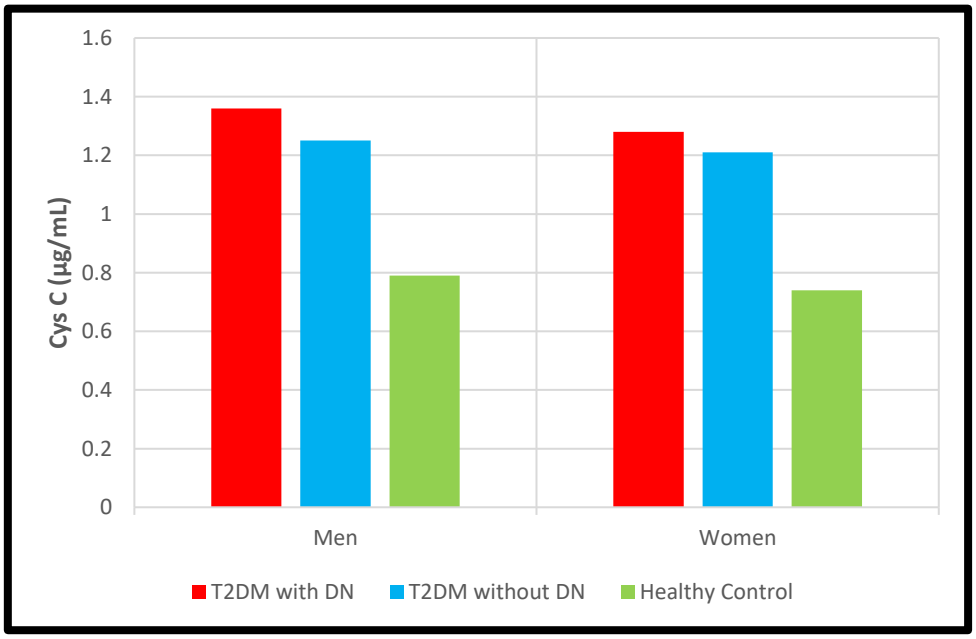


Figure (3.11): Levels of Cys C in T2DM patients with and without DN and control group.

In the current study, the elevation in Cys C level in T2DM patients with and without DN might be due to glucocorticoid therapy, heart failure states, increased

left ventricular mass and its concentricity, higher C-reactive protein, higher white blood cell count, lower serum Alb, liver disease, diabetes mellitus, extreme age, obesity, sex, body mass, hypertension, infection with human immunodeficiency virus (HIV), antihypertensives and insulin treatments, coronary heart disease and steroids (Vichova *et al.,* 2016; Miliku *et al.,* 2017; Kim *et al.,* 2016; Wattanavaekin *et al.,* 2018; Paramanick *et al.,* 2016; Kumar and Kumar, 2015, Elnokeety *et al.,* 2017).

Furthermore, significantly increased level of Cys C in T2DM patients with and without DN could be due to progression of CKD from stages I to III. Also, rise in serum Cys C paralleled progression from normoalbuminuria to microalbuminuria, thereby revealing a positive correlation between serum cystatin C and albumin creatinine ratio (ACR). So, serum cystatin C is a useful in early detection of diabetic nephropathy as it reflects reduction in GFR as well as rise in ACR (Bashier *et al.,* 2015).

From the other hand, the cystatin C levels of serum and urine increased with increasing degree of albuminuria, reaching higher levels in macroalbuminuric patients, indicating that levels of cystatin C in serum/urine might be a useful biomarker for predicting progression of type 2 DN. So, cystatin C measurement in urine and serum is a useful, practical, non-invasive tool for the evaluation of renal involvement in the course of diabetes, especially in normoalbuminuric patients. Thus, being elevated in serum or urine, even before the appearance of albuminuria and creatinine-based estimates, cystatin C may serve as a more sensitive early renal marker for predicting onset of nephropathy in patients with normoalbuminuria (early nephropathy) and its progression in type 2 diabetic patients. In addition, the elevation in Cys C level in T2DM patients with and without DN could be attributed to the tubular phase before glomerular manifestation. This suggests that the cystatin C levels of serum and urine are

related to subclinical tubular impairment and can be an earlier measurable marker of renal involvement before onset of albuminuria (Fiseha, 2015).

Moreover, there are another possible and acceptable explanations for this significantly increasing in Cys C level in T2DM patients with and without DN such as insulin resistance and inflammation, and this may explain the association between cystatin C and cardiovascular disease in type 2 diabetes (Bashier *et al.,* 2015). High levels of cystatin C have been also associated with a hypermethabolic status. Given the various possible mechanisms responsible for the changes in cystatin C levels, it is conceivable that, depending on the clinical setting considered, increased cystatin C levels may variously reflect renal dysfunction, the effects of heart failure as a result of hypertension and/or fluid retention, or CAD associated with inflammation and atherosclerosis (Dandana *et al.,* 2014). The stable production rate of cystatin C and its lack of secretion by the tubular epithelial cells indicated that the renal function is the major determinant of cystatin C and even minimal renal damage will result a significant rise in levels before the appearance of traditional CKD markers (Iacoviello *et al.*, 2015). There is a possibility of introducing cystatin C-based formulas without anthropometric variables to replace creatinine-based equations in predicting GFR (Ajith, 2019).

Finally, there are many advantages of Cystatin-C as a biomarker for detection of renal functions over other available markers because it does not depend on muscle mass, diet, age or gender. In addition, there are no known extra renal routes of elimination and clearance from circulation is only by glomerular filtration. Also, it is sensitive to changes in the so-called creatinine blind GFR range (40-70ml/min/1.73m2). Furthermore, its concentration is not influenced by infections or inflammatory diseases and it can also be used to detect and monitor kidney disease in patients with hepatic disease (Gupta *et al.,* 2017).

3.4.1.11. Level of LC

L-carnitine (LC) is an essential co-factor for fatty acid metabolism and other metabolic pathways, with body stores maintained primarily in skeletal muscle. The majority of the body's LC is supplied in the diet from meats and meat-based foods; however, LC is also synthesized endogenously from lysine and methionine supported by certain vitamins and minerals (Shimizu *et al.*, 2019).

In the present study, there was a high significant decrease ($p<0.01$) in the level of LC in T2DM patients with DN (Men: 29.01±2.58 vs. 47.41±5.34 nmol/mL; Women: 28.25±4.97 vs. 44.97±4.11 nmol/mL) as compared to the healthy controls. While, there was a significant decreasing ($p<0.05$) in levels of LC in T2DM patients without DN, when compared with the healthy group (Men: 37.29±4.49 vs. 47.41±5.34 nmol/mL; Women: 35.39±2.13 vs. 44.97±4.11 nmol/mL), Table (3.15) and Figure (3.12).

Table (3.15): Levels of LC in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. LC (nmol/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		29.01±2.58**	28.25±4.97**	37.29±4.49*	35.39±2.13*	47.41±5.34	44.97±4.11
SE		0.67	1.24	1.16	0.52	1.34	1.00
Range		21.94-36.08	20.15-36.35	30.01-44.57	27.71-39.64	38.71-56.11	38.25-51.69
95% CI	Lower	21.85	14.45	24.83	29.48	32.59	33.56
	Upper	36.17	42.05	49.75	41.3	62.23	56.38

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

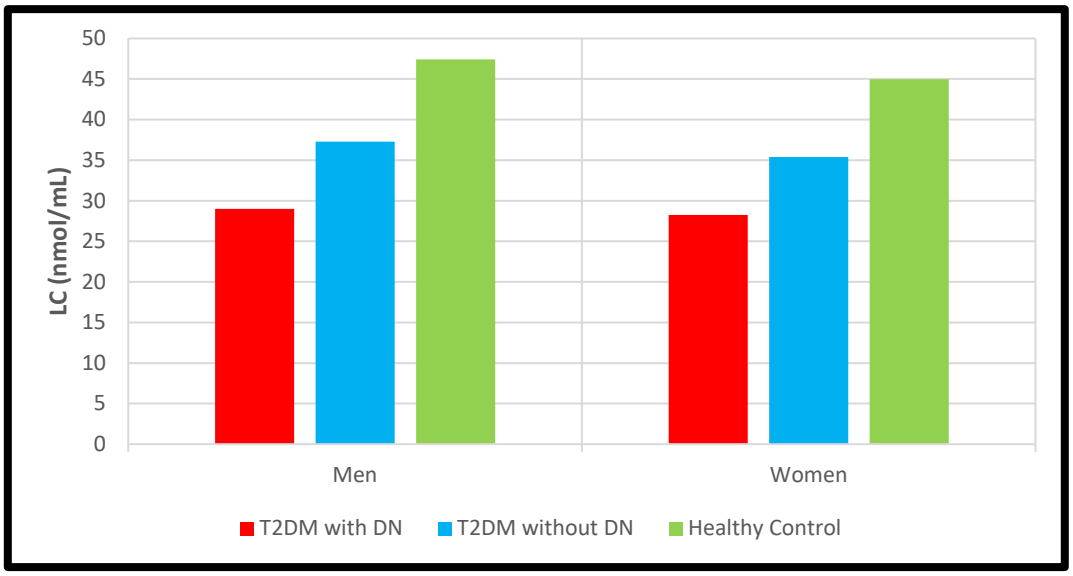


Figure (3.12): Levels of LC in T2DM patients with or without DN and control group.

The significant decreasing in LC level in T2DM patients with and without DN in our study could be due to increased acyl-L-carnitine excretion, decreased renal reabsorption, glomerular inflammation, sclerosis, hyperlipidemia, inflammation, oxidative stress, elevated HbA1c levels and high blood pressure (Bene *et al.,* 2018).

Furthermore, the reduction in some catalysts and co-factors that used by humans to biosynthesize LC from L-lysine and L-methionine via a series of reactions in the liver and, to a lesser extent, in the kidney and brain such as S-adenosylmethionine, α-ketoglutarate, oxygen, ascorbic acid, iron, glycine, vitamin B6 and γ-butyrobetaine hydroxylase could lead to decrease in LC level in T2DM patients with and without DN (Rehman *et al.,* 2017).

From the other hand, the combination of impaired glucose tolerance (IGT) with simple obesity may trigger the acceleration of hepatic ketogenesis in conjunction with an elevated SCAC (short-chain acylcarnitine) and an increasing in carnitine acylation with a reduction in LC levels (Bae *et al.*, 2015).

Moreover, LC is a key substrate in intermediary metabolism and participates in a series of reversible transesterification reactions in which organic acids are

transferred from coenzyme A to the hydroxyl group of LC and vice versa. Acylcarnitines are imported in or exported out of mitochondria and contribute to free fatty acid transport and oxidation, ketogenesis and mitochondrial energy control. In addition, LC, through regulation of availability of free coenzyme A, regulates the activity of key metabolic enzymes, such as pyruvate dehydrogenase and branched-chain alpha-keto acid dehydrogenase. Consequences of LC depletion in T2DM patients with and without DN may therefore involve not only lipid metabolism, but also insulin-mediated pathways of glucose utilisation, regulation of branched chain amino acid catabolism and, ultimately, whole-body protein balance (Bonomini *et al.,* 2019).

3.4.1.12. Level of OPG

Osteoprotegerin (OPG), a soluble glycoprotein composed of 380 amino acid residues, can be found in osteoblasts of the bone, vascular endothelial cells and smooth muscle cells. OPG competes with receptor activator of nuclear factor kappa-B ligands (RANKL) that inhibit osteoclast differentiation and destruct bone absorption (Xia *et al.,* 2015).

In the present study, there was a high significant increase ($p<0.01$) in the level of OPG in T2DM patients with DN (Men: 3.25±0.12 vs. 1.96±0.35 ng/mL; Women: 3.19±0.51 vs. 1.79±0.09 ng/mL) as compared to the healthy controls. While, there was a significant increasing ($p<0.05$) in levels of OPG in T2DM patients without DN, when compared with the healthy group (Men: 2.71±0.15 vs. 1.96±0.35 ng/mL; Women: 2.61±0.24 vs. 1.79±0.09 ng/mL), Table (3.16) and Figure (3.13).

Table (3.16): Levels of OPG in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. OPG (ng/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		3.25±0.12**	3.19±0.51**	2.71±0.15*	2.61±0.24*	1.96±0.35	1.79±0.09
SE		0.03	0.13	0.04	0.06	0.09	0.02
Range		3.04-3.46	2.33-4.01	2.47-2.96	1.93-3.37	1.36-2.5	1.52-2.1
95% CI	Lower	2.92	1.78	2.29	1.94	0.98	1.54
	Upper	3.58	4.61	3.13	3.27	2.93	2.04

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

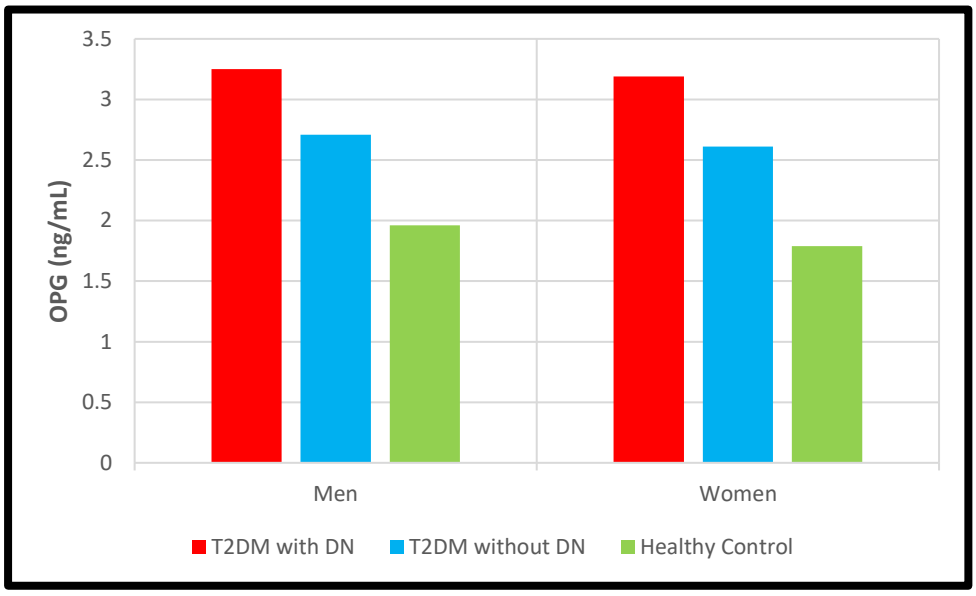


Figure (3.13): Levels of OPG in T2DM patients with or without DN and control group.

The significant elevating in OPG level in T2DM patients with and without DN in our study may be due to ankylosing spondylitis disease, glycolipid metabolic disorder, carotid and peripheral arterial disease, vascular calcification, elevated adiponectin levels, older age, vascular stiffness, the presence of unstable atherosclerotic plaques, acute myocardial infarct, ischemic stroke, cardiovascular mortality, heparin and estrogen treatment, intima-media thickness, brachial flow-mediated vasodilatation, elevated left ventricular mass and hypertrophy, elevated levels of serum troponin I, elevated CRP levels, elevated peripheral blood

mononuclear cells and macrophages, elevated urinary albumin excretion rate (UAER), elevated bone turnover, diabetic angiopathy, diabetic maculopathy, inflammatory bowel disease, ulcerative colitis, Crohn's disease, liver fibrosis, elevated systolic blood pressure and endothelial dysfunction (Wang *et al.,* 2019; El-Saeed *et al.,* 2016; Putri, 2019).

Further, there is another clarification for the significant increasing in OPG level in T2DM patients with and without DN, OPG concentration is increased in the deeper layers of arterial tissue from diabetes patients. This accumulation of OPG may be a part of the generalized matrix changes seen in the arterial wall in diabetes. The arterial accumulation of OPG could relate to the fact that production of the molecule from vascular smooth muscle cells (VSMC) is highly influenced by important factors in the diabetic milieu like pro-inflammatory, hormonal, and metabolic factors such as hypercholesterolemia, obesity and polycystic ovarian syndrome. Thus, TNF-α, interleukin-1 (IL-1), IL-4 and peroxisome proliferator-activated receptor (PPAR) agonists' upregulate the synthesis of OPG (de Ciriza *et al.,* 2015).

Furthermore, obesity could increase the OPG level in T2DM patients with and without DN as OPG expression has been recently confirmed in adipose tissues as well. Abdominal adipose tissue is the largest fat tissue depot in the body and correlates with cardiovascular disease risk, metabolic syndrome and other systemic inflammatory markers and may have an effect on atherosclerosis. Adipose tissue releases several adipokines but also there is increasing evidence that there is a hormonal cross-link between adipose tissue and bone which leading to increase OPG level (Bernardi *et al.,* 2016). In addition, insulin resistance (IR) may have a crucial role in elevating OPG level in T2DM patients with and without DN because it could be the potential mechanism for increased serum lipid levels especially levels of triacylglycerol and this is leading to raise OPG levels (Niu *et al.,* 2015). Also, inflammation may increase OPG level and link it to insulin

resistance. OPG/RANK (Receptor activator of necrosis factor kappa-B)/RANKL (Receptor activator of nuclear factor kappa-B ligand) system is believed to be associated with the regulation of inflammatory and immune responses and directly contributed to the regulation of proinflammatory cytokine production in macrophages. So, it is well established that the OPG/RANK/RANKL system could activate the NF-κB pathway and its downstream players, which are closely related to the pathogenesis of insulin resistance. From the other hand, increased serum OPG levels may be due to an insufficient compensatory self-defensive response to prevent vascular endothelial dysfunction and the progression of atherosclerosis (Duan *et al.,* 2017).

Moreover, elevated creatinine and Cys C levels may raise the OPG level in T2DM patients with and without DN because Cys C is a cysteine proteinase inhibitor that decreases osteoclastogenesis by interfering at a late stage of pre-osteoclast differentiation which leading to decreased protein clearance and decline in renal function and GFR as a potential cause of age-related OPG elevation (Kulcsar-Jakab *et al.,* 2015).

3.4.1.13. Level of SA

Sialic acid (SA) is an essential component of glycoproteins and glycolipids that belongs to the family of acetylated derivatives of neuraminic acid. It has a main role as a co-factor of many cell receptors and is positively associated with most of the serum acute phase reactants (Zhu *et al.,* 2017).

In the current study, there was a high significant increase ($p<0.01$) in the level of SA in T2DM patients with or without DN (Men: 87.68±4.49, 79.46±4.79 vs. 61.08±2.98 mg/dL; Women: 85.78±2.88, 77.31±2.61 vs. 60.39±5.97 mg/dL) as compared with control group, Table (3.17) and Figure (3.14).

Table (3.17): Levels of SA in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. SA (mg/dL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		87.68±4.49**	85.78±2.88**	79.46±4.79**	77.31±2.61**	61.08±2.98	60.39±5.97
SE		1.16	0.72	1.24	0.63	0.75	1.45
Range		80.40-94.96	80.89-90.41	71.69-87.23	69.28-84.51	52.91-69.77	50.63-70.15
95% CI	Lower	75.22	77.78	66.16	70.07	52.81	43.82
	Upper	100.14	93.77	92.76	84.56	69.35	76.96

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

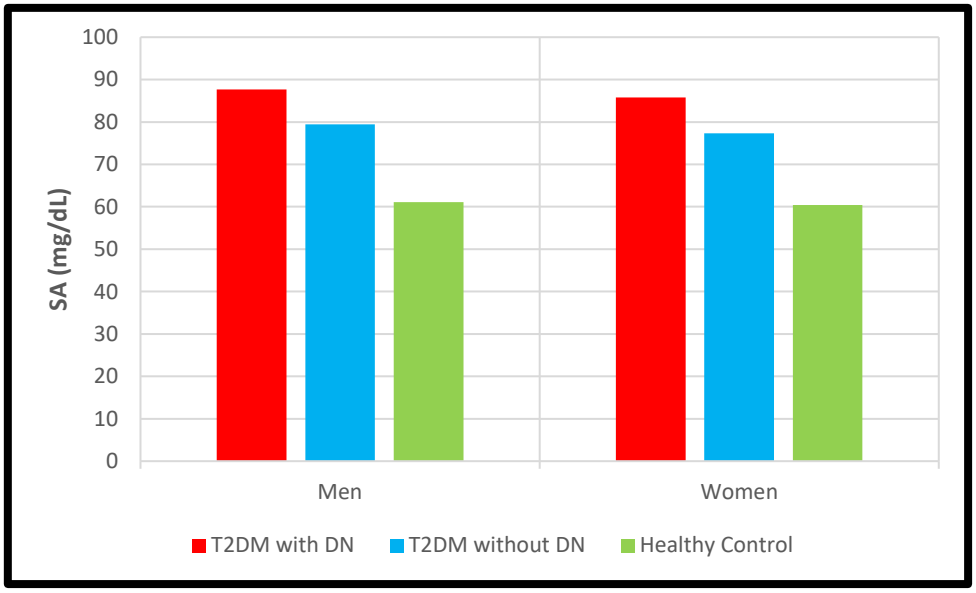


Figure (3.14): Levels of SA in T2DM patients with or without DN and control group.

In the present study, the increased levels of SA in T2DM with and without DN could be attributed to the higher levels of glucose and insulin, IR and obesity, an increase in risk of glucose intolerance, diabetes, renal dysfunction and an increased prevalence of lipid-related abnormalities. Also, some recent studies reported that the percentage of body fat may contribute to the higher levels of SA in blood which clearly observed in patients with T2DM (El-Sayed *et al.*, 2018; Jafri *et al.*, 2017).

Furthermore, the raised level of SA in blood serum of T2DM with and without nephropathy could be attributed to that SA is an important acute phase reactant and maintains the negative charge of renal glomerular basement membrane that is one of the main regulators of membrane permeability. Therefore, vascular endothelium carries a higher level of SA and hence extensive pathological states when there is tissue damage, tissue proliferation and inflammation associated with T2DM accounts for its shedding into the circulation, leading to increased vascular permeability and overall increased SA level (Kumar *et al.*, 2015).

From the other hand, hyperglycemia, obesity and IR could also promote inflammation, and may be factor linking diabetes to the development of diabetic complications. Elevated glucose levels could promote inflammation by increased oxidative stress. Another possibility is that inflammatory response is a result of vascular complications following diabetes. Hence, diabetic vascular complications can cause a severe tissue damage which may stimulates secretion of a large concentrations of local cytokines from cellular infiltrates such as macrophages and endothelial cells. Moreover, this secretion of cytokines may lead to induce an acute phase response with release of acute phase glycoproteins with SA from the liver tissue into the general circulation again leading to increase the level of SA in blood (Ismail *et al.,* 2015). There is another possible and acceptable explanation for this increase in level of SA is that may be result from a difference in the ratio between the two forms of erythrocyte sialidases which are important in maintaining the viability of the erythrocyte and its survival in the circulating blood (Ghosh *et al.,* 2016). A further explanation of our findings, elevated level of SA in DN patients as compared to healthy controls might be resulted from the damage of the vascular endothelial cells of the kidney and it is considered as a newly established potential risk factor for the development of DN. Hence, estimation of serum SA levels may prove to have predictive value and

may have a vital role in prevention of microvascular diseases and their complications in T2DM patients (Jafri *et al.*, 2017).

3.4.2. Determination of Blood Trace Elements

Trace elements play a vital role in human body to perform the functions properly. These elements should be present in the body in appropriate amounts and must be available for reacting with other elements to form critical molecules as well as to participate in various important chemical reactions (Shazia *et al.*, 2012). In this study, trace elements such as, Se, Zn, and Mg were determined in T2MD patients with and without DN and healthy group.

3.4.2.1. Level of Se

Selenium (Se), a trace element, is a major component in glutathione peroxidase (GPx) which is one of the main antioxidant enzymes in the human body and responsible for preventing the production of free radicals, decreasing their activity or destroying them (Al-Fartosy *et al.*, 2017a).

In the current study, there was a high significant decrease ($p<0.01$) in the level of Se in T2DM patients with or without DN (Men: 63.59±3.41, 70.92±1.85 vs. 92.81±1.08 ng/mL; Women: 50.88±1.95, 59.53±1.72 vs. 80.14±3.29 ng/mL) compared with control group, Table (3.18), Figure (3.15).

Table (3.18): Levels of Se in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Se (ng/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		63.59±3.41**	50.88±1.95**	70.92±1.85**	59.53±1.72**	92.81±1.08	80.14±3.29
SE		0.88	0.49	0.48	0.42	0.27	0.80
Range		62.12-67.65	49.27-55.08	69.34-73.64	58.47-64.21	90.05-93.45	74.52-85.86
95% CI	Lower	54.13	45.46	65.78	54.76	89.81	71.01
	Upper	73.06	56.29	76.05	64.31	95.81	89.28

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

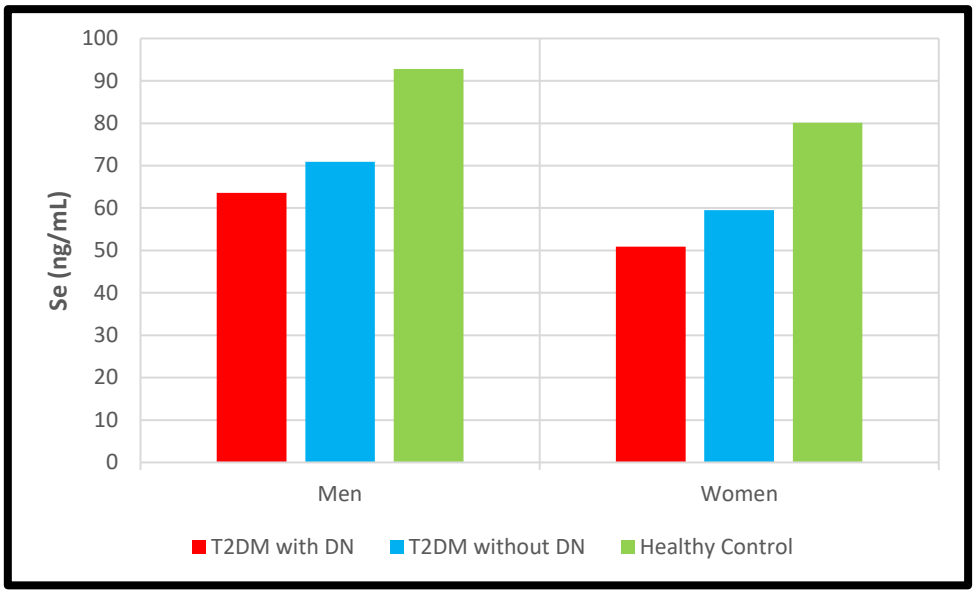


Figure (3.15): Levels of Se in T2DM patients with and without DN and control group.

Selenium (Se) levels were found to be significantly lower in T2DM patients with and without DN as compared to control group. This decrease in Se levels has been described in an earlier study (Onah *et al.*, 2013). Se is known to act as an antioxidant and peroxynitrite scavenger when incorporated into selenoproteins (Blessing *et al.*, 2019). Se is the master element in glutathione peroxidase "GPx" (an active enzyme against oxidative stress) that diminishing the formation of free radicals in the body. This lack in Se levels could participate to oxidative stress and low Se level has been shown to decrease insulin secretion and raised IR in some empirical models, thereby possibly occupying a causal function in the progress and pathogenesis of T2DM. In addition, elevated oxidative stress and glycosylation play a major pathogenic action in diabetic endothelial cell dysfunction in T2DM patients with and without DN (Montali *et al.*, 2015).

Moreover, low level of this element in blood might be an indication of active production of free radical and increased scavenging activity of either Se or GPx (Buha *et al.*, 2017). Experimental evidences from in vitro and in vivo studies in animals suggested that Se may mediate many insulin-like actions and thus enhance insulin sensitivity but this finding is still unconfirmed for patients with T2DM (Ahmed *et al.*, 2015).

3.4.2.2. Level of Zn

Zinc is one of the essential trace elements which are involved in the synthesis, storage, secretion and conformational integrity of insulin. Zn deficiency is associated with many chronic illnesses. Zinc plays an important role in glucose metabolism. It helps in the utilization of glucose by muscle and fat cells. Also, it is required as a cofactor for the function of intracellular enzyme that may be involved in protein, lipid and glucose metabolism. Zinc may be involved in the regulation of insulin receptor initiated signal transudation mechanism and insulin receptor synthesis (Alwan and Hamood, 2017). Zinc is a structural part of key antioxidant enzymes such as superoxide dismutase, and zinc deficiency impairs their synthesis, leading to increased oxidative stress. Zinc has a biphasic effect in that it is required for insulin storage and cellular binding, although high concentrations can lead to a reduction in insulin release (Mohammed *et al.*, 2018).

In the current study, there was a high significant decrease ($p<0.01$) in the level of Zn in T2DM patients with or without DN (Men: 63.59±3.41, 70.92±1.85 vs. 92.81±1.08 µg/mL; Women: 50.88±1.95, 59.53±1.72 vs. 80.14±3.29 µg/mL) as compared with control group, Table (3.19), Figure (3.16).

Table (3.19): Levels of Zn in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Zn (µg/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		0.80±0.26**	0.70±0.22**	0.96±0.16**	0.77±0.18**	1.59±0.30	1.01±0.32
SE		0.07	0.06	0.04	0.04	0.08	0.08
Range		0.50-1.31	0.42-1.12	0.55-1.25	0.53-1.13	1.08-2.06	0.45-1.57
95% CI	Lower	0.08	0.09	0.52	0.27	0.76	0.12
	Upper	1.51	1.32	1.40	1.27	2.42	1.89

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

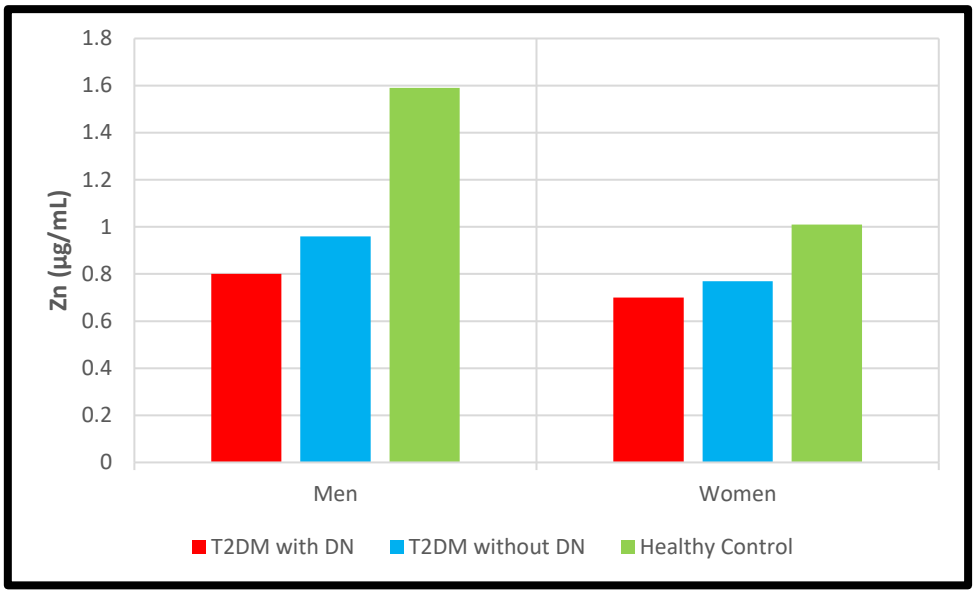


Figure (3.16): Levels of Zn in T2DM patients with or without DN and control group.

Our study indicated that zinc levels in T2DM patients with and without DN were lower than the control group. The possible explanation of the present results comes as the following reasons: In the mammalian pancreas, Zinc is essential for the correct processing, storage, secretion, and action of insulin in beta (β)-cells. Insulin is stored inside secretory vesicles or granules, where two Zn^{2+} ions coordinate six insulin monomers to form the hexameric structure on which maturated insulin crystals are based (Li, 2014). It is also known that like, most other chronic disorders, diabetes increase the excretion of minerals such as zinc in urine than non-diabetics or may be decreased gastrointestinal absorption of zinc (Farooq, 2019). Also, Hyperglycemia in diabetes is usually associated with hyperzincuria, which is of renal origin, and increase urinary loss of Zn^{2+} and decreases of its concentration in total body Zn^{2+} (Feng *et al.*, 2015). Renal tubular defect in handling zinc and glucose-induced, osmotic diuresis are other possibilities. Zn deficiency is associated with metabolic disturbances including impaired glucose tolerance, insulin degradation, and reduced pancreatic insulin content. Furthermore, Zn may improve glycaemia, and a restored Zn status in patients with type 2 diabetes may counteract the deleterious effects of oxidative stress, helping to prevent complication beneficial antioxidant effects in persons

with type 2 diabetes. This is associated with diabetes. Zn has been reported to have is particularly important in the light of the deleterious consequences of oxidative stress in persons with diabetes (Aluwong *et al.*, 2016). Zinc has antioxidant properties; thus, it can stabilize macromolecules against radical induced oxidation (Altoum *et al.,* 2019). Moreover, it plays a key role in the synthesis, secretion and insulin action in both physiological and pathological situations. In addition, recent studies have highlighted zinc's dynamic role as a "cellular second messenger" in the control of insulin signaling and glucose homeostasis. Finally, there was some evidence which shows that zinc acts as an antioxidant. Under Zinc deficiency, free radicals are activated which is due to the impaired antioxidant defence system and due to the imbalances in the production of free radicals and antioxidant defence system. High oxidative stress conditions are created in this situation which is involved in the pathogenesis of diabetes and its related complications (Heidari *et al.*, 2016; Farooq, 2019).

3.4.2.3. Level of Mg

Mg is another trace element which is responsible for maintaining proper body functions. It is required for many enzyme activities and for neuromuscular transmission, and it has a vital role in the immune system, deficiency of this element will lead to hypertension, diabetes, and cardiovascular diseases (Kunutsor *et al.*, 2017).

In the current study, there was a high significant decrease ($p<0.01$) in the level of Mg in T2DM patients with or without DN (Men: 16.71±1.38, 19.93±1.12 vs. 23.42±3.78 µg/mL; Women: 12.95±2.51, 14.94±1.43 vs. 20.15±2.68 µg/mL) as compared with control group, Table (3.20) and Figure (3.17).

Table (3.20): Levels of Mg in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Mg (µg/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		16.71±1.38**	12.95±2.51**	19.93±1.12**	14.94±1.43**	23.42±3.78	20.15±2.68
SE		0.36	0.63	0.29	0.35	0.95	0.65
Range		14.47-18.95	8.71-16.97	18.11-21.75	12.43-17.33	17.06-29.24	17.64-25.65
95% CI	Lower	12.88	5.98	16.82	10.97	12.93	12.71
	Upper	20.54	19.92	23.04	18.91	33.92	27.59

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

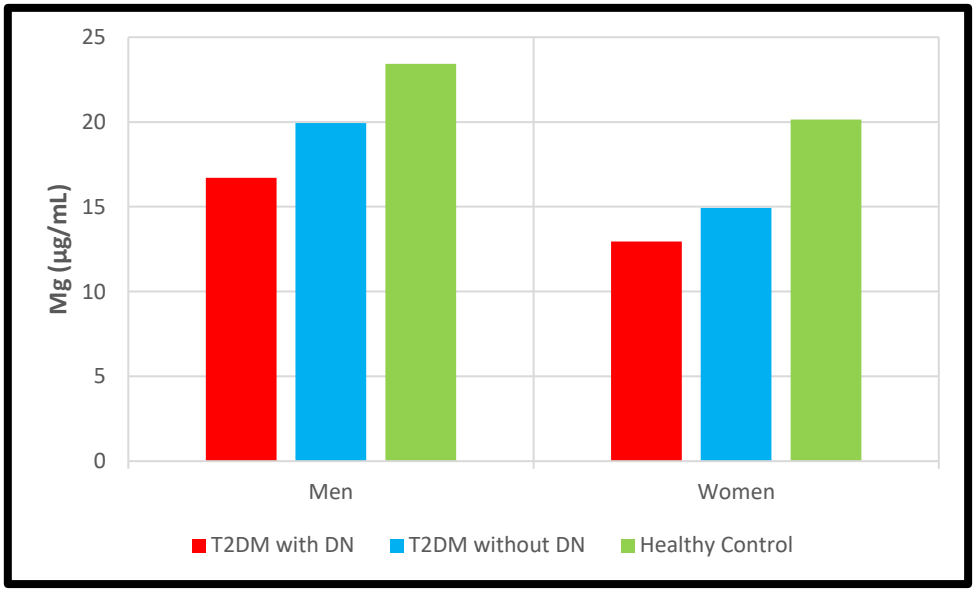


Figure (3.17): Levels of Mg in T2DM patients with or without DN and control group.

In our present study, the serum value of Magnesium showed statistically significant decrease when compared to healthy subjects and T2DM patients with and without DN. Magnesium is necessary for several enzymes that play an important role in glucose metabolism. The hypomagnesaemia in DN is due to poor dietary intake, impaired absorption of magnesium, increased urinary loss due to hyperglycemia, osmotic diuresis, defective Mg reabsorption from renal tubules and loss of plasma protein bound Mg. Magnesium depletion is said to reduce the insulin sensitivity, thereby increasing the risk of secondary

complications. Hyperglycemia leads to decreased cellular Mg levels. Hypomagnesaemia leads to collagen and ADP-induced platelet agreeability and also decreases function of Mg dependent enzymes, kinases and oxidative stress (Al-Jameil *et al.,* 2014). Magnesium deficiency also has a role in the perturbation of lipid metabolism of diabetic patients. Hypomagnesaemia inhibits prostacyclin receptor function, producing an imbalance between prostacyclin and thromboxane effects. Hypomagnesaemia can increase platelet reactivity, increases vascular and adrenal responses to angiotensin II, enhances thromboxane A2 (TXA2) release, and lead to organ damage from free radicals (Khan *et al.,* 2013). Hypomagnesaemia causes dyslipidemia by decreasing activity of lipoprotein lipase, LCAT (Lecithin Cholesterol Acyl Transferase) and increasing HMG CoA reductase enzyme. The lipid changes are attributed to increased Free Fatty Acids flux secondary to insulin resistance (Sharma *et al.,* 2017).

Finally, insulin deficiency and resistance lead to reduced tubular reabsorption of magnesium and ensuing hypomagnesemia favour the onset and progression of diabetic microangiopathy, via a reduction in activity of Na+/K+ ATPase pump (Kumar *et al.,* 2018). So, hypomagnesemia independently prophesies the progression to ESRD in patients with advanced DN (Sakaguchi *et al.,* 2018).

3.4.3. Estimation of Urine Biomarkers

3.4.3.1. Level of Alb

Albumin (Alb), a 65-kDa protein produced in the liver, is the most abundant plasma protein in the body. In normal subjects, a small amount of Alb is filtered in the glomerulus, but almost all of it is reabsorbed by the tubules (Sekulic and Sekulic, 2015).

In the present study, there was a high significant increase ($p<0.01$) in the level of Alb in T2DM patients with DN (Men: 3.25±0.12 vs. 1.96±0.35 ng/mL; Women: 3.19±0.51 vs. 1.79±0.09 ng/mL) as compared to the healthy controls.

While, there was a significant increasing ($p<0.05$) in levels of Alb in T2DM patients without DN, when compared with the healthy group (Men: 2.71±0.15 vs. 1.96±0.35 ng/mL; Women: 2.61±0.24 vs. 1.79±0.09 ng/mL), Table (3.21) and Figure (3.18).

Table (3.21): Levels of Alb in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. Alb (µg/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		29.58±4.62**	28.54±5.25**	11.18±3.11*	10.68±2.59*	6.17±2.13	5.84±2.06
SE		1.19	1.31	0.80	0.63	0.53	0.50
Range		22.09-37.07	19.99-37.09	6.14-16.22	6.44-14.92	4.26-10.38	4.11-13.83
95% CI	Lower	16.75	13.97	2.55	3.49	0.26	0.13
	Upper	42.41	43.11	19.81	17.87	12.09	11.56

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

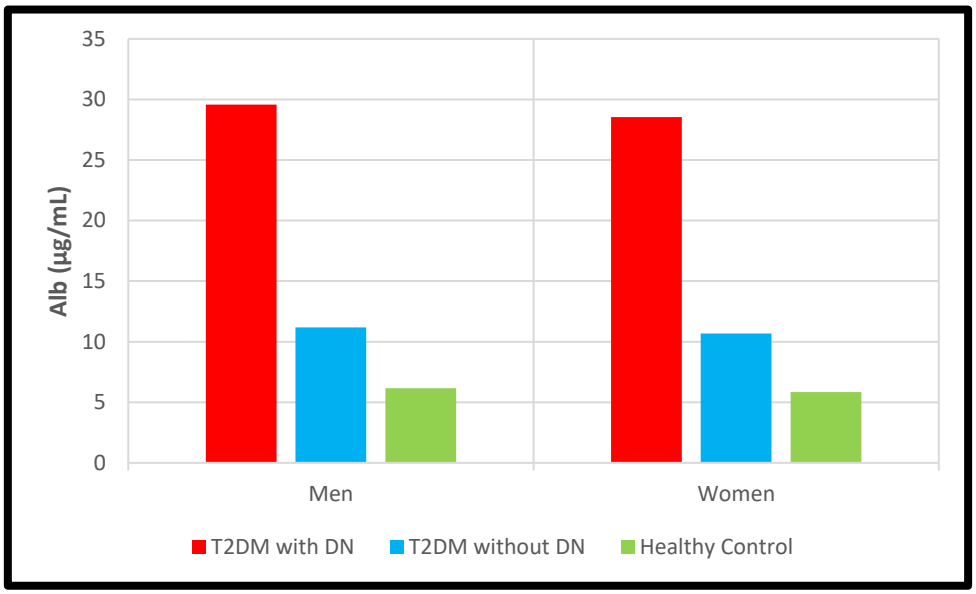


Figure (3.18): Levels of Alb in T2DM patients with or without DN and control group.

The significant increasing in Alb level in T2DM patients with and without DN in our study may be because of elevated urea and creatinine levels, hyperglycemia, insulin resistance, elevated HbA1c level, plasma lipid abnormalities, stress febrile conditions, acute intercurrent disease, urinary tract

infections, heart failure, Plasma prekallikrein activity, diminishing GFR, dyslipidemia, heavy metal poisoning, connective tissue disorders, low HDL-cholesterol levels and elevated uric acid levels (Irshad *et al.,* 2018).

Furthermore, decreasing levels of urinary transthyretin, α1-microglobulin, retinol-binding protein and apolipoprotein (apo) A-I proteins indicates proximal tubular dysfunction and could lead to increase levels of Alb in T2DM patients with and without DN (Kim *et al.,* 2014).

From the other hand, systemic hypertension and glomerular hypertension resulting from glomerular hemodynamic changes, are known to provoke injury to the glomeruli. Therefore, elevated blood pressure can overwhelm normal protection afforded from systemic hypertension to the kidney by autoregulation. Hyperfiltration initiates the renin angiotensin aldosterone system (RAAS), which in turn, increases glomerular permeability and could lead to rise of Alb level in T2DM patients with and without DN (Norris *et al.,* 2018).

Moreover, the volume of the whole kidney and of individual glomeruli is increased with hyperglycemia and glomeruli continue to enlarge later in the disease. Early glomerular enlargement is probably due to enhanced basement membrane production leading to an increased in filtration surface area while later expansion may be caused due to mesangial expansion. The increase in total renal volume is likely to be caused by tubular tissue. So, the enlarged filtration surface area would probably result in greater number of pores (no larger pores have been shown at this stage of diabetes) and influences the filtration of plasma proteins and this leads to raise urinary Alb level in T2DM patients with and without DN (Klemis *et al.,* 2017).

Finally, the term microalbuminuria (MAU) is defined by a urinary Alb excretion (UAE) rate higher than normal but lower than 200 μg/min (Alb excretion in healthy individuals ranges from 1.5–20 μg/min). MAU is the strongest predictor of DN, which is the main cause of morbidity and mortality in

patients with DM. MAU was found in pre-diabetic patients and it was considered a marker of endothelial dysfunction and not only DN. Also, the presence of MAU precedes the development of overt DN by 10–14 years. It is at this stage that one can hope to reverse DN or prevent its progression (Zanetti *et al.*, 2020).

3.4.3.2. Level of NGAL

Neutrophil gelatinase associated lipocalin (NGAL) is a small, 25-kDa, protein that belongs to the lipocalin protein family released from neutrophils and many epithelial cell types including kidney tubular cells. It is representative of the functioning tubular mass and produced as a response to tubular injury (Fiseha and Tamir, 2016).

In the current study, there was a high significant increase ($p<0.01$) in the level of NGAL in T2DM patients with and without DN (Men: 55.26±2.51, 46.38±4.37 vs. 14.78±2.67 ng/mL; Women: 77.42±5.53, 72.09±4.82 vs. 35.73±4.11 ng/mL) as compared to the healthy controls, Table (3.22) and Figure (3.19).

Table (3.22): Levels of NGAL in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. NGAL (ng/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		55.26±2.51**	77.42±5.53**	46.38±4.37**	72.09±4.82**	14.78±2.67	35.73±4.11
SE		0.65	1.38	1.13	1.17	0.67	1.00
Range		53.32-60.60	68.42-86.49	39.35-53.87	64.87-80.04	10.43-19.13	29.01-42.45
95% CI	Lower	48.29	62.07	34.25	58.71	7.37	24.32
	Upper	62.23	92.78	58.52	85.47	22.19	47.14

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [$p>0.05$], A * indicated Significant [$p<0.05$], A ** indicated High Significant [$p<0.01$]) indicated the level of significance in comparison with the corresponding control value.

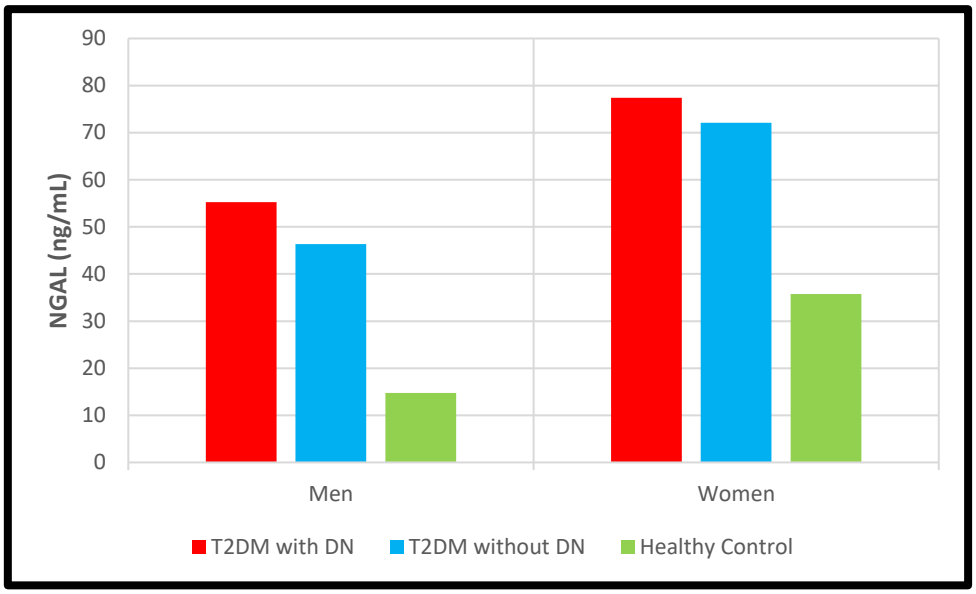


Figure (3.19): Levels of NGAL in T2DM patients with or without DN and control group.

The significant increasing in NGAL level in T2DM patients with and without DN in our study may be due to obesity, insulin resistance, elevated cholesterol and triglycerides levels, ischemia, early pancreatic intraepithelial neoplasia, systemic infections, cardiac surgery, increased creatinine and urea levels, elevated HbA1c and angiotensinogen levels, increased Alb excretion, increased Cys C level, increased level of retinol binding protein, increased urinary white blood cells level, increased levels of TNF-α and IL-18, increased levels of 8-OHdG, declined kidney function, recent treatment with steroids, malignancies, anemia, intoxication, nephrotoxic injury of the kidneys and oxidative stress (Gomez-Chou *et al.*, 2017; Gombert *et al.*, 2018; Wu *et al.*, 2017; Li *et al.*, 2019; Ali *et al.*, 2018; Jagadesan *et al.*, 2019).

Further, the significant elevation in NGAL level in T2DM patients with and without DN in our study could be due to acute kidney injury because NGAL is a protein released by the renal tubules and normally filtered by the glomerulus. In case of kidney injury, the production of NGAL is increased while the absorption is reduced by the kidney tubules resulting an elevation in NGAL levels. Also, high blood pressure could be additional reason for increasing NGAL level in

T2DM patients with and without DN in our study because its relationship with NGAL indicates the endothelial dysfunction in diabetic kidney disease (Siddiqui *et al.,* 2019).

Furthermore, MAU could play a pivotal role in raising levels of NGAL in T2DM patients with and without DN in our study because it activates renal proximal tubular epithelial cells to induce tubulointerstitial inflammation. In contrast, high glucose levels and diabetic substrates, including advanced glycation end-products, carbonyl intermediates, and ultra-filtered growth factors, trigger a number of signalling pathways to promote tubular cell hypertrophy and the interstitial deposition of chemokines, cytokines, growth factors, and adhesion molecules, which are capable of accelerating further inflammation and fibrosis as well as the stimuli that induce epithelial damage which leading to increase NGAL levels (Zeng *et al.,* 2017).

From the other hand, viral infections might be another possible cause for increasing levels of NGAL in T2DM patients with and without DN because of its ability to stimulate NGAL production also in cells not belonging to the immune system. This observation has for the first time raised the hypothesis that this protein could be much more than just a factor involved in nonspecific mechanisms of cellular immunity. This point could be supported by the demonstration that NGAL was able to interact with many ligands other than siderophores such as hepatocyte growth factor, gelatinase-B, different kinases, and proteins of intra- and extracellular matrix. Additionally, NGAL levels could be increased because of some pathological conditions as the result of an increased production and release from several tissues after an injury, for instance, inflammatory diseases involving intestinal epithelium and endothelium, the skin, distal and proximal airway, atherosclerotic plaques and infarcted myocardium (Cernaro *et al.,* 2015).

Moreover, NGAL levels perhaps increased in T2DM patients with and without DN because pathophysiological process of chronic renal diseases such as polycystic kidney disease, glomerulonephritis and several types of renal tumours. Likewise, urinary α1-microglobulin, urinary tract infections or sepsis could be stimulating the overproduction of NGAL. This is indicating that NGAL might not only be elevated in situations of tubular damage, but possibly in glomerulopathies without tubular damage too (Holzscheiter *et al.,* 2014).

So, urine levels of NGAL were significantly higher in microalbuminuria group compared to normoalbuminuria in both diabetes and prediabetes, which suggested that tubular damage may play major role in the development of nephropathy in prediabetes. It was also suggested that NGAL might play an important role in the pathophysiology of renal adaptation to diabetes, and its measurement might become a useful and non-invasive tool for the evaluation of renal involvement in these patients as well as for the early diagnosis of incipient DN (Wu *et al.,* 2017).

3.4.3.3. Level of 8-OHdG

8-OHdG is a product of oxidative DNA damage following specific enzymatic cleavage after reactive oxygen species (ROS) induced 8-hydroxylation of the guanine base in mitochondrial and nuclear DNA. Urinary 8-OHdG appears in the urine without being metabolized and directly related to the DNA oxidation ratio and effectiveness of DNA repair (Hojs *et al.,* 2015).

In the current study, there was a high significant increase ($p<0.01$) in the level of 8-OHdG in T2DM patients with and without DN (Men: 26.92±4.45, 21.43±4.43 vs. 13.41±3.13 ng/mL; Women: 24.47±5.76, 21.09±6.76 vs. 12.86±3.54 ng/mL) as compared to the healthy controls, Table (3.23) and Figure (3.20).

Table (3.23): Levels of 8-OHdG in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. 8-OHdG (ng/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		26.92±4.45**	24.47±5.76**	21.43±4.43**	21.09±6.76**	13.41±3.13	12.86±3.54
SE		1.15	1.44	1.14	1.64	0.78	0.86
Range		19.71-34.13	15.09-33.84	14.25-28.61	10.05-32.13	8.14-18.22	8.75-21.84
95% CI	Lower	14.57	8.48	9.13	2.32	4.72	3.04
	Upper	39.27	40.45	33.73	39.86	22.09	22.69

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

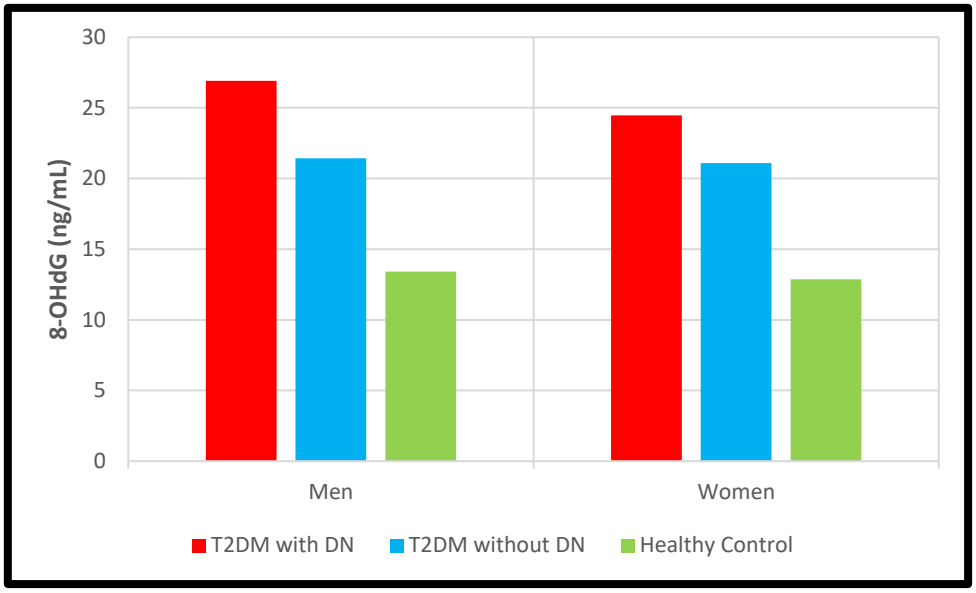


Figure (3.20): Levels of 8-OHdG in T2DM patients with or without DN and control group.

The significant increasing in 8-OHdG level in T2DM patients with and without DN in our study could be due to the severity of the glomerular lesions, increased urinary Alb levels, older age, exposure to different toxic agents, environmental insults, inflammatory bowel disease, stroke, atherosclerotic plaque, vascular recurrence, non-alcoholic fatty liver, non-alcoholic steatohepatitis, heart failure, elevated UACR, elevated HbA1c levels, elevated levels of CRP, IL-6 and TNF, hypertension, hyperlipidemia, hematological disorders, cell lineage, cytokine production other than the grade of malignancies,

proliferation status and tubulointerstitial injury (Zhang *et al.*, 2018; Korkmaz *et al.*, 2018).

Furthermore, production of ROS and lipid peroxidation are increased in diabetic patients, especially in those with poor glycemic control. Oxidative stress may be crucial for development of diabetic vascular complications. Normalizing levels of mitochondrial ROS (MROS) prevents the three major pathways known as the causes of hyperglycemic damage: glucose-induced activation of protein kinase C, increased formation of glucose-derived advanced glycation end products and increased glucose flux through the aldose reductase pathway. So, because intracellular ROS can cause strand breaks in DNA and base modifications which leading to increase levels of 8-OHdG (Ye *et al.*, 2016). From the other hand, oxidative damage yields 8-OHdG after the occurrence of specific enzymatic cleavage when ROS induces 8-hydroxylation in mitochondrial and nuclear DNA. This progressive process clearly depicts the association between increased oxidative stress and T2DM and increased 8-OHdG excreted in urine coincides with increased systemic DNA damage in patients with DN (Chou and Tseng, 2016).

Moreover, insulin resistance could play a central role in producing oxidative stress, free glucose activates aldose reductase activity and the polyol pathway, which decreases NADPH/NADP+ ratios. Elevated intracellular glucose activates PKC through de novo synthesis of diacylglycerol (DAG). Activation of PKC in the glomeruli has been associated with processes increasing mesangial expansion, thickening basement membrane, endothelial dysfunction, smooth muscle cell contraction, and activation of cytokines and transforming growth factor-β (TGF-β). PKC induces oxidative stress by activating mitochondrial NADPH oxidase. Vascular NADPH oxidase consists of multiple subunits including phox47, phox67, and Nox isoforms. ROS generated from Nox isoforms might induce endothelial dysfunction, inflammation, and apoptosis. Excess FFA, mainly

derived from insulin-resistant state, also can increase oxidant production by β-oxidative phosphorylation via mitochondrial metabolism. These mechanisms lead to elevate in 8-OHdG levels significantly in T2DM patients with and without DN (Kong *et al.,* 2017).

Finally, because the increased oxidative stress has a primary role in the pathogenesis of DN, the 8-OHdG in urine could be a useful clinical marker to predict the advanced stage related to the severity of DN (Campion *et al.,* 2017).

3.4.3.4. Level of FN

Fibronectin (FN), a high-molecular-weight protein, is an extracellular matrix (ECM) protein found at high concentrations in a soluble form in blood plasma, and is assembled by cells into elastic, insoluble fibrils. These fibrils form the primordial ECM and are the first matrix proteins assembled by cells during wound healing and embryonic tissue development (Scott *et al.,* 2015). FN is an intrinsic component of the glomerular ECM. It is produced in the liver, vascular endothelia, and platelets. FN is involved in coagulation, platelet function, and tissue repair. In diabetes it may reduce erythrocyte deformity and filterability (Uwaezuoke, 2017a).

In the present study, there was a high significant increase ($p<0.01$) in the level of FN in T2DM patients with DN (Men: 16.41±4.40 vs. 8.98±3.10 ng/mL; Women: 15.38±4.10 vs. 8.07±0.80 ng/mL) as compared to the healthy controls. While, there was a significant increasing ($p<0.05$) in levels of FN in T2DM patients without DN, when compared with the healthy group (Men: 12.27±3.45 vs. 8.98±3.10 ng/mL; Women: 11.32±3.43 vs. 8.07±0.80 ng/mL), Table (3.24) and Figure (3.21).

Table (3.24): Levels of FN in T2DM patients with or without DN and control group. The values are the Mean ± SD.

		F. FN (ng/mL)					
		T2DM Patients				Healthy Controls	
		with DN		without DN			
		Men (15)	Women (16)	Men (15)	Women (17)	Men (16)	Women (17)
Mean ± SD		16.41±4.40**	15.38±4.10**	12.27±3.45*	11.32±3.43*	8.98±3.10	8.07±0.80
SE		1.14	1.03	0.89	0.83	0.78	0.19
Range		9.27-23.55	10.09-24.17	6.67-17.87	5.29-17.05	7.01-16.6	6.81-11.09
95% CI	Lower	4.20	4	2.69	1.80	0.37	5.85
	Upper	28.62	26.76	21.85	20.84	17.58	10.29

Data are presented as mean ± SD, SD: Standard Deviation, SE: Standard Error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence Intervals (Lower and Upper), F.: Fasting, p-value (Non-Significant [p>0.05], A * indicated Significant [p<0.05], A ** indicated High Significant [p<0.01]) indicated the level of significance in comparison with the corresponding control value.

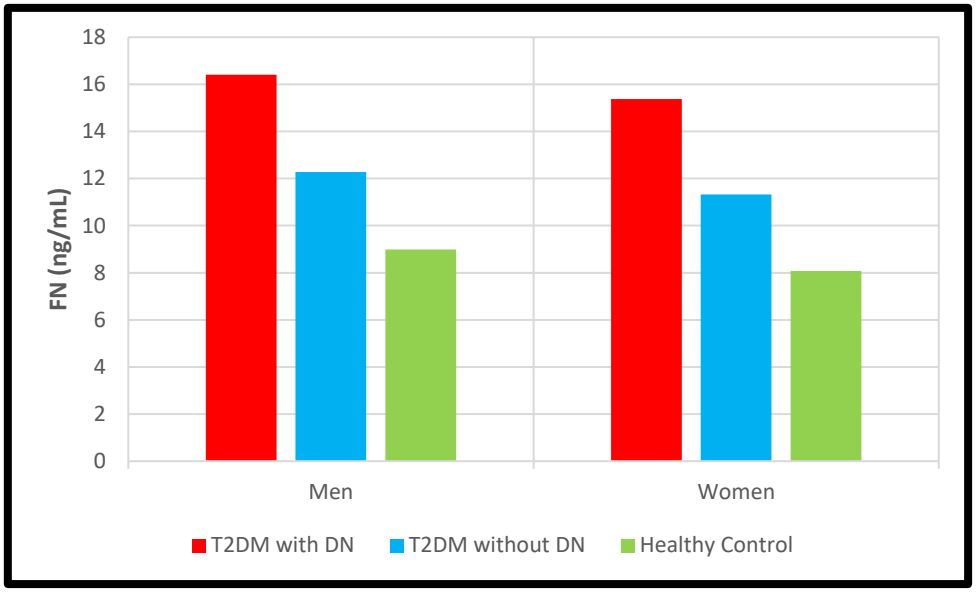


Figure (3.21): Levels of FN in T2DM patients with or without DN and control group.

The significant increasing in FN level in T2DM patients with and without DN in our study could be attributed to obesity, older age, GFR decreasing, increased urinary Alb excretion, nephrotic syndrome, oxidation of glucose and ROS, elevated levels of mitogen-activated protein kinases (MAPK), elevated IGF-1 levels, diabetic microangiopathy, the progression of biopsy proven glomerular diffuse lesions, rheumatoid vasculitis, collagen vascular disorders, acute trauma, sepsis syndrome, ischemic stroke and thrombotic

thrombocytopenic purpura (Caterinoa *et al.*, 2018; Lopes *et al.*, 2019; Lee and Choi, 2015; Klemis *et al.*, 2017).

Furthermore, the significant elevation in FN level in T2DM patients with and without DN in our study could be due to connective tissue growth factor (CTGF) because it plays an important role in glomerular alteration in diabetic sclerosis because this mediator induces transient actin cytoskeleton disassembly in mesangial cells, high production of FN and mesangial cell hypertrophy (Indriani *et al.*, 2020). In addition, protein kinase C (PKC) may play an important role in increasing FN levels in T2DM patients with and without DN through a complex mechanism involving its isoforms (PKC-α, PKC-β, and PKC-ε). These isoforms have been implicated as mediators of renal fibrosis and mesangial expansion through upregulation of vascular endothelial growth factor (VEGF) expression in mesangial cells, as well as transforming growth factor-β (TGF-β) and FN in the glomeruli. Also, NADPH oxidase-driven renal oxidative stress stimulates mesangial expansion and albuminuria by increasing the expression of FN in the kidney (Uwaezuoke, 2017b).

Likewise, the significant increasing in FN level in T2DM patients with and without DN in our study might be due to hyperglycemia because it increases the FN mRNA levels in the kidney cortex and leading to the overproduction of FN in kidney tissues. Hence, the glycemic derangement is an important factor that increases FN synthesis in the kidney, ultimately leading to diabetic glomerular injury. As FN is produced by renal mesangial cells, it was also found that FN in these cells is increased proportionately in the disease states characterized by mesangial expansion including diabetic nodules (Lee and Choi, 2015).

Moreover, there is another possible reason for this significant increasing in FN level in T2DM patients with and without DN attributed to renal fibrosis. The pathologic findings of renal fibrosis are described as glomerulosclerosis, tubule-interstitial fibrosis, and loss of renal parenchyma where tubular atrophy, capillary

loss, and podocyte depletion are prominent. The progression of the disease in the tubule is secondary to that in glomerulus, as the injury of a glomerular segment progress to Bowman's capsule forming synechiae and encroach on the glomerular-tubular junction, leading to a narrowing and obstruction of the urinary orifice. These processes end in a decrease in filtrate delivery, finally depriving the tubule from any workload, causing atrophy and decomposition of the corresponding tubule. The affected kidney tissue undergoes a series of events in attempt to repair from the damage, leading to production of proinflammatory cytokines, monocytes/macrophages, T cells, stimulating mesangial cells, fibroblasts and tubular epithelial cells to undergo phenotypic transition. These processes lead to activation of matrix-producing effector cells and interstitial fibroblasts that induce the production of myofibroblasts, and later tubular epithelial to mesenchymal transition (EMT), with de novo expression of α-smooth muscle actin and overproduction of interstitial matrix components such as fibronectin (Caterinoa *et al.,* 2018). Further, transforming growth factor β (TGF-β) is a central mediator of fibrosis, which induced extracellular matrix expansion (ECM), cell differentiation and proliferation, apoptosis and epithelial to mesenchymal transition (EMT). EMT is regulated by the TGF-β/Smad signalling pathway and leads to morphological and phenotypic changes of the tubular epithelial cells, detachment from the tubular basement membrane and migration into the interstitium, where interstitial cells are transformed into myofibroblasts, inducing the synthesis and deposition of collagens and FN (Lopes *et al.,* 2019).

Finally, FN could be used as a marker of early diabetic kidney disease. It then follows that earlier, more sensitive and specific markers of kidney damage might help in the diagnosis of diabetic kidney. Thus, facilitating the treatment of diabetic kidney disease at an earlier stage to prevent the progression to renal failure (Scherberich *et al*., 2018).

Conclusions:

Based on the previous results, the present study leads to the following conclusions:

1. More than half of patients had T2DM for 13 years or less.
2. Non-statistically significant changes ($p>0.05$) were seen in the level of BMI in T2DM patients with and without DN, as compared to controls.
3. Obesity, a case accompanied by increased levels of insulin and glucose, is a powerful indicator for evaluation of the IR syndrome in diabetic patients (especially with kidney disease) than non-diabetics.
4. Kidney disease is associated with the increased IR and BMI in T2DM patients which had been observed through decreased levels of GFR, CrCl and increased serum urea, creatinine and Hcy.
5. People who have T2DM (with or without DN) tend to have high levels of Hcy and FA which give an indicator on the significant difference in oxidant/antioxidants status and may suggest a possible additional role of oxidative stress mechanisms in pathophysiology of T2DM which causes DN.
6. Serum CysC and creatinine were significantly higher in patients with DN compared to without DN. On the other hand, CysC may be had significantly higher diagnostic accuracy in distinguishing patients with DN than creatinine.
7. Elevated level of serum SA is strongly associated with the presence of DN and it could be representing a predictor of renal dysfunction in DN.
8. The significant increased level of serum OPG ($p<0.01$ in patients with DN and $p<0.05$ in patients without DN) is an independent risk factor for MAU and may be involved in vascular calcification independently of progression of DN in patients with T2DM.

9. LC had renoprotection features through anti-inflammatory and anti-sclerotic effects as well as its ability to improve insulin sensitivity in insulin resistant diabetic patients. Therefore, significantly decreased level of serum LC ($p<0.01$ in patients with DN and $p<0.05$ in patients without DN) may represent a powerful indicator for evaluation of the oxidative stress syndrome in diabetics than non-diabetics.
10. The decreasing in levels of some essential antioxidant trace elements in blood (such as Se, Zn and Mg) is a powerful indicator for evaluation of the oxidative stress syndrome in diabetic patients with and without DN than non-diabetics
11. Urinary Alb, NGAL, 8-OHdG and FN and the combination of all four biomarkers demonstrated an excellent diagnostic value for early-stage of DN in patients with T2DM.

Recommendations:

For future work, the following investigations are recommended:

1. To understand the role of the investigated clinical biomarkers in the physiology and pathophysiology of the development of DN, further studies are required in this area. Other studies should be conducted on patients with other types of DM such as Hybrid Forms, Unclassified and Pregnancy DM in Basrah province and their relationship with other predisposing factors should be assessed.

2. Study the effects of smoking on IR and obesity in diabetic patients with and without DN.

3. Study the correlation of IR with renal dysfunction and oxidative stress in diabetic patients might be an immense research value.

4. There is an involvement of oxidants/antioxidants status in T2DM. So, studies on the dietary supplements should be conducted because they may have a potential application in diagnosis and decrease the development and risks of T2DM and DN.

5. Study the genetic expression of some proteins and mutations in insulin receptors in addition to searching for the genetic analysis obtained in some of the related antioxidant enzymes in patients with T2DM.

6. Regular visits by T2DM patients to optical, cardiovascular and neurological clinics to take early steps to avoid and manage diabetic complications concerning diabetic retinopathy, CVD and diabetic neuropathy.

7. The above investigated clinical and biochemical markers must be used in the hospitals and clinics of Basrah province in the future as new biomarkers for early detection of the development of vascular complications in T2DM patients especially DN.

8. Further studies using larger sample sizes should be performed to establish the diagnostic value of other biomarkers for detection of early-stage DN.
9. Diabetic Nephropathy is a health problem that requires appropriate treatment strategy. Therefore, the Iraqi state must try to understand the problem of this portion of the population and show interest to allocate the necessary resources to solve it. The medical and socio-economic support of patients which means easier access to health education programs, medical services, higher insurance coverage, broader social support, and information are important requirements for resolving the problem.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Appendix

جمهوريه العراق
وزارة الصحة
دائرة صحة البصرة
مكتب المدير العام
مركز التدريب والتنمية البشرية
شعبة ادارة المعرفة/البحوث

العدد : ٥٧٨
التاريخ : ٢٠١٩/١٠/٧

الى / م. الفيحاء التعليمي / مركز الغدد الصم والسكري
م. الموانئ العام
م. الصدر التعليمي

م/ تسهيل مهمة

تحية طيبة...

عقدت لجنة البحوث المركزية اجتماعها يوم الاثنين ٢٠١٩١٩١٣٠ وذلك لدراسة البحث الموسوم (دراسة تأثير مقاومة الانسولين على مؤشرات حيوية خاصة في الدم والادرار للكشف المبكر عن اعتلال الكلية عند المرضى-البدناء لداء السكر غير المعتمد على الانسولين / البصرة – العراق) المقدم من قبل الباحث (سعدون عباس عيدان) طالب الماجستير في كلية العلوم – قسم الكيمياء. وقد تمت الموافقة عليه على ان يتحمل الباحث تكاليف المصروفات والمستلزمات كافة لإجراء بحثه.

..مع التقدير..

الطبيبة الاختصاص
د. رجاء احمد محمود
مديرة مركز التدريب والتنمية البشرية
٢٠١٩/١٠/٧

نسخة منه الى/
مركز التدريب والتنمية البشرية /مع الاوليات.

ثناء

بسمه تعالى
جمهورية العراق

وزارة التعليم العالي والبحث العلمي
جـامعـة البصـرة
كليـة العلـوم
العـدد: ٢٥٧ / ٥ / ٧
التاريخ: ١ / ١ / ١٦

Ministry of Higher Education & Scientific Research
Basra University
College of Science
NO
Date :

إلى / مركز علوم البحار - جامعة البصرة

م/ تسهيل مهمة

تهديكم عمادة كلية العلوم أطيب التحيات .....

يرجـى تفضـلكم بتسـهيل مهمـة طالـب الدراسـات العليـا (**الماجسـتير**) فـي قسـم علـوم الكيميـاء بكليتنـا **سـعدون عبـاس عيـدان** وذلـك لغـرض اجـراء بعـض التحاليـل المختبريـة بجهـاز (FAAS) لحاجتـه الماسـة لإنجـاز بحثـه العلمي الخاص برسالته شاكرين تعاونكم معنا .

. مـع التقديـر .....

الأستاذ الدكتور
عـلاء حسـن عبـد الله
م . العميد للشؤون العلمية والدراسات العليا وشؤون الطلبة
٢٠١٦ / ١ / ٦

نسخة منه ////
- الدراسات العليا
- الصادرة

سميرة / عار/١٥ /١/ ٢٠٢٠

بسمه تعالى
جمهورية العراق

وزارة التعليم العالي والبحث العلمي
جـامعـة البصـرة
كليـة العلـوم
العـدد: ٢٠٨/٥٤/٧
التاريخ: ٢٠١٨/١/١٦

Ministry of Higher Education & Scientific Research
Basra University
College of Science
NO
Date :

إلى/ كلية الطب البيطري / جامعة البصرة

م/ تسهيل مهمة

تهديكم عمادة كلية العلوم أطيب التحيات .....

يرجـى تفضـلكم بتسـهيل مهمـة طالـب الدراسـات العليـا **(الماجسـتير)** فـي قسـم علـوم الكيميـاء بكليتنـا **سـعدون عبـاس عيـدان** وذلـك لغـرض اجـراء بعـض التحاليـل المختبريـة بجهـاز (ELISA) لحاجتـه الماسـة لإنجـاز بحثـه العلمي الخاص برسالته شاكرين تعاونكم معنا .

. مـع التقديـر .....

الأستاذ الدكتور
عـلاء حسـن عبـد الله
م. العميد للشؤون العلمية والدراسات العليا وشؤون الطلبة

نسخة منه ////
- الدراسات العليا
- الصادرة

سميرة / عمار/١٥ /١/ ٢٠٢٠

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Osteoprotegerin and Some Trace Elements in Type 2 Diabetic Patients with or without Nephropathy: Effect of Insulin Resistance

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ABSTRACT— We aimed to evaluate the effect of insulin resistance on serum effect of IR on serum OPG and trace elements levels in diabetic nephropathy patients in province of Basrah-Iraq. From 63 volunteers who suffering from T2DM, 31 patients with nephropathy and 32 patients without nephropathy, while 33 normal voleteers were taken as controls. Their fasting insulin hormone and osteoprotegerin were determined by ELISA methods. BMI, glucose urea, creatinine, GFR and Cr.Cl., and homeostasis model assessment for determined of insulin resistance (IR). Mg and Zn were measured by flame atomic absorption spectrometry, while Se in whole blood was determined using hydride generation method. A non-significant change ($p>0.05$) was seen in the level of BMI, significantly decreased ($p<0.01$) in levels of Se, Zn and Mg, while a higher significantly changes ($p<0.01$) were seen in the levels of glucose, insulin, HOMA-IR, urea, creatinine in subjects of type 2 diabetic patients with and without nephropathy, as compared to healthy group. On the other hand, levels of OPG, GFR and Cr.Cl., were highly significantly ($p<0.01$) changes in diabetic patients with nephropathy and significantly ($p<0.05$) changes in patients without nephropathy, compared to the healthy control. Elevated serum OPG and decreased trace elements (Se, Zn and Mg) levels are strongly associated with BMI, insulin resistance and physical activity which can be used as a biomarker of renal dysfunction in diabetic nephropathy thus decreasing the mortality and morbidity.

KEYWORDS: Osteoprotegerin, Trace Elements, Diabetic Nephropathy, Insulin Resistance, Oxidative Stress.

1. INTRODUCTION

Diabetes Mellitus can be illustrated as a group of metabolic morbidness or a metabolic disorder which results from various etiologies in which any diabetic person has hyperglycemia may be due to the pancreas does not make adequate insulin hormone, or because cells do not response to the insulin [1]. The term of insulin resistance (IR) is represented the inability of cells response to the insulin action in transporting glucose from the bloodstream into muscle and tissues. Therefore, it may be developing with obesity and diabetes mellitus, especially with type 2 diabetes [2]. Obesity is usually linked with abnormality in secretion of insulin hormone. Also, it has an ability to increases the resistance to the cellular actions of insulin which may be led to losing the ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle [3; 4]. Diabetes is still an important health problem as a rising number of patients with chronic and poorly maintained diabetes develop diabetic nephropathy "DN" [5]. DN is a kidney disease linked with long-standing hyperglycemia, first discovered in 1936 by P. Kimmelstiel and C. Wilson as inter-capillary glomerulonephritis. The main lineaments of diabetic nephropathy contain the nephrotic syndrome with extravagant filtration of protein into the urine (proteinuria), high blood pressure, and advanced failure of kidney function. In acute cases, DN drives to kidney failure and end-stage renal disease (ESRD) with the

الخلاصـــة

يُعد مرض اعتلال الكلية السكري (DN) أحد المضاعفات الوعائية الدقيقة والحادة لمرض السكري من النوع الثاني (T2DM) الذي يمكن أن يؤدي إلى الإصابة بأمراض الكلى في نهاية المرحلة (ESRD) . ومن ثم ، فإن الهدف من هذه الدراسة هو دراسة تأثير مقاومة الأنسولين (IR) على مؤشرات محددة في الدم والادرار في التنبؤ المبكر بالاعتلال الكلوي لدى الرجال والنساء من مرضى السكري من النوع الثاني الذين يعانون من السمنة المفرطة من سكان محافظة البصرة (جنوب العراق).

شارك في هذه الدراسة 186 متطوعا من الرجال والنساء. تمت متابعة 63 من المرضى الذين يعانون من مرض السكري من النوع الثاني [31 مريضا يعانون من اعتلال الكلية السكري (15 رجلا و 16 امرأة) و 32 مريضا لا يعانون من اعتلال الكلية السكري (15 رجلا و 17 امرأة)] و 33 شخصا سليما (16 رجلا و 17 امرأة) لمدة 8 أشهر، حتى إنتهاء الدراسة. في حين تم استبعاد 90 متطوعًا (50 مريضًا و 40 سليما) من الدراسة لعدم التمكن من متابعتهم. تم مطابقة المجموعات الثلاث لمؤشر كتلة الجسم BMI مع قياس بعض المؤشرات الحيوية في مصل الدم مثل سكر الدم glucose، الانسولين insulin، تقييم نموذج متوازن لمقاومة الانسولين HOMA-IR، معدل الترشيح الكبيبي GFR، تنقية الدم من الكرياتنين CrCl، اليوريا urea، الكرياتينين creatinine، الهوموسستين Hcy، الفركتوز أمين FA، السيستاتين سي CysC، الكارنيتين LC، الأوستيوبروتيجيرين OPG، حامض السياليك SA، السيلينيوم Se، الزنك Zn والمغنيسيوم Mg. كما تم قياس بعض المؤشرات الحيوية في الادرار مثل الألبومين ALB، نيوتروفيل جيلاتيناز المرتبط بالليبوكالين NGAL، 8-هيدروكي ديوكسي كوانوسين 8-OHdG والفايبرونكتين FN.

مقارنة بالأشخاص السليمين، أشارت النتائج إلى أن مرضى السكري من النوع الثاني مع أو بدون اعتلال الكلية السكري كان هناك زيادة معنوية كبيرة ($p<0.01$) في مستويات سكر الدم، الانسولين، تقييم نموذج متوازن لمقاومة الانسولين، الهوموسستين، الفركتوز أمين، حامض السياليك، نيوتروفيل جيلاتيناز المرتبط بالليبوكالين و 8-هيدروكي ديوكسي كوانوسين. علاوة على ذلك ، كان لدى المرضى الذين يعانون من مرض السكري من النوع الثاني مع اعتلال الكلية السكري زيادة معنوية كبيرة ($p<0.01$) و لدى المرضى الذين يعانون من مرض السكري من النوع الثاني بدون اعتلال الكلية السكري زيادة معنوية ($p<0.05$) في مستويات اليوريا، الكرياتينين، السيستاتين سي، الأوستيوبروتيجيرين، الألبومين والفايبرونكتين، مقارنة مع الاشخاص الاصحاء. من ناحية أخرى، أظهرت النتائج أن معدل الترشيح الكبيبي و تنقية الدم من الكرياتنين وكذلك الكارنيتين انخفضت بفارق معنوي كبير ($p<0.01$) في مرضى مرض السكري من

النوع الثاني الذين يعانون من اعتلال الكلية السكري وبفارق معنوي ($p<0.05$) في المرضى الذين لا يعانون من اعتلال الكلية السكري. في حين أن مستويات السيلينيوم، الزنك و المغنيسيوم كانت منخفضة بفارق معنوي كبير ($p<0.01$) لدى مرضى السكري من النوع الثاني مع اعتلال الكلية السكري أو بدونه مقارنة بالمجموعة السليمة. علاوة على ذلك، أشارت النتائج التي تم الحصول عليها إلى أنه كان هناك تغيير غير معنوي ($p>0.05$) في مستوى مؤشر كتلة الجسم في مرضى مرض السكري من النوع الثاني مع اعتلال الكلية السكري وبدونه مقارنة بالمجموعة السليمة.

نستنتج أن مقاومة الأنسولين ترتبط ارتباطًا وثيقًا بالسمنة ولها دور مهم في التسبب في المرض وزيادة تعقيد العديد من الأمراض البشرية، مثل مرض السكري الذي يمكن استخدامه كمؤشر حيوي للخلل الكلوي في اعتلال الكلية السكري وبالتالي تقليل الوفيات ونسبة الاصابة بالمرض. وبالتالي، مع الأخذ في الاعتبار الجمع بين السمنة ومقاومة الأنسولين و ومرض السكري غير المعتمد على الانسولين قد أظهرت مؤشرا ممتازا لاعتلال الكلية السكري في مرحلة مبكرة في المرضى الذين يعانون من مرض السكري من النوع الثاني.

جامعة البصرة
كلية العلوم
قسم الكيمياء

دراسة تأثير مقاومة الانسولين على مؤشرات حيوية خاصة في الدم والادرار للكشف المبكر عن اعتلال الكلية عند المرضى-البدناء لداء السكر غير المعتمد على الانسولين / البصرة – العراق

رسالة مقدمة الى
كلية العلوم – جامعة البصرة
كجزء من متطلبات نيل درجة الماجستير في
علوم الكيمياء

من قبل
سعدون عباس عيدان السالمي
بكالوريوس ٢٠١١

أ.د. عدنان جاسم محمد الفرطوسي
المشرف

أ.د. ناظم عبدالنبي عواد
المشرف

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