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Some biochemical and immunological aspects in patients with type 2 diabetic nephropathy

A Thesis

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بسم الله الرحمن الرحيم

قُل كلٌّ يعملُ على شاكلتهِ فربكُمُ اعلمُ بمنْ هوَ أهدى سبيلًا (84) ويسألونكَ عنِ الروحِ قلٌ الروحُ منٌ أمرِ ربي وما أُوتيتمُ منَ العلمِ إلّا قليلا(85).

صدق الله العظيم

﴿سورة الإسراء- الآيات 84 و 85﴾

Dedication

I would like to dedicate my thesis to whom given me love and care that makes difference to my life

My parents

Huda

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At the beginning, thanks to great Allah, Lord of the whole creation who gave me the faith and strength to accomplish this work. Mercy and peace on the Prophet Mohammad and his relatives and companions.

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I am sincerely grateful to my father, my mother and my brother Bashar for their help and support throughout my study.

Huda

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Summary

Diabetic mellitus (DM) is a metabolic disorder group of carbohydrate metabolism in which glucose is underused, producing hyperglycemia. Different statistics have led to diabetes being described as one of the main threat to human health in the 21st century. DM is the major cause of renal morbidity and mortality, and diabetic nephropathy is a leading cause chronic kidney failure. This study aimed to asses of different biochemical and immunological markers in diabetic patients with renal impairment and to find out the tests that can assess the renal function in those patients.

Forty –five Type 2 diabetic nephropathy patients attending to the Abd- Almajid private hospital between December 2013 and July 2014 were recruited for this study. For the purpose of comparison, twenty –nine control subjects matched for age were also included. The patients were characterized by microcalbuminuria or increase in serum creatinine above the normal reference and history of diabetes which refers to early stage of diabetic nephropathy.

Random blood glucose (RBG), total cholesterol (TC), HDL-cholestrol, LDL-cholesterol, triglyceride (TG), glycosylated hemoglobin (HbA1c), urea, creatinine , immunoglobulins and some inflammatory markers were measured in blood . Microalbumin , creatinine and ceruloplasmin were measured in urine .

The results demonstrated a significant increase glycosylated hemoglobin ,RBG , blood urea , s.creatinine and decrease in eGFR in Type 2 diabetic nephropathy patients compared with healthy control.

The high – sensitive C-reactive protein (hs-CRP) showed a significant increase in type 2 diabetic nephropathy patients in comparison with healthy control (32.63 mg/dl *vs* 11.76 mg/dl) .

The Transforming growth factor –beta 1 (TGF- β1) and tumor necrosis factor – alpha (TNF –α) showed a non significant difference between the type 2 diabetic nephropathy and healthy control.

Urine markers including microalbumin and ceruloplasmin showed a significant increase in level in Type 2 diabetic nephropathy patients when compared with the healthy control.

In conclusion, the study suggests that hyperglycemia and advanced glycation end product could increase the kidney damage. hs-CRP , microalbumin and ceruloplasmin could be a good markers for the assessment of early kidney damage.

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List of abbreviation

BP : Blood pressure

CP : Ceruloplasmin

DAG-PKC: Diacylglycerol

DKD: Diabetic Kidney disease

DM: Diabetic mellitus

DMP- 7 : Bone morphogenic protein 7

DN: Diabetic nephropathy

ECM : Extracellur matrix

ECM :extracelluar matrix

EDTA: Ethylenediaminetetraacetic acid

EGFR : estimated glomerular filtration rate

ELISA : Enzyme linked immunosorbent assay

ESRD :End stage renal disease

HbA1c :Glycosylated hemoglobin

HDL : High density lipoprotein

Hs –CRP : High sensitive C-reactive

IL-1 : Interlukin -1

IL-2 : Interlukin -2

LDL : Low density lipoprotein

MALB : Microalbumin

NADPH : Nicotinamide adenine dinucleotide phosphate

PDGF: Platelet derived growth factor

PKC :Protein Kinase C

RBG : Random blood glucose

RNA: Ribonucleic acid

ROS : Reactive oxygen species

s.TG : serum triglyceride

T.Chol :Total cholesterol

T2DN : Type 2 diabetic nephropathy

TGF-β : Transforming growth factor –β

TMB: Tetramethylbenzidine

TNF –α : Tumor necrosis factor –α

UAE : Urine albumin excration

VEGF: Vascular endothelial growth factor

Chapter One

Introduction and Literature Review

1.1 Introduction

Diabetes militues is a metabolic disorder characterized by chronic hyperglycemia with disturbances in metabolism of carbohydrate, fat, and protein that results from the defect in insulin secretion, insulin action, or both (Anderson and Deaths, 2001). Chronic hyperglycemia is an associated with long-term dysfunction, and failure of various organs, especially the kidneys, eyes, heart, nerves, and blood vessels (American Diabetes Association, 2009). In worldwide the prevalence of Type 2 diabetes already reached to 366 million in 2011 according to the international Diabetes Federation (IDF), so the total number of people with Type 2 diabetes is expected to reach 552 million people in 2030 (Wild *et al*., 2004 ; International Diabetes Federation , 2011).

Diabetic nephropathy (DN) is the most microvascular complication of diabetes including kidneys damage (Long and Dagogo-Jack, 2011), it is the leading cause of kidney disease in patients starting renal replacement therapy and affects 40% of type 2 diabetic patients (Jorge *et al.,* 2005).

Diabetic nephropathy has many pathways for development such as glomerular hyperfiltration, advanced glycation end products, activation of the polyol pathway, upregulation of growth factors and increased oxidative stress (Ohgas *et al.,* 2004). Classical factors contributing to the pathology of diabetic nephropathy include, hyperglycemia, hypertension, hypoinsulinemia, and hyperlipidemia (Miyata *et al.,* 2009).

The Diabetic nephropathy is also characterized by early elevation of arterial blood pressure, albuminuria with gradual decline in glomerular filtration rate (GFR) (Wilmer *et al.,* 1999) , rise in serum creatinine, a well-accepted marker for the progression of DN, (creatinine value 1.4 to 3.0 mg/dl) is the indicator for impaired renal function (National Kidney Foundation , 2002).

The early medical treatment and lifestyle adjustments have been shown to reduce the progression from microalbuminria to macroalbuminuria and end stage of renal failure. Therefore, the early detection of microalbuminuria as possible in the course of the disease is very important. In the developing countries, this is even more so because of the economic constraints, renal transplant therapy is seldom an option (Hasslacher *et. al.,* 1993).

1.1.1 Aims of study

1. Assessment of different biochemical and immunological markers in diabetic patients with renal impairment.
2. Searching for the tests used to assess renal function in Type 2 diabetic nephropathy patients .
3. Study the correlation between the biochemical and immunological parameters in Type 2 diabetic nephropathy patients .

1.2 Literature Review:

1.2.1 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. (American Diabetes Association, 2010).

Four major types of diabetes have been defined by the National Diabetes Data Group (NDDG) and the World Health Organization (WHO): insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM), gestational diabetes mellitus (GDM), and diabetes secondary to other conditions. (National Diabetes Data Group, 1979; WHO, 1985).

Type 2 diabetes mellitus (T2DM) has become one of the most important chronic public health problems and is a growing cause of mortality and morbidity. One of its complications, the diabetic nephropathy (DN), is the leading cause of premature deaths in diabetic patients due to renal failure (Marshall and Bangert, 2004). Mortality due to kidney failure in diabetic patient is 20–40 times more than those without diabetes (Burtis *et al.*, 2006).

1.2.2 Diabetic nephropathy:

Diabetic nephropathy is defined as a progressive decline in glomerular filtration rate, accompanied by proteinuria and other end-organ complications such as retinopathy (O'Connor and Schelling 2005). Diabetic nephropathy progresses to end stage renal disease via a number of stages including normoalbuminuria, microalbuminuria/incipient

diabetic nephropathy, macroalbuminuria and finally end-stage renal disease (O'Connor and Schelling, 2005). Progression to end stage renal disease is enhanced by hyperglycaemia, hypertension and proteinuria, which are all common in diabetes (Wolf , 2007). Renal disease in diabetic patients is characterised by hemodynamic (hyperfiltration and hyperperfusion) as well as structural abnormalities (glomerulosclerosis, alterations in tubulointerstitium including interstitial fibrosis) and metabolic changes (Cooper, 2001). The more important, it appears that all renal cell types are affected by hyperglycaemic injury including glomerular podocytes, mesangial and endothelial cells, tubular epithelial cells, interstitial fibroblasts, and vascular endothelia (Kanwar, *et al.,* 2008). Within glomeruli, there is thickening of basement membranes, mesangial expansion and hypertrophy and glomerular epithelial cell (podocyte) loss (Bohlender *et al.*, 2005). In conjunction, disease progression is also seen in the tubulointerstitial compartment causing expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis and arteriosclerosis (Marshall, 2004).

1.2.3 Epidemiology

The prognostic value of a small amount of albumin in urine for the development of kidney damage in patients with type 1 or 2 DM was confirmed in the early 1980’s. This stage of kidney damage was called the microalbuminuria stage or initial nephropathy (Viberti *et al.,* 1982). Approximately 20-30% of the patients develop microalbuminuria after 15 years of disease duration and less than half develop real nephropathy (Mogensen, 1984). The European Diabetes (EURODIAB) Prospective Complications Study Group (Orchard *et al.*, 1990) and 18-year Danish study (Chaturvedi *et al.,* 2001) showed that the overall occurrence of microalbuminuria in patients with type 1 and 2 DM is 12.6% (after 7.3

years) and 33%, respectively. According to the United Kingdom Prospective Diabetes Study (UKPDS), the annual incidence of microalbuminuria in patients with type 2 DM in Great Britain is 2% and the prevalence is 25% ten years after the diagnosis (Adler *et al.*, 2003). Proteinuria develops in approximately 15-40% patients with type 1 DM, usually after 15-20 years of DM duration (Hovind *et al.*, 2004). In patients with type 2 DM, the prevalence varies between 5% and 20% on average (Adler *et al.*, 2003). Diabetic nephropathy is more frequent in African Americans, Asian Americans, and Native Americans (Young *et al.*, 2003). In Caucasians, the progressive kidney disease is more frequent in patients with type 1 than type 2 DM, although its overall prevalence in the diabetic population is higher in patients with type 2 DM because this type of DM is more prevalent. The occurrence of diabetic nephropathy in Pima Indians is very interesting, according to a study published in 1990, around 50% of Pima Indians with type 2 DM developed nephropathy after 20 years of the disease, and 15% of them were already in the terminal stage of kidney failure (Craig *et al.,* 2003). In the United States, the occurrence of diabetic nephropathy in patients beginning kidney replacement therapy doubled in the 1991-2001 period (Young *et al.*, 2003).Fortunately, the trend has been decreasing, most likely due to the better prevention and earlier diagnosis and treatment of DM (Caramori *et al.,* 2002).

1.2.4 Risk Factors of the diabetic nephropathy:

The two main risk factors for DN are hyperglycemia and arterial hypertension. However, DN develops in only about 40% of patients, even in the presence of hyperglycemia and elevated BP for long periods of time. This observation raised the concept that DN will develop only in a susceptible subset of patients (Krolewski , 2001). Furthermore, family

studies have confirmed a genetic contribution for the development of DN in both type 1 and type 2 DM (Canani *et al.,* 1999). Once DN is present, progression factors may act, favoring evolution to more advanced stages. There is evidence that some factors involved in the development of proteinuria are also common to the loss of GFR, but others are unique to each one of them (Placha *et al.,* 2005).

1.2.4.1 Hyperglycemia

Hyperglycemia is a significant risk factor for the development of microalbuminuria, both in type 1 and in type 2 DM.A reduction of 1% in HbA1c is associated with a 37% decrease in microvascular endpoints (Stratton *et al.*, 2000). In the presence of micro- and macroalbuminuria the role of metabolic control is less defined, even though some studies showed a deleterious effect of high glucose levels on GFR (Hovind *et al.*, 2003). Moreover, it was demonstrated that pancreas transplantation reversed renal damage in type 1 DM patients with mild to advanced DN lesions (Fioretto *et al.*, 1998). A large trial also reinforced the importance of intensive treatment of DM to decrease the microvascular complications (Patel *et al.*, 2008).

1.2.4.2 Arterial Hypertension

Arterial hypertension is a main risk factor for the development of DN (Park *et al.*, 1998), and probably the best known relevant factor related to its progression. Analysis of UKPDS showed that every 10 mmHg reduction in systolic BP is associated with a 13% reduction in the risk of microvascular complications, with the smallest risk among those patients with systolic BP <120 mm Hg (Adler *et al.,* 2000) .

1.2.4.3 Dyslipidemia

In type 2 DM, elevated serum cholesterol is a risk factor for the development of DN. In type 1 DM patients increased serum triglycerides, total and LDL-cholesterol were associated with micro- and macroalbuminuria (Jenkins *et al.,* 2003). High serum cholesterol also seems to be a risk factor for GFR loss in macroalbuminuric type 1 diabetic subjects (Mulec *et al.*, 1993).

1.2.4.4 Proteinuria

Proteinuria itself could lead to progression of DN. Proteinuria >2 g/24 h is associated with a greater risk of ESRD (Ruggenenti and Remus, 1998). Increased leakage of albumin may induce glomerular damage probably through activation of inflammatory cascades (Remuzzi *et al.*, 1997). This would be a reason to target decreased urinary albumin excretion in DN treatment.

1.2.4.5 Genetic risk factors

The exact genetic model underlying DN susceptibility is uncertain, but theoretically few genes with a major contribution and some with minor interaction with the environment could cause DN (Krolewski *et al.,* 2001). Unfortunately, no gene with a major effect had been identified so far. The knowledge of which gene(s) predisposes to DN will allow the identification of patients at high risk for this complication, and adoption of preventive measures. In genetic studies the clear definition of the phenotype, DN, is very important. DN could be defined by different parameters: for instance, the presence of microalbuminuria, macroalbuminuria, ESRD or decreased GFR. Some genes probably are involved in the development of proteinuria, others with decline in GFR and some will be involved in both situations (Krolewski *et al.,* 2001).

Therefore, a more comprehensive definition of DN used in the genetic studies is important to make the results more comparable. A familial aggregation of DN has been demonstrated in studies of sibling-pairs (Canani *et al*., 1999), parent-offspring pairs or studies of extended families. One practical application of the studies with diabetic siblings is that the chance of having DN increases 2-3 times if the subject's sibling has DN when compared to the subject who has a normoalbuminuric sibling, either in type 1 or type 2 diabetes (Canani *et.al.,* 1999).

Recent advances in technology make easier to look for regions in whole genome linked to different DN phenotypes (Krolewski *et al*., 2006; Pezzolesi *et al*., 2009; Rogus *et. al*., 2008). This approach identified regions and putative genes not previously known to be associated with DN and it could raise new candidate genes. Moreover, new targets for drug development may come into sight, since some of the genes found are novel and have not been previously implicated in the pathogenesis of DN.

Association studies of candidate genes have been performed aiming to identify polymorphic variants associated with DN or with different degrees of renal disease. Often, genes that play a role in the expression of proteins that are related to the modulation of cytokines, proteins involved in the glycol and lipid metabolism, in the formation of extracellular matrix, in blood pressure homeostasis, and in insulin sensitivity, have been considered candidates for the development of DN (Gross *et al*., 2005). However, the studies have not been successful in identifying genes that consistently show an association with DN. Replication studies have demonstrated conflicting results (Boright *et al*., 2005). The evaluation of 360 thousand polymorphisms in patients with type 1 DM, with and without DN, showed a total of 13 polymorphisms located at 4 loci in two independent cohorts of subjects strongly associated with the presence of

DN (Pezzolesi *et al.,* 2009). Some of these polymorphisms are located in genes highly expressed in the kidney with DN, and its development over time. Another approach that has been used to investigate the genetics of DN involves the study of microRNAs role on this process. These are non-encoding short RNAs that induce post-transcriptional protein modifications. Little is known about these molecules and their role in DN. In a study, microRNA mirR-192 expression was increased in the glomeruli of rodents with DM (Brosius, 2008). Their induction by TGF-β in mesangial cells caused increased collagen synthesis and suggests that this type of molecule may be implicated in the development of DN, opening up a new prospect of research in elucidating the pathogenesis of this DM complication. The replication of this finding and this type of approach must be better explored in studied conducted in human beings. As previously stated, Brazilians of African descent have more aggressive renal disease as people of European ancestry (Gerchman *et al.*, 2008). This could be due to several reasons, such as the presence of different risk factors, different access to medical attention, and socioeconomic differences. However, none of the assessed known risk factors were different between African and Europeans (Kramer *et al.*, 2009) make unclear an explanation for the different rates of DN between black and white subjects. Unfortunately, data on socioeconomic status were unavailable. An alternative explanation for this observation, but hard to prove, would be a different genetic susceptibility.

1.2.5 Pathogenesis of diabetic nephropathy

The resident and nonresident renal cells are stimulated by hyperglycemia and produce humoral mediators, cytokines and growth factors that are responsible for structural alterations such as increased deposition of ECM and functional alterations such as increased

permeability of glomerular basement membrane, which contribute to the pathogenesis of DN. Moreover, the development and progression of DN include various hyperglycemia-induced metabolic and hemodynamic derangements that involve increased formation of AGEs, enhanced ROS generation and PKC activation, polyol pathway and RAS (Zelmanovitz *et al.*, 2009) .

1.2.5.1 Hyperglycemia and advanced Glycosylation end products:

Glycated haemoglobin is defined as haemoglobin that is irreversibly glycated at one or both N-terminal valines of the beta chains. This definition does not exclude haemoglobin that is additionally glycated at other sites on alpha or beta chains (Kobold *e.t al.,* 1997). HbA1c has been the most widely used and accepted test for monitoring the glycaemic control in individuals with diabetes. Once a haemoglobin molecule is glycated, it remains in the red blood cell for the rest of its life-span (120 days). As such, it provides information about the degree of long-term blood glucose control. The HbA1c level does not reflect an exact mean blood glucose; rather, it is weighted proportionally towards recent levels (Tahara and Shima, 1995).

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening (Tahara and Shima, 1995).

In vitro studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production (Heilig *et al.*, 1995) and

mesangial cell apoptosis (Mishra *et al*., 2005; Lin *et al*., 2006). Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells (Heilig *et al*., 1995).

Hyperglycemia might also upregulate VEGF expression in podocytes, (Wolf and Ziyadeh, 2007) which could markedly increase vascular permeability (Wolf *et al*., 2005; Chen *et al*., 2007).

Hyperglycemia, however, does not account fully for the risk of DN , as shown by studies in which kidneys from nondiabetic donors were transplanted into patients with diabetes and nephropathy developed irrespective of the glucose control (Mauer *et al*., 1983).

Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage. Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway (Friedman, 1999). Oxidative stress seems to be a theme common to all three pathways (Brownlee, 2001).

Glycosylation of tissue proteins contributes to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins. This non enzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. T These advanced products can be involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alteration in the level of

soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency, since they are normally excreted in the urine. The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications (Singh *et.al*., 1998). Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner (Hogan *et. al*., 1992).

1.2.5.2 Role of cytokines in diabetic nephropathy

Cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects with characteristic features (Coppack, 2001). These molecules cluster into several classes (i.e., interleukins, tumor necrosis factors, interferons, colony-stimulating factors, transforming growth factors and chemokines), which are relevant humoral mediators in a highly complex, coordinated network regulating inflammatory immune responses with the participation of different cytokine-associated signaling pathways .

In addition, they exert important pleiotropic actions as cardinal effectors of injury (Aldhahi, 2003). Cytokines are produced by a wide variety of cells in the body, playing an important role in many physiological responses that have a therapeutic potential (Balwilk and Burke, 1989). It is recognized that chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of diabetes mellitus (Pickup and Crook, 1998; Crook, 2004; Pickup, 2004). Increasing evidence suggests that individuals who progress to diabetes mellitus display features of inflammation years

before the disease onset (Dandona *et al.,* 2004). Moreover, population-based studies suggest that inflammatory parameters, including inflammatory cytokines, are strong predictors of the development of diabetes (Spranger *et al.,* 2003). The main cytokines involved in the pathogenesis of diabetes are IL-1, TNF-α, and IL-6 (Alexandrak *et al.,* 2006). In addition, studies in recent years have shown that inflammation, and more specifically inflammatory cytokines, are determinant in the development of microvascular diabetic complications, including neuropathy, retinopathy, and nephropathy (Skundric and Lisak, 2003; Navarro and Mora, 2005; Mora and Navarro, 2006).

Hyperglycemia stimulates increased expression of different growth factors and activation of cytokines, which overall contributes to further kidney damage (Hohenstein *et al*., 2006; Navarro-Gonzalez and Mora-Fernandez, 2008).

In the kidney biopsy samples from patients with type 2 DM, a significant increase in platelet derived growth factor (PDGF) expression was found. Moreover, the site of expression of this factor is adjacent to the areas of interstitial fibrosis, which is important in the pathogenesis of fibrosis in kidney injury (Langham *et al.,* 2003).

Hyperglycemia also increases the glomerular expression of TGF-beta; matrix proteins are specifically stimulated by this growth factor (Wolf and Ziyadeh, 1999). Furthermore, the expression of bone morphogenic protein 7 (BMP-7) in DM is decreased, and the expression of profibrinogenic TGF-beta is increased (Wang *et al.* 2006; Turk *et al.*, 2009). In particular, the proinflammatory IL-1 increases the expression of chemotactic factors and adhesion molecules, enhances vascular endothelial permeability, and stimulates the proliferation of mesangial cells and matrix synthesis (Rivero *et al.,* 2009). Renal IL-1 expression is

found increased in diabetic animals and correlates with albuminuria and macrophage content (Navarro *et al*., 2005).

Specific blockade of IL-1 activity by the IL-1 receptor antagonist reduced the release of inflammatory cytokines and chemokines in pancreatic islet from diabetic rats, and also decreased hyperglycemia and improved insulin sensitivity (Ehses *et al.,* 2009). In type 2 diabetic patients, anakinra improved glycemia and beta-cell secretory function and reduced markers of systemic inflammation (Larsen *et al.,* 2007). Further studies are needed to demonstrate the biological effects of this compound on diabetic kidneys. IL-6 is a pleiotropic cytokine secreted by renal cells in response to a diabetic milieu (Min *et al.,* 2009; Tang *et al.,* 2010) that stimulates mesangial cell proliferation, affects extracellular matrix dynamics in renal cells, and enhances endothelial permeability (Navarro-Gonzalez and Mora- Fernandez, 2008). Serum IL-6 levels are significantly increased in patients with type 2 DN compared to levels observed indiabetic patients without nephropathy (Navarro-Gonzalez and Mora-Fernandez, 2008), and studies in renal biopsies revealed a significant association between the severity of diabetic glomerulopathy and the expression levels of IL-6 in glomerular cells (Suzuki *et al.,* 1995), thus suggesting a role for IL-6 in the pathogenesis of DN.

There are no direct data of treatment against elevated IL-6 levels in DN, however there are indirect evidences. In patients with incipient and established DN, the treatment with pentoxyfylline, a methylxanthine derivate and nonselective phosphodiesterase inhibitor, caused a decrease in the urinary albumin excretion, and this renoprotective effect was attributable in part to reduced levels of IL-6 among other proinflammatory mediators (Navarro *et al*., 2005).

1.2.5.3 Transforming growth factor –beta (TGF-β)

Human TGF-β1 is involved in a number of key developmental, immunologic, and homeostatic processes (Ruscetti and Bartelmez, 2001).

TGF-β1 has a wide range of activities, During an immune response, TGF-β1 impacts antibody production by preferentially inducing IgA production in both mouse and human (Stavnezer, 1995). It also regulates dendritic cell chemotaxis by altering the expression of chemokine receptors (Sato *et al.,* 2000). It can downmodulate an inflammatory response by dampening macrophage activity and proinflammatory secretion (Wahl *et al.,* 2000).

TGF-β is a well-known profibrogenic factor which controls synthesis and degradation of extracellular matrix proteins by stimulating transcription of extracellular matrix genes in renal cells and reducing collagenase production, eventually inhibiting matrix turnover.

Furthermore TGF-β is involved in tubuloglomerular sclerosis and podocyte apoptosis in diabetes. TGF-β gene and protein levels are significantly increased in glomeruli and tubulointerstitium of type 1 and 2 diabetic patients and animals (Yamagishi and Matsui, 2010). Factors that regulate TGF-β expression in renal cells include hyperglycemia, AGEs, endothelin, lipids and products of oxidative stress. TGF-β is also modulated by the RAAS (Goh *et al.,* 2008).

In fact, angiotensin II stimulates TGF-β and its inhibition reduces serum and urinary levels of TGF-β (Wolf, 2007). In diabetic patients, treatment with the ACE inhibitor perindopril reduced the intrarenal TGF-β expression and activity (Langham *et al.,* 2003). Furthermore, the antifibrotic agent N-acetyl-serylaspartyl- lysyl-proline, which reduced TGF-β -induced extracellular matrix production and prevented renal fibrosis and albuminuria in diabetic db/db mice, conferred an additional renoprotective effect when combined with the angiotensin II receptor

antagonist losartan (Sugaru *et al.,* 2006). A range of novel compounds has been recently examined to inhibit TGF-β and TGF- β - dependent pathways in diabetes. Several blocking Abs against TGF-β effectively reduce mesangial matrix accumulation and glomerulosclerosis in diabetic mouse models (Goh *et al.,* 2008), and particularly the TGF-AY1 Ab is in clinical development for the treatment of chronic kidney disease, with focus on DN (Cortinovis *et al.,* 2008). In addition, the soluble human TGF-β type II receptor (sT RII-Fc), a high-affinity TGF-β1 binding molecule, has been proposed as a potential new agent for the treatment of fibrosis and albuminuria in DN (Russo *et al.,* 2007).

Potential therapeutic approach is the use of micro RNA (miRNA)-based strategies. The miRNAs are short noncoding nucleotides that regulate target messenger RNAs at the posttranscriptional level and are involved in many biological processes (Lorenzen *et al.,* 2011).

Studies have identified miRNA-mediated circuits controlling auto-upregulation of TGF-β1 and amplification of TGF-β1 signaling that accelerate chronic fibrotic kidney diseases including DN (Kato *et al.,* 2011). In particular, miRNA-92c and miRNA-192 are induced in renal cells by high glucose and TGF-β, and mediate cell apoptosis and extracellular matrix accumulation (Kato *et al*., 2011; Long *et al*., 2011). Renal expression of these miRNAs increased in type 1 and type 2 diabetic animals, and *in vivo* knockdown prevented progression of DN. Their widespread and distinct expression patterns under normal and disease states make miRNAs attractive molecular therapeutic targets for human diseases. Different miRNA modulators (such as antagomirs and locked nucleic acid antimiRs) have been developed for specific targeting of miRNAs and respective downstream gene networks (Lorenzen *et al.,* 2011).

1.2.5.4 Tumor necrosis factor –alpha (TNF - α)

Tumor necrosis factor alpha (TNF-α), also known as cachectin, is the prototypic ligand of the TNF superfamily (Idriss and Naismith, 2000). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (Hehlgans and Pfeiffer, 2005 ; Chen et al., 2007 ; Salek-Ardakani and Croft, 2010; Van Herreweghe *et al.,* 2010). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (Hehlgans and Pfeffer , 2005 ; Berry *et al.,* 2007 ; Leung and Cahill 2010 ; Tzanavari *et al.,* 2010 ; Wu and Zhou, 2010).

TNF-α, are strongly associated with the risk of developing diabetic complications (Shikata and Makino, 2004). ILs comprise a large group of cytokines secreted by leukocytes and other body cells that can be classified as proinflammatory and anti-inflammatory.

TNF-α is a pleiotropic cytokine produced mainly by monocytes / macrophages that is involved in systemic inflammation. TNF-α exerts cytotoxic effects on renal cells (Min *et al*., 2009), and it has been shown to participate in renal damage development in experimental models of renal disease including lupus nephritis, glomerulonephritis, nephropathy, hypertension, and diabetes (Elmarakby and Sullivan, 2010). A role for TNF-α in DN is supported by the finding that urinary albumin excretion significantly correlates with renal TNF-α levels and urinary TNF-α excretion in streptozotocin-induced diabetic rats (Navarro *et al.,* 2005). TNF-α also contributes to sodium retention and renal hypertrophy, which are early characteristic signs of streptozotocin-induced DN (DiPetrillo *et al*., 2003). Renal TNF-α expression, particularly in the glomerulus and

tubulointerestitium, is increased in streptozotocin diabetic rat kidneys, and serum TNF- α is increased in type 2 diabetic patients (Navarro *et al.,* 2005). Therefore, TNF-α plays an important role in the incidence and progression of DN and renal TNF-α levels correlate with markers of DN.

Strategies to inhibit TNF-α have been successfully used in experimental diabetes. DiPetrillo and others in (2003) reported that treatment of diabetic rats with the anti-TNF-α agent TNFR:Fc, a soluble TNF-α receptor fusion protein, reduced urinary TNF- α excretion and prevented sodium retention and renal hypertrophy. Similarly, TNF-α inhibition with infliximab, a chimeric monoclonal Ab directed against TNF-α, significantly reduced both albuminuria and urinary TNF-α in streptozotocin-induced diabetic rats (Moriwaki *et al.,* 2007). Unfortunately, no other parameters such as structural changes or hemodynamics were studied. A recent retrospective study evaluated the effects of anti–TNF-α agents on control of type 2 diabetes in patients with rheumatoid arthritis and Crohn's disease. Anti- TNF treatment improved glucose tolerance and control, although future prospective studies are needed to solidify these results (Gupta-Ganguli *et al*., 2011)

1.2.5.5 The role of oxidative stress in diabetic nephropathy

Numerous studies clearly indicate that both diabetic state and insulin resistance play a central role in producing oxidative stress; free glucose activates aldose reductase activity and the polyol pathway, which decreases NADPH/NADP+ ratios (Tesfamariam, 1994). Elevated intracellular glucose activates PKC through *de novo* synthesis of diacylglycerol (DAG) (Ishii, 1996). 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) (Koya and King, 1998). PKC induces oxidative stress by activating mitochondrial NADPH oxidase (Bedard and Kraus, 2007). Vascular NADPH oxidase consists of multiple subunits including phox47, phox67, and Nox isoforms (Bedard and Kraus, 2007). ROS generated from Nox isoforms might induce endothelial dysfunction, inflammation, and apoptosis (Cave *et al.,* 2006). Excess FFA, mainly derived from insulin-resistant state, also can increase oxidant production by β oxidative phosphorylation via mitochondrial metabolism (Duckworth, 2001). Studies using rodents indicate that increases in oxidative stress could be responsible for developing DN; inhibition of the polyol pathway with aldose reductase inhibitors could reduce the effects of hyperglycemia on DN (Dunlop, 2000). Further, administration of vitamin C or E has been shown to be effective in ameliorating rodent model of DN (Bursell and King 1999). Another study has also shown that high doses of vitamin E normalized parameters of oxidative stress and inhibited vascular abnormalities caused by DAG-PKC activation in the kidney (Koya *et al*., 1997).

1.2.6 Immunoglobulines

Antibodies play key roles in humoral adaptive immune response by binding to specific antigens and linking them to the innate immune system. The ability of antibodies to harness such immunity is attributed to their unique structure, and has been successfully exploited in development of therapeutic treatments for many disease types, including cancers, autoimmune diseases, and inflammatory disorders (Chang *et al*., 2007; Scott *et al*., 2012).

Human immunoglobulins can be categorized into five classes (IgG, IgA, IgD, IgE, and IgM). IgG and IgA antibodies are further separated into four (IgG1-4) and two subclasses (IgA1-2), respectively.

Immunoglobulin G (IgG) is a protein synthesized and secreted by plasma cells. It has a molecular weight of 150 kDa, which is larger than albumin. Urinary IgG excretion is higher in diabetic patients compared to healthy controls, and its excretion in diabetic patients with normoalbuminuria predicts the development of microalbuminuria (Narita *et al*., 2006).

Urinary IgG excretion correlates with the progression of glomerular diffuse lesions. One IgG isoform (IgG4) has been used more specifically as a marker of glomerular charge selectivity impairment.

Only IgG4 excretion is elevated in patients with microalbuminuria, while the excretion of both IgG and IgG4 are increased in patients with macroalbuminuria compared with normoalbuminuric patients. One study found that urinary concentration of IgG2 in patients with normoalbuminuria was significantly higher than in healthy control, whereas further elevation of IgG2, IgG4, and IgA was more pronounced in patients with microalbuminuria. Fractional excretion of IgG2 was the highest among all immunogloubins, which indicated that elevation of those particular immunogloubin subtypes was a contribution of novel mechanisms in early DKD, different from charge and size barrier impairment (Gohda *et al.,* 2012). One systemic review, including 13 studies, indicated urinay IgG was a good marker for predicting onset of nephropathy (Hellemons *et al*., 2012).

Immunoglobulin M (IgM), secreted by plasma cells, is the largest antibody in the human. Due to its large molecular radius, the appearance of IgM in urine indicates that a large, nonselective pore exists in the glomerular capillary wall. One study showed that urine excretion of IgM was significantly higher in type 2 DM compared to type 1 DM, and patients with type 2 DM with nephrosclerosis had significantly higher urine excretion of IgM compared to the age-matched healthy subjects

(Bakoush *et al*., 2002). Another study found renal survival of type 2 diabetic patients was inversely associated with urine IgM excretion, which indicated that higher urinary IgM excretion was a better predictor of decline in kidney function than albuminuria in type 2 DM. However, urinary IgM excretion has not been regarded as an early marker of DKD, since its excretion in urine is associated with severe injury of the glomerular capillary wall, while it is also a promising marker which may predict the eventual need for renal replacement therapy (Tofik *et al*., 2009).

1.2.7 Screening and diagnosis

Screening for diabetic nephropathy must be initiated at the time of diagnosis in patients with type 2 diabetes (American Diabetes Association, 2004), since 7% of them already have microalbuminuria at that time (Adler *et al*., 2003). For patients with type 1 diabetes, the first screening has been recommended at 5 years after diagnosis (American Diabetes Association, 2004). However, the prevalence of micro-albuminuria before 5 years in this group can reach 18%, especially in patients with poor glycemic and lipid control and high normal blood pressure levels (Stephenson and Fuller, 1994). Furthermore, puberty is an independent risk factor for microalbuminuria (Schultz *et al*., 1999). Therefore, in type 1 diabetes, screening for microalbuminuria might be performed 1 year after diabetes diagnosis, especially in patients with poor metabolic control and after the onset of puberty. If microalbuminuria is absent, the screening must be repeated annually for both type 1 and 2 diabetic patients (American Diabetes Association, 2004).

The first step in the screening and diagnosis of diabetic nephropathy is to measure albumin in a spot urine sample, collected either as the first urine in the morning or at random, for example, at the medical visit. This

method is accurate, easy to perform, and recommended by American Diabetes Association guidelines (American Diabetes Association, 2004). Twenty-four–hour and timed urine collections are cumbersome and prone to errors related to collecting samples or recording of time. The results of albumin measurements in spot collections may be expressed as urinary albumin concentration (mg/l) or as urinary albuminto- creatinine ratio (mg/g or mg/mmol) (Eknoyan *et al.*, 2003). Although expressing the results as albumin concentration might be influenced by dilution/concentration of the urine sample, this option is still accurate and cheaper than expression as albumin- to-creatinine ratio .The cutoff value of 17 mg/l in a random urine specimen had a sensitivity of 100% and a specificity of 80% for the diagnosis of microalbuminuria when 24-h timed urine collection was the reference standard (Zelmanovitz *et al.*, 1997) This value is similar to the cutoff value of 20 mg/l recommended by the European Diabetes Policy Group (European Diabetes Policy Group, 1999). All abnormal tests must be confirmed in two out of three samples collected over a 3- to 6-month period (Eknoyan *et al.*, 2003) due to the known day-to-day variability in UAE. Screening should not be performed in the presence of conditions that increase UAE, such as urinary tract infection, hematuria, acute febrile illness, vigorous exercise, short-term pronounced hyperglycemia, uncontrolled hypertension, and heart failure (Mogensen *et al.*, 1995). Samples must be refrigerated if they are to be used the same day or the next day, and one freeze is acceptable before measurements (Eknoyan *et al.*, 2003). Immunoassays routinely used for albumin measurements present adequate diagnostic sensitivity for detection of diabetic nephropathy. However, it was demonstrated that conventional immunochemical- based assays did not detect unreactive fraction of albuminuria, underestimating UAE (Comper *et al.*, 2004). High-performance liquid chromatography measures total

albumin, including immunoreactive and immunounreactive forms, and may allow early detection of incipient diabetic nephropathy. In situations where specific UAE measurements are not available, semiquantitative dipstick measurements of albuminuria, such as Micral Test II, can be used (Mogensen *et al*., 1997). Another alternative is to use a qualitative test for proteinuria (dipstick) (Sacks *et al*., 2002) or a quantitative measurement of protein in a spot urine sample (Eknoyan *et al*., 2003). The presence of a positive dipstick (Combur M; Boehring Manheim) or a urinary protein concentration 430 mg/l has a sensitivity of 100% for both tests and a specificity of 82 and 93%, respectively, for the diagnosis of proteinuria (Zelmanovitz *et al*., 1998). An abnormal result should be confirmed by measurement of total protein in a 24-h urine sample.

Although the measurement of UAE is the cornerstone for the diagnosis of diabetic nephropathy, there are some patients with either type 1 or type 2 diabetes who have decreased glomerular filtration rate (GFR) in the presence of normal UAE (MacIsaac *et al*., 2004). In patients with type 1 diabetes, this phenomenon seems to be more common among female patients with longstanding diabetes, hypertension, and/or retinopathy (Caramori et al., 2003). For patients with type 2 diabetes in NHANES III (Third National Health and Nutrition Examination Survey; *n* = 1,197), low GFR (<60 ml · min−1 · 1.73 m−2) was present in 30% of patients in the absence of micro- or macroalbuminuria and retinopathy (Kramer *et al.,* 2003).

Although renal biopsy was not performed, this observation was probably related to renal parenchymal disease other than classical diabetic glomerulosclerosis. These studies indicate that normoalbuminuria does not protect from a decrease in GFR in type 1 and type 2 diabetic patients Therefore, GFR should be routinely estimated

and UAE routinely measured for a proper screening of diabetic nephropathy.

GFR can be measured by specific techniques, such as inulin clearance, 51Cr-EDTA, 125I-iothalamate, and iohexol (Gaspari *et al.*, 1997). The clearance of endogenous creatinine is commonly used, despite its limitations. In clinical practice, GFR can be estimated by prediction equations that take into account serum creatinine concentration and some or all of the following variables: age, sex, race, and body size (Levey *et al.*, 2003).

1.2.8 Estimated glomerular filtration rate (GFR)

GFR is measured as the urinary or plasma clearance of an ideal filtration marker such as inulin or of alternative exogenous markers such as iothalamate, EDTA, diethylene triamine pentaacetic acid, and iohexol. Measuring clearance with the use of exogenous markers is complex, expensive, and difficult to do in routine clinical practice. (Mohanram and Toto, 2005) Furthermore, research studies have reported a measurement error of 5 to 20 percent (variation within a single clearance procedure or between clearance procedures on different days) (Gaspari *et al.*, 1998).The variation is greater in the higher ranges of GFR on the absolute scale (Levey *et al.*, 1993).

1.2.9 Creatinine with GFR

Creatinine is an amino acid derivative with a molecular mass of 113 D that is freely filtered by the glomerulus. Many studies support the similarity of creatinine clearance to GFR and its reciprocal relationship with the serum creatinine level (Stevens and Levey, 2005). Creatinine is secreted by proximal tubular cells as well as filtered by the glomerulus; thus, the creatinine clearance exceeds the GFR. Tubular secretion of

creatinine varies among and within individual persons, especially in those with a mild to- moderate reduction in the GFR (Levey, 1999). Some drugs, including trimethoprim and cimetidine, inhibit creatinine secretion, thereby reducing creatinine clearance and elevating the serum creatinine level without affecting the GFR (Levey, 1990).The generation of creatinine is determined primarily by muscle mass and dietary intake, which probably accounts for the variations in the level of serum creatinine observed among different age, geographic, ethnic, and racial groups (Jafar *et al*., 2003). Extrarenal elimination of creatinine may be increased at low levels of GFR; this increase is mainly related to the degradation of creatinine by intestinal bacteria and can be affected by the use of antibiotics (Stevens and Levey, 2005). For these reasons, the relationship between the levels of serum creatinine and GFR varies substantially among persons and over time. The use of a single reference range for serum creatinine to distinguish between a normal GFR and an abnormal one can be misleading (Stevens and Levey, 2005; Myers *et al*., 2006).

1.2.10 Microalbumin (urine)

Albuminuria is a well-known predictor of poor renal outcomes in patients with type 2 diabetes and in essential hypertension (Keane *et al*., 2003). Albuminuria has also been shown to be a predictor of cardiovascular outcomes in these populations. There is emerging data that reduction of albuminuria leads to reduced risk of adverse renal and cardiovascular event (Brenner *et al*., 2001; Ibsen *et al*., 2005). It has become increasingly clear that albuminuria should not only be measured in all patients with type 2 diabetes and hypertension, but also that steps should be taken to suppress albuminuria in order to prevent future renal and cardiovascular adverse events. Albuminuria may reflect underlying

renal expression of vascular damage, endothelial dysfunction, hypertension, (Stehouwer *et al*., 2004) and low-grade of inflammation (Ramachandran, 2005).

1.2.11 C- reactive protein

C-reactive protein (CRP) is an acute-phase protein that serves as an early marker of inflammation or infection. The protein is synthesized in the liver and is normally found at concentrations of less than 10 mg/L in the blood. During infectious or inflammatory disease states, CRP levels rise rapidly within the first 6 to 8 hours and peak at levels of up to 350–400 mg/L after 48 hours CRP binds to phosphocholine expressed on the surface of damaged cells, as well as to polysaccharides and peptosaccharides present on bacteria, parasites and fungi (Ballou and Kushner, 1992). This binding activates the classical complement cascade of the immune system and modulates the activity of phagocytic cells, supporting the role of CRP in the opsonization (i.e. the process by which a pathogen is marked for ingestion and destruction by a phagocyte) of infectious agents and dead or dying cells (Ballou and Kushner, 1992). When the inflammation or tissue destruction is resolved, CRP levels fall, making it a useful marker for monitoring disease activity (Young *et al.*, 1991).

CRP has been most widely measured using enzyme-linked immunosorbent assays (ELISA), immunoturbidimetry, or antibody-based nephelometric assays, which are typically sensitive to concentrations of 5–20 mg/L. Recent awareness of the utility of measuring CRP as a risk factor for cardiovascular disease has led to the development of high-sensitivity CRP (hs-CRP) assays to detect lower levels of CRP; these assays are sensitive to 0.5–10 mg/L (Ledue *et al*., 2003). CRP levels are unaffected by anemia, protein levels, red blood cell shape or patient age

or sex (Clyne and Olshaker, 1999). However, in women, CRP concentrations tend to be higher late in pregnancy.

1.2.12 Ceruloplasmin

Ceruloplasmin, is the major copper-carrying protein in the blood. It is more negatively charged than albumin and therefore more difficult to be filtered by the glomerulus.

Urinary ceruloplasmin excretion is higher in type 2 diabetic patients compared to controls (Narita *et. al.,* 2004), even in the normoalbuminuric phase (Narita *et al.,* 2001 ; Narita *et al.,* 2008). It correlates well with albumin excretion rate (Shi *et al*, 2001) and predicts the development of microalbuminuria in normoalbuminuric patients (Narita *et al*., 2006). Glycemic control (Narita *et al*., 2001) and low-dose losartan (Narita *et al*., 2008) revert the increased urinary ceruloplasmin excretion in normoalbuminuric patients. And diurnal changes in the systolic blood pressure significantly correlate with urinary ceruloplasmin excretion, but not with UAE (Hosoba *et al*., 2009).

The ceruloplasmin/creatinine ratio is higher in diabetic nephropathy compared to nondiabetic nephropathy patients (Qin *et al*., 2004). It has been reported that urine ceruloplasmin/ creatinine ratio has a sensitivity of 90-91%, specificity of 61–66% and 75% concordance, in diagnosing diabetic nephropathy (Shi *et al*, 2001; Qin *et al*., 2004).

Chapter Two

Subjects Materials and Methods

Materials and Methods

2.1 Materials

2.1.1 Equipment

The equipment used and their sources are given in table (2-1).

Table (2-1): The equipment used and their sources.

Name of Equipment	Company	Origin
Automatic micropipettes	Dragon MeD	China
Automatic micropipettes (0.5-10)	Eppendorf Research plus	Germany
Blood Mixer	KJMR2	China
Centrifuge	Remi	India
Comblizyer (urinelyzer)	Human	Germany
Disposable gel and clot activator tube	AFCO-DISPO	Jordan
EDTA K2(2.5 ml)	AL-Hanof factory	Jordan
Eppendorf	CETOTEST	china
Microplate reader	AWARENESS	USA
Nycocard reader	Nycocard	Sweden
Pipette tips		china
Plain tube	AFCO-DISPO	Jordan
Plastic disposable syringes; 5ml	QJECT	Qatar
Printer	EPSON	UK
Reflotron plus	Roche	Germany
Refrigerator and freezer (-20°C)	LG	Korea

Rule		China
Spectrophotometer	SHIMADZU	Japan
Squirt bottle		china
Volumetric cylinders	Volac	England
Water bath	Elektro.mag	

2.1.2 Chemicals

The chemicals used and their sources are given in table (2-2).

Table (2-2): The used chemicals and their sources.

Name of chemicals	**Origin**
Blood urea kit	BioMerieux
Cholestrol kit	Biolabo
C-reactive protein (CRP) kit	Nycocard
Creatinine strip (for serum)	Roche
Glucose kit	Spinreact
Glycosylated hemoglobin (HbA1c) kit	Nycocard
HDL cholesterol strip	Roche
Human ceruloplasmin ELIZA kit	Usbiological
Human Transforming growth factor-Beta (TGF-β) ELISA kit	Raybio
Human Tumor necrosis factor-alpha (TNF-α) ELIZA Kit	R&D systems
Immunoglobulin G radial immunodiffusion plate	LTA
Immunoglobulin M radial immunodiffusion plate	LTA
Microalbumin ELIZA kit	Orgentic
Triglceride kit	Biolabo
Urine strip (COMNBINA13)	HUMAN

2.2 Methods

2.2.1 Typ2 Diabetic nephropathy patients:

The study was conducted on 45 type 2 diabetic nephropathy patients, 23 females and 22 males, their ages mean (64.49 ± 10.74)years. The patients were randomly selected from those attending the Al-fajer laboratory at Abdalmajid private hospital between December 2013 and July 2014.

The patients having microalbuminuria or elevated serum creatinine were selected to this study. The information about the age , the presence of hypertension and having treatment were documented from the patients.

2.2.2 Control subjects

For the purpose of comparisons, 29 healthy control subjects comparable to diabetes mellitus patients in respect to age (55.41 ± 14.58) year and gender (12 females and 17 males), were included in the study. The controls were selected among subjects who were apparently healthy in terms of being non-diabetic, with no other endocrine disorders or metabolic kidney diseases and were free of acute illness or infection at time of sampling.

2.3 Collection of samples

2.3.1 Collection of blood samples:

Five ml of blood were obtained from patients and control subjects by venipuncture, using a 5 ml disposable syringe, 3ml of blood and dispensed in a gel tubes and centrifuged at 3000 rpm for 10 minutes to collect serum. The serum was divided into aliquots in Eppendorf tubes for

measuring the RBG, B. urea , s.creatinine, cholesterol and Triglyceride and stored in deep freezer at (-20◦C), other 2 ml were collected into EDTA tubes for measuring HbA1c and EDTA plasma for HDL chol.

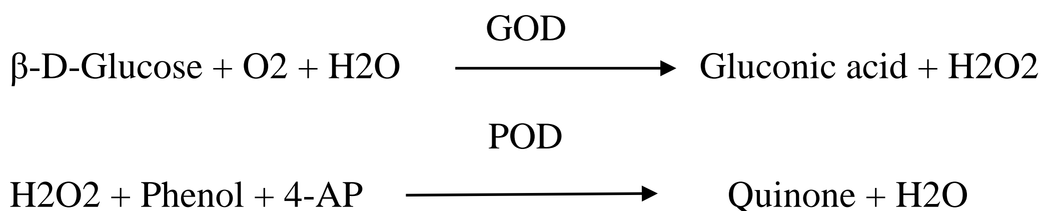
2.3.2 Collection of urine samples:

Urine was collected two times by spot urine collection. The urine was collected into sterile cups without any blood or urinary tract infection.

2.4 Blood glucose determination using glucose kit (spin react).

2.4.1 Principle of the method

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H_2O_2) , is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD):



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

$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{POD} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the glucose concentration in the sample.

2.4.2 Assay procedure:

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, then it were mixed well and incubated at 37ºC for 10 min.

b) An aliquot (10µl) of the standard was added to tubes containing 1ml of reagent R , then it were mixed well and incubated at 37ºC for 10 min (Standard).

c) An aliquot 1ml of reagent R was added to the tubes and incubated at 37ºC for 10 min (Blank).

d) The samples and standard, were read at (505 nm) wavelength against the Blank. The color is stable for at least 30 minutes.

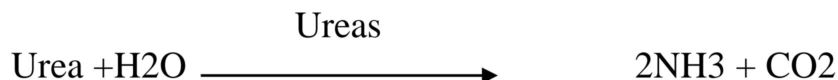
e) The Glucose concentration (mg/dl) was calculated in the sample as follow:

Glucose mg/dl = (A) sample / (A) standard x 100 (standard conc.).

2.5 Blood urea determination using blood urea kit (BioMerieux).

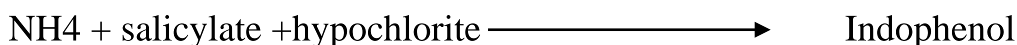
2.5.1 Principle of the method

Urea- kit enables end point enzymatic determination of urea concentrations (Urease – modified Berthelot reaction) in human urine, serum or plasma. Urease hydrolyzes urea by producing ammonium:



In an alkaline medium, the ammonium ions react with the salicylate and hypochlorite to forma green colored indophenol (2, 2 dicarboxy lindo -phenol).

The reaction is catalyzed by the sodium nitroprusside.



The color intensity is proportional to the urea concentration in the sample.

2.5.2 Assay Procedure

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of working reagent (WR), mixed well and incubated at 25ºC for 5min .

b) An aliquot (10µl) of standard was added to tubes containing 1ml of working reagent (WR), mixed well and incubated at 25ºC for 5min .

c) An aliquot of 1ml working reagent was added to tubes and incubated at 25ºC for 10 min.

d) An aliquot of 200 µl of reagent 4 (R4) was added to each sample and standard, then it were mixed well and incubated at 25ºC for 10 min.

e) The sample and standard , were read at (580 nm) wavelength against the blank. The color intensity is stable in dark for two hours at 20-25ºC.

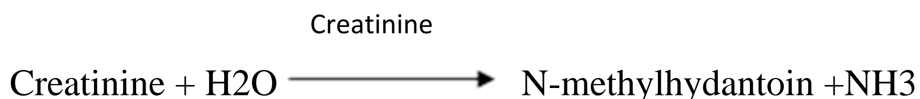
f) The urea concentration was calculated in samples as follow:

Blood urea mg /dl = (A) sample / (A) standard x 50 (standard concentration).

2.6 Serum creatinine determination using refloteron strips (Roche).

2.6.1 Principle of the method

After application to the test strip the sample flows into the reaction zone. In the case of blood after separation of the erythrocytes from the plasma. In a reaction catalyzed by creatinine iminohydrolase creatinine is hydrolyzed to N- methylhydantoin , with release of ammonia in further reaction steps hydrogen peroxide is formed which reacts with an indicator to from a blue dye which is proportional to the creatinine concentration in the sample:



Sarcosine oxidase

N-methylhydantoinase

Carbamoylsarcosine hydrolase

At a temperature of 37°C the dye formed is measured at 642nm the creatinine concentration displayed after about 120 seconds in mg/dl or µmol/l depending on how the instrument that been set.

2.6.2 Assay Procedure

a) An aliquot (30µl) of serum was added by used Reflotron pipette to the center of red application zone as a drop on creatinine strips.

b) Creatinine test strip was placed in reflotron instrument.

c) Creatinine concentration was calculated automatically and the result was displayed on screen of the reflotron instrument.

2.7 Determination of Estimated Glomerular filtration rate (eGFR)(Levey *et al.*, 2000).

The following formula was used for calculated estimated GFR:

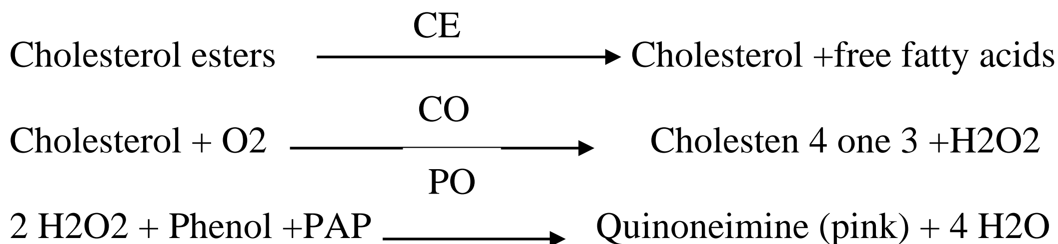
$$eGFR = 186.3 \times [sCr]^{-1.154} \times age^{-0.203}$$

Age and sCr = serum creatinine concentration was measured in years. This formula is for white males. For females was multiplied by 0.742.

2.8 Serum cholesterol determination using Cholesterol kit (Biolab)

2.8.1 Principle of the method

Enzymatic method which reaction scheme is as follows:



2.8.2 Assay procedure:

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, mixed well and incubated at 37ºC for 5 min.

b) An aliquot (10µl) of the serum was added to tubes containing 1ml of Standard, then it were mixed well and incubated at 37ºC for 5 min (Standard).

c) An aliquot (10µl) of distilled water was added to tube containing 1ml of reagent R and incubated at 37ºC for 5 min (Blank).

d) The samples and standard were read at (500 nm) wavelength against the Blank. The color is stable for 1 hour.

e) The Cholesterol concentration (mg/dl) was calculated in the sample as follow:

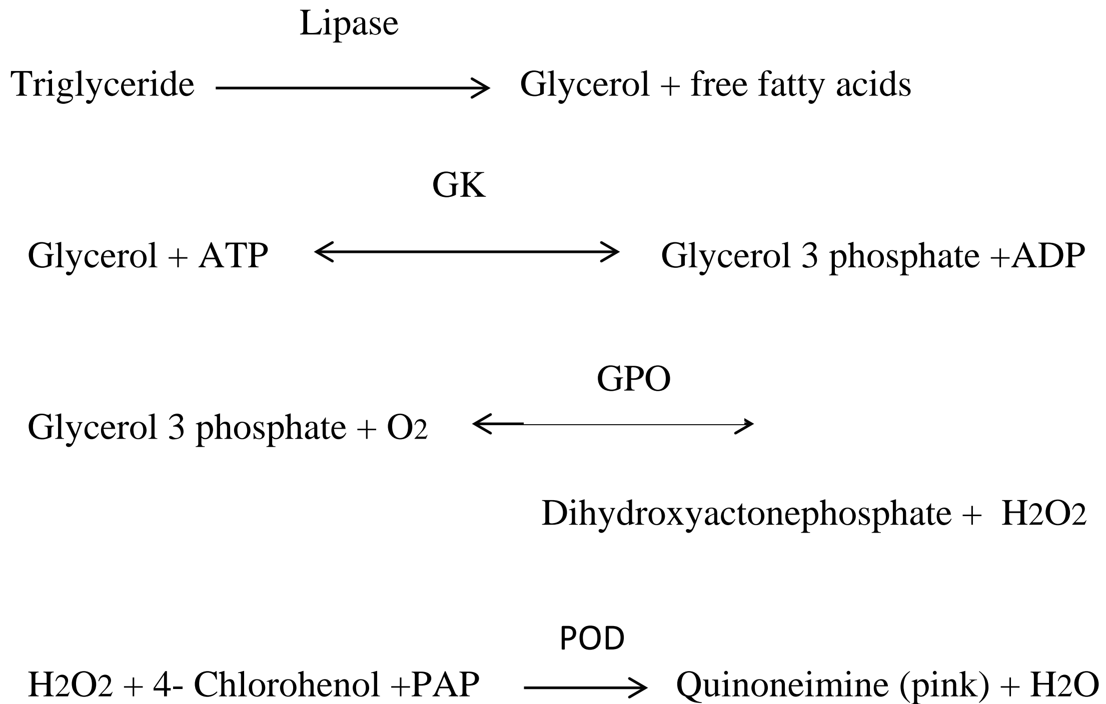
Cholesterol = (A) sample / (A) standard x 200 (standard conc.).

2.9 Serum triglycerides determination using triglycerides kit (Biolab).

2.9.1 Principle of the method

Fossati and Prencipe method associated with Trinder reaction .

Reaction scheme is as follows :



The absorbance of the colored complex (quinoneimine), proportional to the amount of the triglyceride in the specimen, is measured at 500 nm.

2.9.2 Assay procedure:

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, then it were mixed well and incubated at 37ºC for 5 min.

b) An aliquot (10µl) of the serum was added to tubes containing 1ml of Standard, then it were mixed well and incubated at 37ºC for 5 min (Standard).

c) An aliquot (10µl) of distilled water was added to tubes containing 1ml of reagent R and incubated at 37ºC for 5 min (Blank).

d) The samples and standard were read at (500 nm) wave length against the Blank. The color is stable for 1 hour.

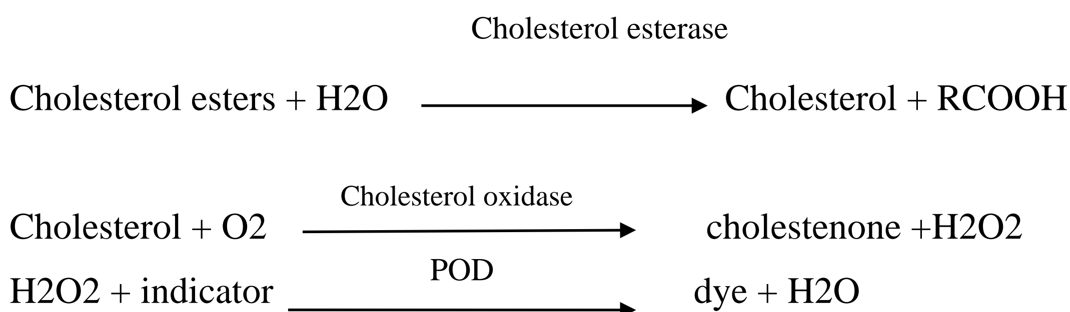
e) The Triglyceride mg/dl was calculated in the sample as follow:

Triglyceride (mg/dl) = (A) sample / (A) standerd x 200 (standard conc.)

2.10 Determination of High Density Lipoprotein Cholesterol level (HDL Cholesterol) (HDL Cholesterol Reflotron).

2.10.1 Principle of the method

The dextran sulphate /Mg^2 on the test strip precipitates the chylomicrons, VLDL and LDL, leaving HDL in the sample. The cholesterol concentration of this HDL is then determined enzymatically. The cholesterol esters are cleaved into the corresponding fatty acid and cholesterol which is then oxidized to cholesterol and hydrogen peroxide in the presence of oxygen. In a further reaction step catalyzed by the enzyme peroxide a redox indicator, resulting in a blue dye which is proportional to the cholesterol concentration in the sample:



At the temperature of 37°C the dye formed is measured at 642 nm and the HDL cholesterol concentration is displayed about 150 seconds in mg/dl .

2.10.2 Assay procedure:

a) The test strip was removed from the container.

b) un warp the strip , taking care not to bend it

c) Using the reflotron pipette, draw the sample material into the pipette (avoiding bubbles) and apply as a drop to the center of the yellow application zone. Take care not to touch the application zone with the pipette tip. Required volume of specimen for application (30 µl).the sample required is EDTA plasma. With the sliding cover open, the test strip was placed on to guide within 15 sec and slide forward horizontally until it locks into place. The sliding cover was closed.

The instrument displays HDL to confirm that it has correctly read the test specific magnetic code. The display shows the number of second left before the result s display. The HDL cholesterol concentration is calculated automatically from readings.

2.11 Determination the Low Density Lipoprotein Cholesterol level (LDL) (Tietz, 1995).

LDL cholesterol= Total cholesterol _ Triglyceride /5_HDL Cholesterol

2.12 Glycosylated hemoglobin determination using HbA1c kit (Nycocard).

2.12.1 Principle of the method

NycoCard HbA1c is a boronate affinity assay. The kit contains test devices with a porous membrane filter, test tubes pre-filled with reagent and a washing solution. The reagent contains agents that lyse erythrocytes and precipitate hemoglobin specifically, as well as a blue boronic acid conjugate that binds cis-diols of glycated hemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse. All hemoglobin

precipitates. The boronic acid conjugate binds to the cis-diol configuration of glycated hemoglobin. An aliquot of the reaction mixture is added to the test device, and all the precipitated hemoglobin, conjugate-bound and unbound, remains on top of the filter. Any excess of colored conjugate is removed with the washing solution. The precipitate is evaluated by measuring the blue (glycated hemoglobin) and the red (total hemoglobin) color intensity with the NycoCard® READER II, the ratio between them being proportional to the percentage of HbA1c in the sample.

2.12.2 Assay procedures

a) An aliquot (5µl) of whole blood was added to test tube with R1/Reagent, and mixed well .The tube was leaved for minimum 2 min , maximum 3 min. The R1/ Reagent was equilibrated to room temperature (20-25ºC) before used.
b) Remixed to obtain a homogenous suspension. (25µl) of the mixture was applied to a TD/ test device ,the pipette was holed approximately 0.5 cm above the test well and empty the pipette quickly in the middle of the test well. The mixture was allowed to soak completely into the membrane. Then wait for maximum 10 sec. Air bubbles was avoided.
c) 25µl of R2/washing solution was applied to the TD/test device, the reagent was allowed to soak completely into the membrane. The wait for minimum 10 sec. Air bubbles was avoided.
d) The test result was read within 5 min using the NycoCard reader , the NycoCard reader instruction manual was followed.

2.13 C-reactive protein determination using CRP kit (Nycocard).

2.13.1 Principle of the method

Nycocard CRP single test is a solid phase, sandwich-format, immunometric assay. In the test well of the device there is a membrane coated with immobilized CRP-specific monoclonal antibodies , A diluted sample is applied to the test device .When the sample flows through the membrane , the C-reactive proteins are captured by the antibodies . CRP trapped on the membrane will then bind the gold antibody conjugate added, in a sandwish-type reaction. Unbound conjugate is removed from the membrane by washing the solution. A paper layer underneath the membrane absorbs excess liquid .In the presence of a pathological level of CRP in the sample, the membrane appears red-brown with colour intensity proportional to the CRP concentration of the sample. The colour intensity is measured quantitatively with the NycoCard READER.

2.13.2 Assay procedure:

a) An aliquot (5µL) of serum was filled in capillary, and capillary was drop into the tube with dilution liquid (R1). Close the tube and mix well for 10 seconds. Air bubbles were avoided in the capillary and excess sample on the outside of the capillary.
b) An aliquot (50µL) of diluted serum was applied to the test device (TD).the sample was allowed to soak into membrane approximately 30 seconds. Air bubbles were avoided on the membrane.
c) One drop conjugate (R2) was applied to the test device (TD). The reagent was allowed to soak into the membrane approximately 30 seconds. The droplet bottle was held vertically, about 1cm above membrane.

d) One drop washing solution (R3) was applied to the test device (TD). The reagent was allowed to soak into membrane approximately 20 seconds. The droplet bottle was held vertically, about 1cm above the membrane.
e) The result was read within 5 minutes using the NycoCard READER. The READER user instruction manual was followed.

2.14 Determination of serum IgG and serum IgM protein by using radial immunodiffusion plate.

2.14.1 Principle of the method

The examined protein, diffusing in agarose gel containing a specific antibody will form an immune-complex, visible as a ring around the well. The ring diameter is direct proportional to the concentration of the analyzed protein .The proportion corresponds to the diffusion time. At the end of 72 hour the square of diameter will be in linear proportion to the concentration of the sample with the plate is supplied a reference table in which each diameter of the halo is associated a concentration.

2.14.2 Assay procedure:

The plate was removed from its envelope and leaved to stand at room temperature for few minutes so that any condensed water in the wells can evaporated. The wells was filled with (5 µl) of samples and wait it was be completely adsorbing before handling the plate. The plate was closed and place it in refrigerator for 72 hrs. for IgG plate (96 hrs. for IgM plate).

The precipitating ring was measured with appropriate ruler .Read on enclosed reference table the concentration value corresponding to precipitating ring diameter.

2.15 Determination of transforming growth factor beta-1 (TGF-β1) (RayBio ELIZA kit).

2.15.1 Principle of the method

The RayBio Human TGF- β1 ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TGF-β1 in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for human TGF- β1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TGF- β1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated antihuman TGF- β1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TGF- β1 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2.15.2 Assay procedure:

Before carrying out the assay procedure, the kit was left at room temperature (18-25 ℃) to equilibrate, as suggested by manufacturer. The assay was carried out of the following the instruction in kits leaflet:

1. Serial concentration (0, 0.0823, 0.247, 0.741, 2.222, 6.667, 20 and 60) ng/ml of standard were made using the diluent.
2. An aliquot (100 µl) of each standard and serum samples was added into appropriate wells. The wells was covered and incubated for 2.5 hours at room temperature with gentle shaking.
3. The solution was discarded and wash 4 times with 1x wash solution. Each wells was washed by filling with wash buffer (300

µl) pipette was used. After the last wash any remaining wash buffer was removed by aspirating or decating. The plate was inverted and blot against clean paper towels.

4. An aliquot (100 µl) of 1x prepared biotinylaed antibody to each well. The plate was incubated for 1 hour at room temperature with gentle shaking.
5. The solution was discarded and wash four times as in (step 3).
6. An aliquot (100 µl) of prepared streptavidin solution was added to each well. The plate was incubated for 45 minutes at room temperature with gentle shaking.
7. The solution was discarded and wash four times as in (step 3).
8. An aliquot (100 µl) of TMB substrate reagent was added to each well. The plate was incubated for 30 minutes at room temperature in dark with gentle shaking.
9. An aliquot (50 µl) of stop solution was added to each well. Then was read at 450 nm immediately.
10. Calculation : the sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample Figure (2.1) Using curve equation

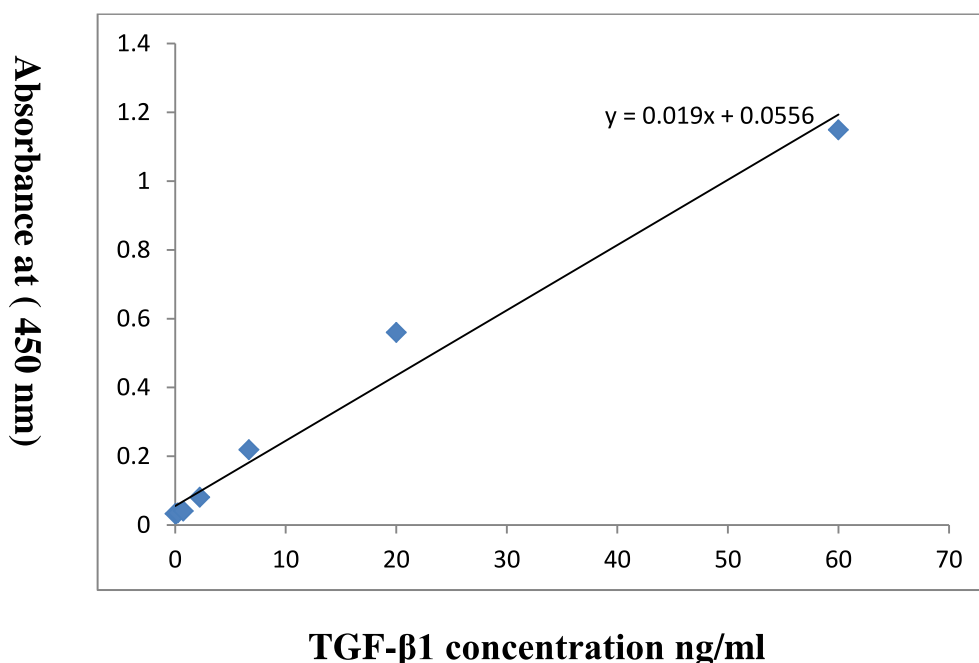


Figure (2.1) Standard curve of TGF-β1

The result was in ng/ml then it was converted to Pg/ml.

2.16 Determination of Tumor necrosis factor –alpha (TNF-α) (R&D systems EILZA kit).

2.16.1 Principle of the method

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.16.2 Assay procedure:

1. All reagent and samples was left at room temperature before use.
2. An aliquot (50 µl) of assay diluent was added to each well. The assay diluent was mixed well before and during use.
3. An aliquot (200 µl) of standard and serum sample was added to each well. Then was covered with adhesive strip and incubated for 2 hours at room temperature.
4. Each well was aspirated and washed. The process of washing was repeated four time each wells was filed with wash buffer (400 µl) squirt bottle was used. After the last wash any remaining wash buffer was removed by aspirating and decanting. The plate was inverted and blot it against clean paper.
5. An aliquot (200 µl) of TNF-α conjugate was added to each well. Then was covered with a new adhesive strip and incubated for 2 hours at room temperature.
6. The wells were aspirated and washed as in step 4.
7. An aliquot (200 µl) of substrate solution was added to each well , then were incubated for 20 minutes at room temperature . The plate was protected from light.
8. An aliquot (50 µl) of stop solution was added to each well.
9. The optical density of each well was determined within 30 minutes by set the microplate reader to 450 nm and calculated the results(Figure 2.2).

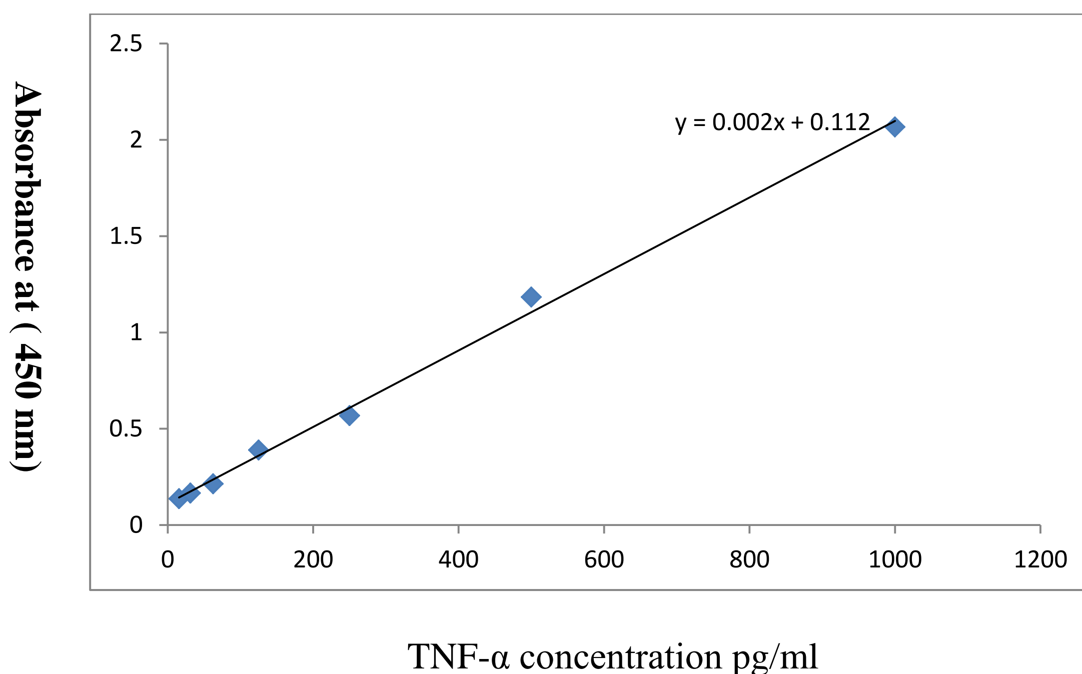


Figure (2.2) Standard curve of TNF-α

2.17 Creatinine and microalbumin detection by urine test strip (COMBINA 13).

Two methods were used for the determination of microalbumin by urine strip and ELISA technique , the microalbumin strip was used as a screening test for microalbuminuria .

Assay procedure:

1. Only well mixed, non-centrifuged urine was used which should not be older than two hours.
2. The test strip was immersed in the urine for approximately 2 sec, so that all reagent are covered. The excess urine was removed from the strip by wiping the edge of the strip on the urine container or on absorbent paper.
3. The urine strip was reflectometric read with Combilyzer.

2.18 Determination of Microalbumin concentration in urine (Orgentic ELIZA kit).

2.18.1 Principle of the method

Highly purified human albumin is bound to micro wells. Calibrators, controls and undiluted patient samples are pipetted together with anti-human-albumin-peroxidase conjugate in the wells. Microalbumin, if present in diluted urine, will compete with coated albumin for binding of the anti-albumin-conjugate. Washing of the microwells removes unspecific components. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of color is inversely proportional to the concentration of albumin present in the original sample.

2.18.2 Assay procedure:

1. Sufficient number of microplate modules were prepared to accommodate calibrators, controls and patient samples.
2. An aliquot (20 µl) of calibrators, controls and undiluted patient samples was added into the wells.
3. An aliquot (100 µl) of enzyme conjugate solution was added into each well.
4. The microplate was incubated for 30 minutes at room temperature (20-28ºC) .
5. The contents of the micro wells were discarded and washed 3 times with (300µl) of wash solution.
6. An aliquot (100µl) of TMB substrate solution into each well.

7. The microplate for 15 minutes at room temperature.
8. an aliquot (100µl) of stop solution was added to each well of the modules and it was leaved untouched for 5 minutes.
9. the optical density was read at 450 nm and the results was calculated. The developed color is stable for at least 30 minutes.
10. The result was calculated depend on the stander curve which is shown in the figure (2.3).

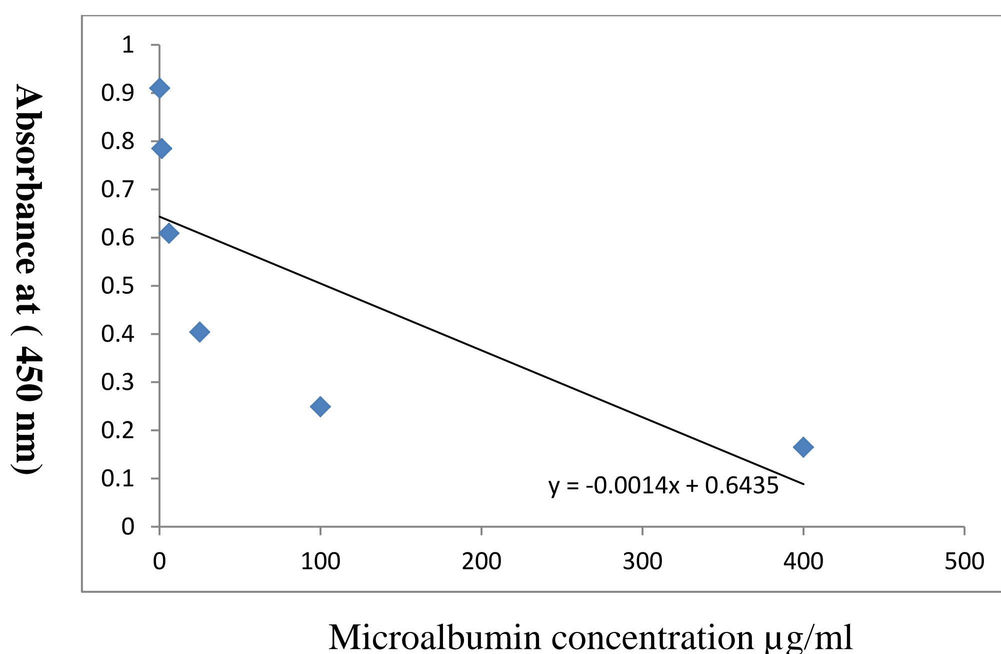


Figure (2.3) Standard curve of Microalbumin

2.19 Determination of Ceruloplasmin concentration in urine using human ceruloplasmin ELISA kit (USBiological)

2.19.1 Principle of the method

The wells of the microtiter strips provided in this kit have been pre-coated with an antibody specific to CP standards or samples are then added to the appropriate wells with a biotin-conjugated antibody specific to next, avidin conjugated to horseradish peroxides (HRP) is added to each well and incubated. After TMB substrate solution is added, only

those wells that contain CP, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometically at a wavelength of 450nm .

The concentration of CP in the samples were determined by comparing the O.D. of the samples to the standard curve.

2.19.2 Assay procedure:

a) The number of the wells required for standard, and samples was determined.
b) An aliquot (100µl) of each standard and urine samples were added into the wells, then the plate was sealed and incubated for 2 hours at 37ºC. The liquid of each well was removed without washing.
c) an aliquot (100µl) of detection reagent A working solution was added to each well , then the plate was sealed and incubated for 1hour at 37ºC.
d) The solution of each well was aspirated then washed with 350 µl of washing solution by using squirt bottle. The washing was repeated 3 times, then any remaining wash buffer was removed completely by decating, the plate was inverted and blot against absorbent paper.
e) An aliquot (100µl) of detection reagent B working solution to each well, then the plate was sealed and incubated for 30 minutes at 37ºC.
f) The washing of each well was repeated 5 times in process as conducted in step (e).
g) An aliquot (90 µl) of TMB substrate was added to each well, then covered with new plate sealer and incubated for 25 minutes at 37ºC in the dark .
h) An aliquot (50 µl) of stop solution to each well was added.

i) The absorbance was read at wavelength of 450 nm immediately by ELISA reader and the result was calculated depended on standard curve (figure 2.4).

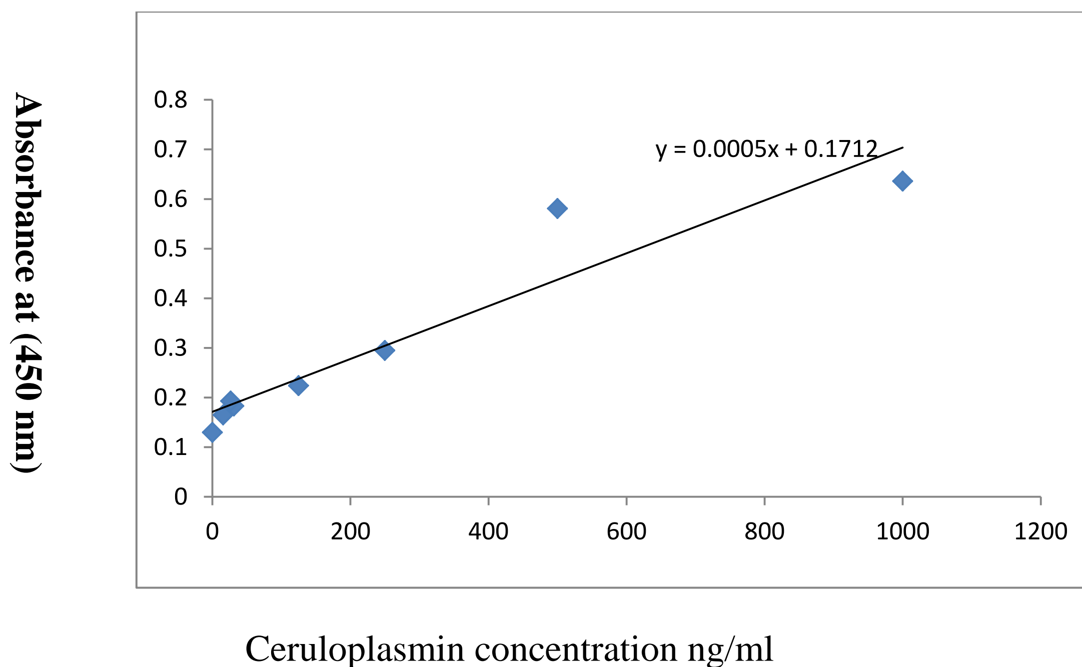


Figure (2.4) Standard curve of ceruloplasmin

2.20 Statistical Analysis

Statistical analysis was carried out using statistical package (SPSS v.20). Students t-test was used and the differences were considered significant at the level of $P \leq 0.05$.

The values of the studied parameters were given in term of mean ± standard deviation.

Chapter Three

Results and Discussion

3 Results and Discussion

3.1 Biochemical parameters and urine parameters in diabetic nephropathy

The result of Random blood glucose (RBG) was 91.90 mg/dl *vs* 49.52 mg/dl , Blood urea was 38.28 mg/dl *vs* 56.60 mg/dl , Serum ceratinine was 0.81 mg/dl *vs* 1.82 mg/dl , eGFR was 91.90 ml/min/1.73m^2 *vs* 49.52 ml/min/1.73m^2, low density lipoprotein (LDL) was 102.48 mg/dl *vs* 115.66 mg/dl and the glycosylated hemoglobin A1C was 5.64 *vs* 7.90 shown a significant ($P \leq 0.05$) increase in type 2 diabetic nephropathy patients as compared to healthy controls (Table 3.1). While the result of cholesterol 177.52 mg/dl *vs* 191.05 mg/dl , the triglyceride mg/dl 162.48 *vs*. 181.60 mg/dl and high density lipoprotein (HDL) was 42.45 mg/dl *vs* 39.66 mg/dl showed no significant ($P > 0.05$) difference between the Type 2 diabetic nephropathy patients and healthy controls, Also the patients age showed no significant ($P > 0.05$) difference between the patients and healthy controls which is shown in table (3.1) (Figure 3.1) .

The levels of microalbumin were 32.54 μg/ml vs 200.7 μg/ml increased significantly ($p \leq 0.05$) in Type 2 diabetic nephropathy patients compared with healthy control. Also, urine ceruloplasmin levels were 18.96 ng/ml vs 60.72 ng/ml shown a significant increase in Type 2 diabetic nephropathy compared healthy controls . Albumin / creatinine ratio were 2.46 mg/mmol vs 7.57 mg/mmol significantly increase in Type2 diabetic compared with healthy control. But urine creatinine shown non significant difference (14.16 vs 13.17) between the Type 2 diabetic patients and healthy controls as shown in table (3.2).

Table (3.1) :The biochemical parameters in Type 2 diabetic nephropathy and healthy controls.

Parameters	Healthy Control (n=29)	T2 DN patients (n=45)	p-value
RBG (mg/dl)	94.90 ± 11.43	154.91 ± 61.65	**< 0.05**
Blood .Urea (mg/dl)	38.28 ± 3.85	56.60 ± 24.53	**< 0.05**
S.Creatinine (mg/dl)	0.81 ± 0.20	1.82 ± 1.07	**< 0.05**
GFR (ml/min/1.73m^2)	91.90 ± 20.48	49.52 ± 28.26	**≤ 0.05**
HbA1c %	5.64 ± 0.35	7.90 ± 1.70	**< 0.05**
S.Cholestrol (mg/dl)	177.52 ± 34.25	191.06 ± 54.50	N.S.
S.TG (mg/dl)	162.48 ± 98.66	181.60 ± 57.73	N.S.
HDL (mg/dl)	42.45 ± 5.55	39.66 ± 5.50	N.S.
LDL (mg/dl)	102.48 ± 29.98	115.66 ± 52.18	**≤0.05**

N.S.: non- significant

Values are expressed as mean ± SD.

The increase in RBG and HbA1c , seems to agree with Aggarwal and Kumar (2014) who reported an increase in glycosylated hemoglobin with the presence of microalbuminria.

Glucose is the risk factors for microalbuminuria. Gupta *et al.* (1991) demonstrated that HbA1c was associated with microalbuminuria.

John *et al* .(1991) reported that poor glycemic control, and raised blood pressure as risk factors of microalbuminuria. A long and permanent derangement of carbohydrate metabolism, that is, in decompensation stage of diabetes mellitus and in the absence of adequate treatment, the level of HbA1c increases.

Table (3.2) : Parameters in Urine of Type 2 diabetic nephropathy and healthy control.

Parameters	**Healthy control (n=29)**	**T2 DN patient (n=45)**	p-value
U.Creatinine (mmol/L)	14.16 ± 7.23	13.17 ± 7.60	N.S.
MALB strip (mg/L)	35.71 ± 19.87	99.78 ± 65.45	**< 0.05**
MALB (μg/ml)	32.54 ± 25.22	200.70 ± 52.73	**< 0.05**
Albumin /creatinine ratio (mg/mmol)	2.46	7.57	**<0.05**
U.Ceruloplasmin (ng/ml)	18.96 ± 5.46	60.72 ± 10.93	**< 0.05**

MALB strip :urine microalbumin measured by strips . MALB : urine microalbumin measured by ELISA.

N.S.: non- significant

Values are expressed as mean ± SD

At the same time, hemoglobin and other body proteins undergo an enzymatic glycosylation. These can cause receptor dysfunction, thickening of membranes and metabolic disorders, which are typical for progression of diabetes mellitus (Verma *et al.* , 2006).

The increase in urea level is seen when there is damage to the kidney or the kidney is not functioning properly. Elevation in of blood urea level and blood sugar level clearly indicates that the increase blood sugar level causes damage to the kidney. Research conducted by Anjaneyulu *et al*. (2004) found that the increase urea and serum creatinine in diabetic rats indicates progressive renal damage , while Vijay *et al (1994)* reported duration of diabetes, systolic and diastolic

blood pressure, age of the patient, and serum creatinine to be associated with proteinuria.

Measurement of serum creatinine is convenient and inexpensive for assessing renal function and consistently elevated levels indicate chronic kidney disease. However, some patients have a substantial decrease in glomerular filtration rate, while their serum creatinine concentration remains within the normal range. Hence it is a poor screening test for mild kidney dysfunction. Minimizing microalbuminuria and having a tight glycemic control is an important treatment goal for patients with DM. (Sheikh ,2009)

Strong relationship was found between blood urea level and blood sugar level. To monitor the diabetic patients, estimation of blood urea level along with blood sugar level could be important. Shrestha *et al.*(2008) found the gender was not a determining factor for the diabetes. There was not relationship between gender and the blood sugar levels like wise significant relation between gender and urea level was also not observed .

There no significant differences in cholesterol ,Triglyceride and HDL while LDL showed significant difference . This result was agreed with Iranparvar *et al.* (2006) which found serum cholesterol level, HDL and LDL-cholesterol were within the normal range in both groups and no significant differences were found between them, Because the patients were currently taking statins and did not find any relation between serum cholesterol level and microalbumin .

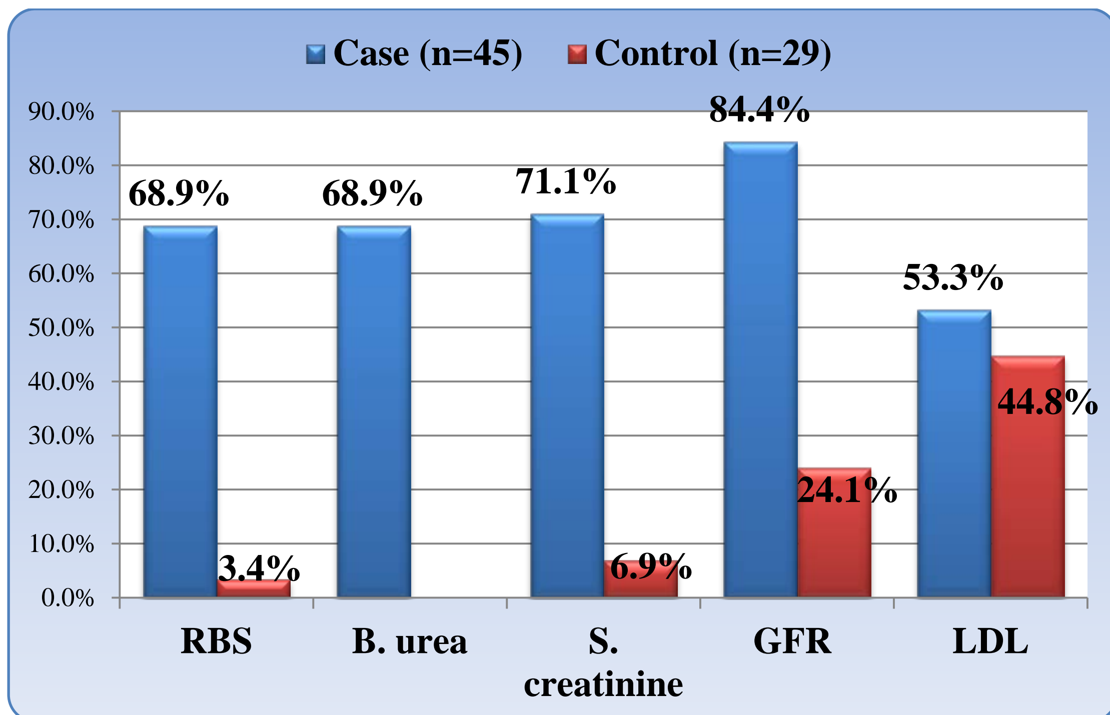


Figure (3.1) : the percentages of biochemical significant variables showing significant changes in Type 2 diabetic nephropathy patients compared with the healthy control .

Microalbuminuria was described for more than three decades ago as a predictor of nephropathy and associated with higher cardiovascular risk (Khosla *et al*., 2006). However, it has also been considered as the first indication of renal injury in patients with diabetes. Thus screening for microalbuminuria is currently recommended for all patients with diabetes or kidney disease (Molitch *et al.,* 2004).

Measurement of albumin in urine has important role in secondary prevention, to decide treatment and monitor response to treatment. The measurement of albumin in urine is not standardized. There is a large variation for estimation of albumin in urine between different laboratories and between different methods. Furthermore, there is no consistency among laboratories regarding sample type, units of reporting, and

reference intervals or cutoff values (McQueen and Don-Wauchope, 2008).

Ahmedani *et al*. (2005) stated that microalbuminuria was the first clinical detectable sign of involvement of the kidney. Microalbuminuria affects between 20-40% of subjects, 10-15 years after the onset of DM. Once microalbuminuria is present, it progresses over 5-10 years to proteinuria in 20-50% of subjects. With microalbuminuria, the decline in renal functions varies but average reduction in glomerular filtration is around 10-12 ml/min/year. Progression to end stage renal disease is accelerated by hypertension. The process of renal involvement is step wise and microalbuminuria (also referred to as incipient nephropathy) is potentially reversible. Microalbuminuria is also strongly associated with traditional cardiovascular risk factors and cardiovascular complications.

Glycemic control can prevent progression to microalbuminuria. Preventing the progression of each step of renal disease in patients with diabetes microalbuminuria, diabetic nephropathy, and end stage renal disease or death can be achieved with blood pressure control and the use of antiangiotensin inhibiting therapies such as angiotensin-converting-enzyme (ACE) inhibitors and angiotensin II receptor blockers . Primary prevention (preventing microalbuminuria) can be achieved through good glycemic and blood pressure control and through the use of an ACE inhibitor in both type 1 and type 2 diabetes.

Intensive and multifactorial interventions are recommended for patients with microalbuminuria to delay progression of cardiovascular disease . Despite the clear association of microalbuminuria with cardiovascular risk, limited studies have been performed to evaluate strategies to reduce microalbuminuria and evaluate the impact on cardiovascular events. The prevention of renal and vascular endstage disease intervention trial is the only randomized trial to study the effect of albuminuria lowering in

microalbuminuric, otherwise healthy individuals (Asselbergs *et al.,* 2004).

These variability in the results of urinary and serum creatinine may be attributed to the occurrence of low glomerular filtration rate, which result from renal hypertrophy, progressive mesangial deposition of extracellular matrix (ECM) and progressive glomerular capillary occlusion. The factors responsible for the deposition and accumulation of extracellular matrix material within the kidney are hyperglycemia, glycated proteins, vasoactive hormones, systemic and glomerular hypertension, proteinuria, growth factors, and cytokines which have been implicated in the pathogenesis of diabetic nephropathy (Reeves and Andreoli, 2000).

Urinary ceruloplasmin excretion is higher in type 2 diabetic patients compared to controls that agreed with Narita et.al (2004), it could be predicts for the development of microalbuminuria in normoalbumin patients (Narita *et al.,* 2006). Glycemic control effected on increased urinary ceruloplasmin excretion in normoalbumin patients. And diurnal changes in the systolic blood pressure significantly correlate with urinary ceruloplasmin excretion (Hosoba *et al.,* 2009).

3.2 Serum IgM and serum IgG level in the Type 2 diabetic nephropathy patients :

Table (3.3) shows no significant ($P > 0.05$) difference in serum IgM concentration (144.51 mg/dl *vs* 148.71 mg/dl) and serum IgG concentration (924.37 mg /dl *vs* 1094.40 mg/dl) between Type 2 diabetic nephropathy patients and healthy controls.

Table (3.3): Serum IgM and IgG concentration in Type 2 diabetic nepthropthy patients and healthy controls .

Parameters	Healthy Control (n=29) Mean ± SD	T2 DN patients (n=45) Mean ± SD	P-value
IgM (mg/dl)	144.51 ± 43.69	148.71 ± 37.73	N.S.
IgG (mg/dl)	924.37 ± 224.55	1094.40 ± 290.95	N.S.

N.S.: non- significant

Values are expressed as mean ± SD.

The normal values of IgM is 60 – 280 mg/dl , IgG 800 -1800 mg/dl.

The non significant difference between the mean of serum IgM and IgG concentration in Type 2 diabetic nephropathy patients and healthy control could be explain to transport of proteins across the glomerular capillary walls. The increased urine excretion of IgG and IgM that accompany albuminuria in type2 diabetic nephropathy patients suggests that the dominated pathophysiological mechanism of proteinuria in type 2 diabetic nephropathy may be due to alteration in size selective properties of the glomerular capillary wall (Bakoush *et al*. 2002).

On the other hand, Rodriguez-Segade *et al*.(1991) recorded that raised globulin levels have been reported in studies that described abnormalities in serum immunoglobulin concentrations in patients with diabetes, It has been hypothesized that elements of the innate immune system, such as cytokines or the acute phase reactants that they may stimulate, may contribute to the development of obesity and type 2 diabetes mellitus.

3.3 Serum level of high sensitive C-reative protein (hs-CRP) , Transforming growth factor –beta1 (TGF-β1) and Tumor necrosis factor alpha (TNF-α)

The hs-CRP levels were 11.76 mg /dl *vs* 32.63 mg/dl showed a significant ($p<0.05$)increase in the Type 2 diabetic nepthropahty patients compared with healty control as shown in table 3.4.

The serum levels of TGF-β1 were 129.83 pg/dl *vs*. 153.30 pg/dl , and serum levels of were TNF-α 12.14 pg/dl *vs*. 14.5 pg/dl demonstrated no significant difference between Type 2 diabetic nephropathy patients compared with healthy controls as shown in (Table 3- 4).

Table (3-4) : High sensitive- CRP ,Transforming growth factor -β1 and Tumor necrosis factor -α in Type 2 diabetic nephropathy patients and healthy controls.

Parameters	Healthy control (n=29)	T2 DN Patient (n=45)	p-value
hs-CRP	11.76 ± 8.14	32.63 ± 16.93	<0.05
TGF-β1(pg/dl)	129.83 ± 74.05*	153.30 ± 36.34	N.S.
TNF-α (pg/dl)	14.5 ± 4.14	12.14 ± 4.91	N.S.

N.S.: non- significant

Values are expressed as mean ± SD.

* The highest value 1645 pg/dl. The lower value 37 pg/ml.

The normal values of high sensitive is <0.5 mg/dl , TGF-β1 is 82-102 pg/ml and TNF –α is < 15.6 pg/ml .

Some studies suggest a significant role of inflammation cytokines in the pathogenesis and progression of diabetic nephropathy (Juan *et al.*, 2008). However, inflammation in diabetic nephropathy is activated by metabolic, biochemical, and haemodynamic disorders which progressively and persistently lead to kidney injury (Akira ,2013) .

The availability of highly sensitive assays resulted in that hs-CRP has become an exquisite marker of chronic subclinical inflammation.
In other study, hs-CRP levels in type-2 diabetic patients with microalbuminuria and were significantly greater than that in diabetic patients without albuminurea and healthy individuals. These results are in agreement with the results of Navarro et al,(2003) who compared 65 type-2 diabetic patients and 21 healthy controls and found that serum and urine TNF-α and hs-CRP were significantly higher in diabetic than in control subjects .

TGF-β1 did not change significantly which could be explain due to the percentage of patient that having hypertension , in patients with type I diabetes treated with either a placebo or the ACE-I captopril, the reduction in circulating TGF-β levels was inversely correlated with the rate of decline of the glomerular filtration rate (Sharma *et al.,*1999).

The inability of ACE-I treatment to completely halt the progression of diabetic nephropathy may relate to the incomplete suppression of TGF-b production by these agents. Border and Noble (1998) found that ACE-I or AII receptor antagonists reduced TGF-β production by only about 50%. Likewise, in the captopril study mentioned above ,serum TGF-β levels were reduced only 14% by captopril. Thus, it will be important to develop more effective approaches for suppressing TGF-β production or activity and to determine whether these approaches are more effective in halting the progression of kidney disease (Border and Noble,1997).

Many studies showed a significant increase in TGF-β concentration in diabetic patients with albuminuria whether macro or microalbuminuria (diabetic patients with nephropathy) not with diabetic normoalbuminuria (diabetic patients without nephropathy) or control subject who were hypertensive, these results were supported by Yaqiu *et al.* (2014) who concluded that the serum concentration of TGF-β1 has started to increase in the early stages of diabetic nephropathy and with the development of diabetic nephropathy the serum level of TGF-b1 significantly increased.

Benigni et al. (2003) suggest that anti–TGF-β antibody added to a background of chronic ACE inhibition therapy fully protects from the development of proteinuria and renal injury of overt diabetic nephropathy. Combining TGF-β antibody and ACEI may represent a novel route to therapy and remission of disease for diabetic patients who do not fully benefit ACEI treatment.

Of clinical interest is the fact that ACEI therapy protected the kidney by lowering the levels of TGF-β. Van den Heuvel *et al.*(2009) showed that, in captopriltreated patients, the decrease in the circulating TGF-β level predicted a better preservation of the glomerular filtration rate.

Angiotensin II is a potent stimulus for TGF-b production by kidney cells and acts in synergy with elevated glucose concentrations to stimulate matrix production by renal epithelial cells .Thus, it is likely that some of the beneficial effects of ACEI in diabetic nephropathy (and perhaps other kidney diseases) are related to the suppression of TGF-b production . However, the inability of ACEI treatment to completely halt the progression of diabetic nephropathy may be related to the
incomplete suppression of TGF-b production by these agents .

ACEI effectively limit renal fibrosis with no effect on systemic blood pressure. In addition, ACEI significantly slow the progression of renal failure even in the absence of high blood pressure . Studies have shown that in hypertensive type 2 diabetic patients, ACEI or ARB
treatment reduced proteinuria independent of lowering of blood pressure . The classical view of angiotensin II as a vasoactive agent has been changed to consider it as a true cytokine with an active role in the renal pathology and as a renal growth factor that modulates cell growth and ECM production. The effects of angiotensin II stimulating mesangial cell matrix expression, renal tubular hypertrophy, and renal interstitial fibrosis seem to be mediated by release of TGF-β.

In other studies positive significant correlations between both glucose concentration and glycated hemoglobin and both urinary and serum TGF-β1, and these results agreed with Hellmich *et al*. (2000), Hefini *et al*. (2007) and El Mesallamy *et al*. (2012). Significant positive correlations were found between the concentration of TGF-β1 in urine and both proteinuria and albuminuria in patients with type II diabetes, these results agreed with Rivarola *et al*. (1999) and Hefini et al. (2007).
In other study stepwise regression analysis demonstrated that TNF-α was an independent predictor of urinary albumin excretion while IL-6 was not (Zahran *et al*.,2012). Ng Dp *et al*,.(2008) demonstrated that TNF-α system is likely to exert independent effects on albuminuria and renal function in type 2 diabetes while C reactive protein and IL-6 did not show that . Serum and urine TNF-α were found to be independently and significantly associated with ACR in diabetic patients (Refaat *et al.,* 2010 ; Navarro *et al.,* 2006).

Serum and urine TNF-α and CRP levels are significantly elevated in this group of diabetic patients, and correlate positively with severity of

proteinuria. This suggests a significant role for TNF-α in the pathogenesis and progression of renal injury in diabetes mellitus (Zahran *et al.,* 2012).

3.4 The correlation between parameters

There is a positive correlation between HbA1c and hs-CRP , hyperglycemia can cause increase in glycosylated hemoglobin which led to form advanced glyaction end product (AGE) . AGE may exert an effect on hs-CRP which is considered one of inflammatory proteins .

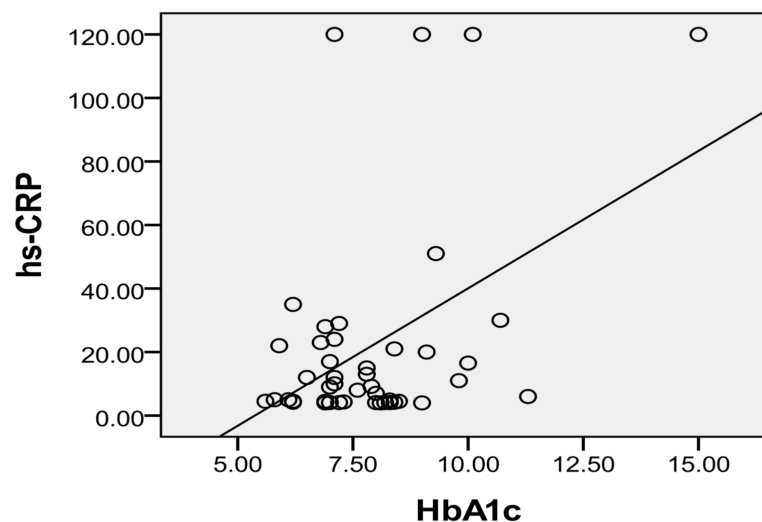


Figure (3.2): Correlation between HbA1c and hs-CRP in type 2 diabetic nephropathy patients (r = 0.452 P <0.05)

There was no correlation between hs-CRP and Microalbumin . Bashir *et al*.(2014) concluded that there is a significant association between the level of serum CRP and microalbuminuria in type 2 diabetics.

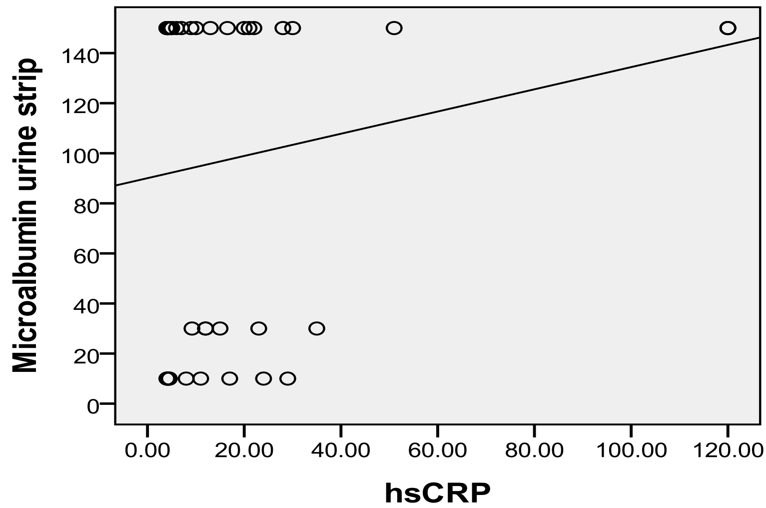


Figure (3.3): No correlation between hs-CRP and Microalbumin urine strip in Type 2 diabetic nephropathy (r=0.221 P >0.05).

In our data shown no correlation between the hs-CRP and TGF-β1,that could be to ACE which have anti –TGF-β1 activity .

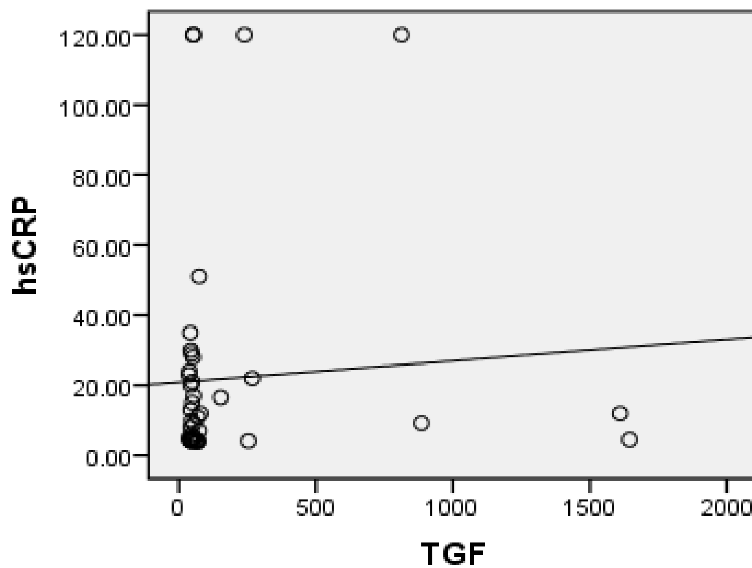


Figure (3.4): no correction between TGF-β and hs-CRP in Type 2 diabetic nephropathy patients . (r=0.06 P >0.05).

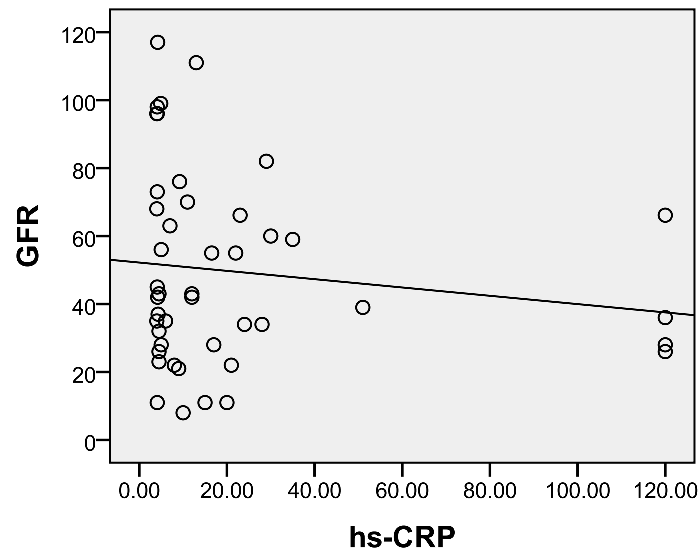


Figure (3.5): No correlation between hs-CRP and GFR in diabetic nephropathy patients (r=- 0.141 P > 0.05)

In figure (3.6) shown asignificant positive correlaction between HbA1c and cerulopalsmin in urine, the advanced glycolyaction end product formed by hyperglycemia that might have effect on increase ceruloplasmin secretion.

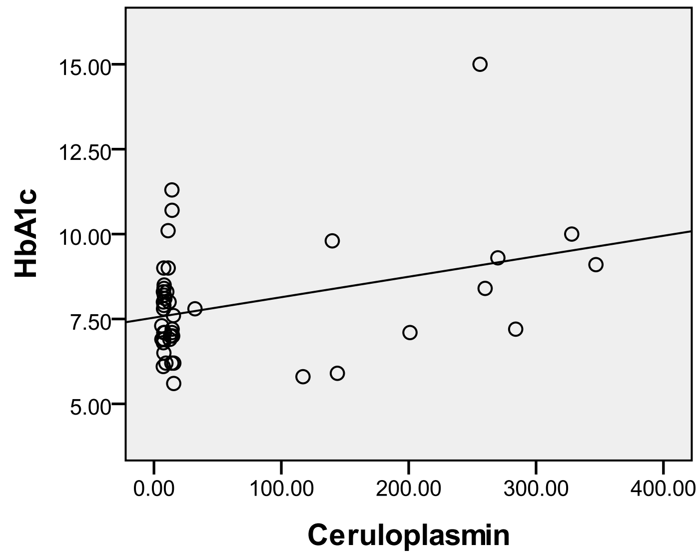


Figure (3.6): Correlation between Ceruloplasmin and HbA1c(r = 0.357 **P < 0.05**).

there was no significant correlation between hs-CRP and ceruloplasmin also no correlation between microalbumin in urine and ceruloplasmin .

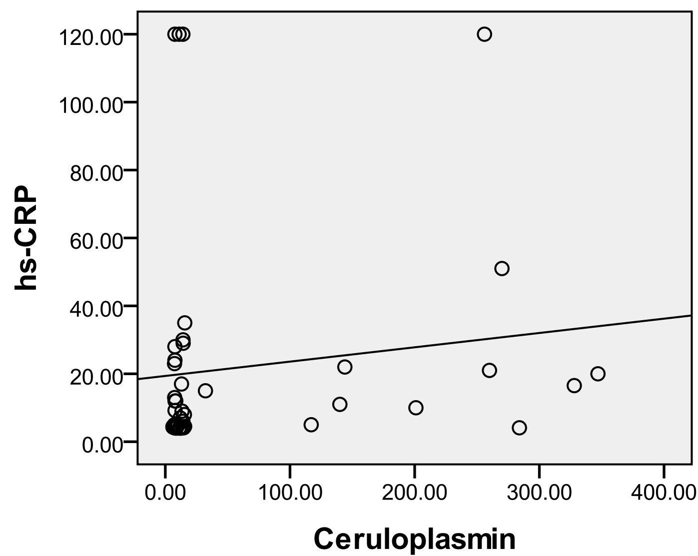


Figure (3.7): No correlation between Ceruloplasmin and hs-CRP in diabetic nephropathy patients (r = 0.130 P > 0.05).

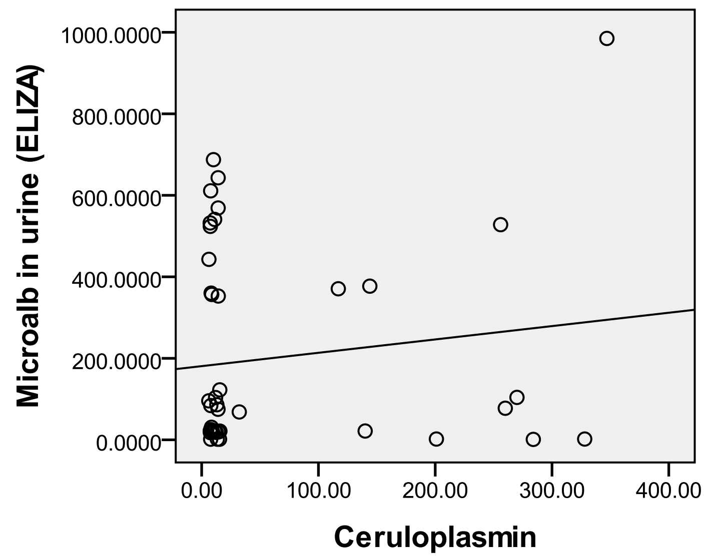


Figure (3.8): No correlation between Ceruloplasmin and microalbumin in urine (ELIZA) in diabetic nephropathy patients ($r = 0.131$ $P > 0.05$).

Conclusions

and

Recommendations

Conclusions:

1. Activation of inflammatory pathways in progression of kidney disease as represented by high sensitive C-reactive protein suggests that measurement of serum CRP can be useful for diagnosis of early stages of diabetic nephropathy .
2. Elevated ceruloplasmin in urine of diabetic nephropathy patients could be a good urinary marker for predicted diabetic nephropathy.
3. Poor glycemic control in diabetic patients can led to increase the complications of diabetes including nephropathy .

Recommendations

1. Evaluation of cystatin C for calculation of eGFR .
2. Carrying out a molecular study for diabetic nephropathy.
3. Assessment of inflammatory marker Like IL-10 , IL-17 ,IL-18 in diabetic nephropathy patients.
4. Further study on Immunoglobulin secretion in urine.
5. Study the complement as immunological marker like C3, C4 in diabetic nephropathy patients.
6. Study oxidative stress in diabetic nephropathy by measuring malondialdehyde and carbonylated protein.

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الخلاصه

يمثل داء السكر اضطرابا لمتلازمه أيض الكاربوهيدات . ويعد ارتفاع السكر في دم مرضى داء السكر من اكبر المشاكل لصحه الانسان في القرن الحادي والعشرون.

ان مرض داء السكر هو السبب الرئيسي لاصابه الكلى وان الفشل الكلوي المزمن هو احد الامراض الناتجه من اعتلال الكلى المصاحب لداء السكر .

ان الهدف من هذه الدراسه هو لتقييم بعض العوامل الكيمائيه الحياتيه والمناعيه للاشخاص المصابين باعتلال الكلى المصاحب لداء السكر ولايجاد فحوصات للكشف عن عمل الكلى لمرضى داء السكر.

تضمنت الدراسة خمسه واربعون مريض مصابين باعتلال الكلى المصاحب لداء السكر النوع الثاني من المراجعين لمستشفى عبد المجيد الاهلي للفتره من كانون الاول 2013 ولغايه تموز 2014 ولغرض المقارنه تم جمع تسع وعشرون عينه لاشخاص اصحاء متوافقين بالاعمار مع المصابين .

و ان المرضى الذين تم اخذ عيناتهم يمتازون بارتفاع مستوى المايكرو البومين في البول او مستوى الكرياتين في الدم عن المستوى الطبيعي والتاكد من اصابه المريض بداء السكر .

قد تم قياس السكر العشوائي في الدم والكوليسترول والدهون ذات الكثافه العاليه والدهون ذات الكثافه المنخفضه والدهون الثلاثيه والسكر التراكمي واليوريا والكرياتينين وكذلك الاجسام المضاده في الدم وتم قياس بعض العوامل المناعيه في مصل الدم .

وتم قياس مستويات المايكروالبومين والكرياتنين والسيريولوبلازمين في البول .

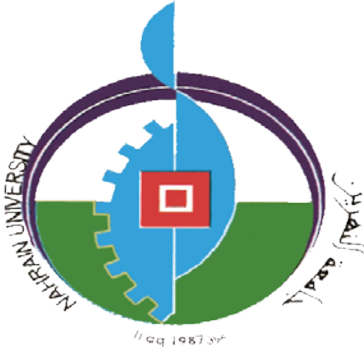
ووجد في هذه الدراسه ان زيادة معنويه في مستوى السكر العشوائي والسكر التراكمي واليوريا والكرياتينين ومعدل الترشيح الكبيبي في الاشخاص المصابين باعتلال الكلى المصاحب لداء السكر النوع الثاني بالمقارنه مع الاشخاص الاصحاء.

ولوحظ وجود زيادة معنويه في hs-CRP (32.63 vs 11.76)والذي يعد أحد البروتنيات المصاحبه للالتهابات بالمقارنه مع الاشخاص الاصحاء .

لوحظ عدم وجود فروق معنويه في ال TGF –β1 وال TNF-α بين الاشخاص المصابين باعتلال الكلى المصاحب لداء السكر النوع الثاني بالمقارنه مع الاشخاص الاصحاء .

كما ولوحظ وجود زيادة معنويه في المايكروالبومين والسيريولوبلازمين في البول في المرضى المصابين بداء السكر بالمقارنه مع الاشخاص الاصحاء .

استنتج من هذه الدراسه ان ارتفاع نسبه السكر والبروتينات المرتبطه بالسكر تؤدي لزياده تلف الكلى وان hs-CRP والمايكروالبومين والسيريولوبلازمين هم عوامل تخمين جيده للكشف المبكر عن اعتلال الكلى .



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

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رسالة

مقدمة الى كلية العلوم/جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير علوم/تقانة احيائية

من قبل

هدى منذر مهدي

بكلوريوس علوم/تقانة احيائية/كلية العلوم/ جامعة النهرين,2012

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