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STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY

Dissertation Submitted for

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CHENNAI

MAY - 2018

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The work done by **DR.C.ANBUMANI** on **"STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY"** is under my supervision and I assure that this candidate will abide by the rules of the Ethical Committee.

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This is to certify that dissertation titled "**STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY"** is a bonafide work done by **Dr.C.ANBUMANI** under my guidance and supervision in the Department of Biochemistry, Thanjavur Medical College, Thanjavur during her post graduate course from 2015 to 2018.

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DECLARATION

I, **Dr. C.ANBUMANI** hereby solemnly declare that the dissertation title "**STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY"** was done by me at Thanjavur Medical College and Hospital, Thanjavur under the Supervision and Guidance of my Professor and Head of the Department **Dr.N.Sasivathanam, M.D(Bio).,DGO.** This dissertation is submitted to the Tamil Nadu Dr. M.G.R Medical University, towards partial fulfillment of requirement for the award of M.D. Degree (Branch –XIII) in Biochemistry.

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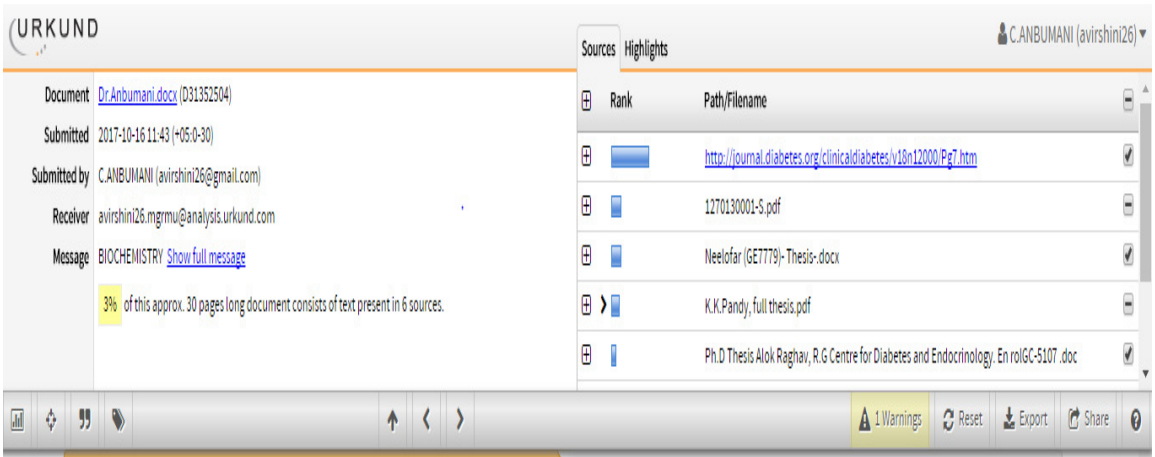

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of Albumin Creatinine Ratio (ACR) [1]; There is also marked day-to-day variability in Urine albumin excretion, so that at least two to three urine specimens done should show elevated levels before a patient is designated as having microalbuminuria.

In type 1 diabetes mellitus, microalbuminuria is rarely present at diagnosis, but persistent and untreated microalbuminuria will progress to albuminuria in 30%-80% of individuals over 10-15 yrs, and of those, 50%-78% will progress to Diabetic nephropathy over the next 10-18 yr [2]. Hypertension usually develops as a complication of nephropathy. In type 2 diabetes mellitus, microalbuminuria and even albuminuria may be present at or soon after diagnosis, in part due to the fact that diabetes has often already been present for years. If left untreated, 20%-40% of such patients will develop overt nephropathy, with only approx 20% of those patients progressing to DN over the next 20 yr [3]. Hypertension is frequently already present at the time of diagnosis of diabetes, often as part of the metabolic syndrome. Based on the current evidence that early intervention may slow the progression of diabetic kidney disease, it is now the standard of care to do annual screening because microalbuminuria, in type 1 diabetes starts at puberty or 5 yr after diagnosis, and in type 2 diabetes begins at diagnosis [4].

However, cross-sectional studies have found decreased GFR in the absence of increased urine

CERTIFICATE - II

This is to certify that this dissertation work titled **STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY** of the candidate **Dr. C.ANBUMANI** with registration Number **201523151** for the award of **M.D.,** in the branch of **BIOCHEMISTRY.** I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **4** percentage of plagiarism in the dissertation.

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CONTENTS

INTRODUCTION

Diabetic nephropathy (**DN**) is the chronic loss of kidney function occurring in those with diabetes mellitus. It is a serious complication, affecting approximately one-quarter of adult diabetics in the United States. It is usually slowly progressive over years.

Pathophysiologic abnormalities in DN begin with long-standing poorly controlled blood glucose levels. This is followed by multiple changes in the filtration units of the kidneys, the nephrons. (There are normally about 3/4-1 1/2 million nephrons in each adult kidney).Initially, there is constriction of the efferent arterioles and dilatation of afferent arterioles, with resulting glomerular capillary hypertension and hyperfiltration; this gradually changes to hypofiltration over time. Concurrently, there are changes within the glomerulus itself: these include a thickening of the basement membrane, a widening of the slit membranes of the podocytes, an increase in the number of mesangial cells, and an increase in mesangial matrix. This matrix invades the glomerular capillaries and produces deposits called Kimmelstiel-Wilson nodules.

The mesangial cells and matrix can progressively expand and consume the entire glomerulus, shutting off filtration. As this process affects more and more glomeruli, filtration in both kidneys progressively declines: the pathological process called nephrosclerosis.

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease. Albuminuria is the most commonly used marker to predict onset of diabetic nephropathy (DN) without enough sensitivity and specificity to detect early DN.

Cyclophilin A (CypA) is an 18-kDa protein with ubiquitous characteristics. It is mostly distributed in the cytoplasm and facilitates protein folding and protein trafficking.

Interestingly, sCypA was also detected in plasma of diabetic patients and was shown to be secreted by monocytes in response to hyperglycemia, indicating that sCypA could be a potential secretory marker in type 2 DM. As a product directly produced by kidney, urine could be best measure for detection of renal injury. Therefore in this study the urinary level of Cyp A in the patients with Diabetic nephropathy were assayed.

REVIEW OF LITERATURE

Definition and Natural History

It is known that DN can be detected before the onset of decreased glomerular filtration rate (GFR) in most patients by detecting abnormal amounts of albumin in the urine. Two stages have been designated: microalbuminuria (defined as urine albumin between 30 and 300 mg/24 h, 20-200 μg/min on a timed sample, or spot urine albumin to creatinine ratio 30-300 mg/g) and albuminuria, also termed clinical albuminuria, macroalbuminuria, and overt nephropathy (>300 mg/24 h, >200 μg/min on a timed sample, or spot urine albumin to creatinine [ACR] ratio >300 mg/g). Short-term hyperglycemia, exercise, urinary tract infections, marked hypertension, heart failure, and acute, febrile illness can cause transient elevations of Albumin Creatinine Ratio (ACR) [1]; There is also marked day-to-day variability in Urine albumin excretion, so that at least two to three urine specimens done should show elevated levels before a patient is designated as having microalbuminuria.

In type 1 diabetes mellitus, microalbuminuria is rarely present at **diagnosis**, but persistent and untreated microalbuminuria will progress to albuminuria in 30%-80% of individuals over 10-15 yrs, and of those, 50%-78% will progress to Diabetic **nephropathy** over the next 10-18 yr [2]. Hypertension usually develops as a complication of nephropathy. In type 2 diabetes mellitus, microalbuminuria and even albuminuria may be present at or soon after diagnosis, in part due to the fact that

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However, cross-sectional studies have found decreased GFR in the absence of increased urine **albumin**excretion (UAE) in a substantial percentage of adults with type 2 diabetes [5]. In the Third National Health and**Nutrition** Examination Survey, which collected demographic and health information from a nationally representative sample of the US population, 13% of adults with type 2 diabetes had a GFR <60 mL/min/ 1.73 m2. Among this population, an absence of increased UAE (defined in this study as a spot urine ACR ≥17 mg/g in men and ≥25 mg/g in women) was noted in approx. 40%, whereas absence of both increased UAE and diabetic retinopathy was noted in 30% [5]. Decreased GFR in the absence of increased UAE among adults with both type 1 and 2 diabetes have also been reported in other studies [6]. In follow-up, the rates of decline of GFR in those with type 2 diabetes with initial GFR levels <60 mL/min/1.73 m2 were similar, regardless of the presence or absence of **albuminuria** [7]. Thus, these studies demonstrate that substantial declines in GFR may be noted in adults with type 1 and 2 diabetes in the absence of increased UAE. Because these studies did not perform kidney biopsies, investigators could only speculate on the etiology of decreased GFR in the

absence of increased UAE. Pathological evidence of DN has been documented in adults with diabetes even in the absence of increased UAE [8,9]. In addition, older patients with type 2 diabetes may also have vascular and tubulo-interstitial changes owing to the presence of comorbid conditions, including long-standing **hypertension** and renal vascular disease, and potential senescence of glomeruli owing to aging itself [10,11]. Conversely, studies have also reported a range of biopsy findings from normal to typical diabetic changes and frequently other **kidney diseases** in adults with type 2 diabetes and increased UAE [12]. Therefore, in addition to yearly screening for albuminuria, the yearly measurement of serum creatinine with estimation of GFR using the adjusted modification diet in renal disease (MDRD) [13] or other formulae [14] should also be carried out.

Diabetes is the most common cause of end-stage renal disease (ESRD) in the United States today.(15) Approximately 40% of patients with type 1 diabetes and 5 - 15% of patients with type 2 diabetes eventually develop ESRD, although the incidence is substantially higher in certain ethnic groups.(16)

This is thought to be a potentially preventable calamity. Sensitive tests are available to identify patients with renal involvement early in the clinical course, when preventive measures may have greatest impact.

The pathophysiologic mechanisms of diabetic nephropathy are incompletely understood but include glycosylation of circulating and intrarenal proteins, hypertension, and abnormal intrarenal hemodynamics. The earliest demonstrable abnormalities include intrarenal hypertension, hyperfiltration (increased glomerular

filtration rate [GFR]), and microalbuminuria. Clinically, the most important screening tool for identifying early nephropathy is detection of microalbuminuria.

Risk factors for development of diabetic nephropathy include hyperglycemia, hypertension, positive family history of nephropathy and hypertension, and smoking. Key elements in the primary care of diabetes include glycemic control, blood pressure control, and screening for microalbuminuria. In general, the goal for glycemic control is a blood glucose level as close to normal and HbA_{1c} <7% as possible without causing hypoglycemia. Blood pressure control is important as glucose control, especially after the onset of renal damage, and blood pressure should be consistently <130/85. Screening for diabetic nephropathy involves monitoring at least yearly for urinary albumin excretion >30 mg per day.

Diabetic Kidney Disease was previously known as diabetic nephropathy and is defined as diabetes with albuminuria (ratio of urine albumin to creatinine $\geq$ 30 mg/g), impaired glomerular filtration rate (<60 mL/min/1.73 m^2), or both and is the single strongest predictor of mortality in patients with diabetes [17]. Today, DKD encompasses not only diabetic nephropathy but also atheroembolic disease, ischemic nephropathy, and interstitial fibrosis that occurs as a direct result of diabetes.

Diabetic nephropathy (DN) is one of the major causes of end-stage renal failure worldwide [18]. Clinically, microalbuminuria is an important index to assess the progression of DN [19]. However, it is not accurate to evaluate the severity or prognosis simply based on the degree of proteinuria. It is now well recognized that not all diabetic

patients who develop renal function failure have massive albuminuria [20]. Therefore, nephrologists and endocrinologists should be aware of the significance of pathological changes of DN in their clinical practice. Specifically, nondiabetic renal disease (NDRD), which might commonly be superimposed with diabetic renal lesions in some patients with type 2 diabetes, could only be confirmed and excluded by biopsy [21].

Etiology and Epidemiology

WHO estimates that 347 million people worldwide suffer from diabetes. In the region of Africa, around 20 million are living with diabetes. This figure is expected to double by 2030. In comparison to other world regions, Africa has the highest percentage of undiagnosed diabetes cases reaching 62% and the lowest diabetes related health expenditure.

Globally, an estimated 422 million adults are living with diabetes mellitus, according to the latest 2016 data from the World Health Organization (WHO).Diabetes prevalence is increasing rapidly; previous 2013 estimates from the International Diabetes Federation put the number at 381 million people having diabetes. The number is projected to almost double by 2030.Type 2 diabetes makes up about 85-90% of all cases. Increases in the overall diabetes prevalence rates largely reflect an increase in risk factors for type 2, notably greater longevity and being overweight or obese.

Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however, occurring in low- and middle-income countries including in Asia and Africa, where most patients will probably be found by 2030.The increase in incidence in developing countries follows the trend of urbanization and lifestyle changes, including

increasingly sedentary lifestyles, less physically demanding work and the global nutrition transition, marked by increased intake of foods that are high energy-dense but nutrient-poor (often high in sugar and saturated fats, sometimes referred to as the Western pattern diet). The risk of getting type 2 diabetes has been widely found to be associated with lower socio-economic position across countries.

Diabetes is fast gaining the status of a potential epidemic in India with more than 62 million diabetic individuals currently diagnosed with the disease. In 2000, India (31.7 million) topped the world with the highest number of people with diabetes mellitus followed by China (20.8 million) with the United States (17.7 million) in second and third place respectively. According to Wild et al. the prevalence of diabetes is predicted to double globally from 171 million in 2000 to 366 million in 2030 with a maximum increase in India. It is predicted that by 2030 diabetes mellitus may afflict up to 79.4 million individuals in India, while China (42.3 million) and the United States (30.3 million) will also see significant increases in those affected by the disease. India currently faces an uncertain future in relation to the potential burden that diabetes may impose upon the country. Many influences affect the prevalence of disease throughout a country, and identification of those factors is necessary to facilitate change when facing health challenges.

Diabetic nephropathy is one of the leading causes of chronic renal failure in India. It has been reported that among 4837 patients with chronic renal failure seen over a period of 10 years, the prevalence of diabetic nephropathy was 30.3% followed by chronic interstitial nephritis (23.0%) and chronic glomerulonephritis (17.7%)

Pathological Changes of DN

The most significant and consistent pathological changes identified in renal biopsies of clinical DN patients are the glomerular lesions [22] which are, especially, diffuse and nodular mesangial expansion and Glomerular Basement Membrane (GBM) thickening [23]. Diffuse mesangial expansion, which develops at as early as 5th year since the onset of diabetes, is the earliest observable change by light microscopy [24]. The mesangial fractional volume [Vv(Mes/glom)] is correlated with albumin excretion rate (AER) and Glomerular Filtration Rate (GFR) in both type 1 [25] and type 2 diabetes [26]. As the disease advances, diffuse mesangial expansion progressively develops into nodular accumulations of mesangial matrix in the late stage of the DN. These nodular lesions, also known as Kimmelstiel-Wilson nodules, can be observed in about 25% of patients with advanced DN [27]. Nodular lesions and diffuse lesions are two stages of DN. Compared to the patients with diffuse mesangial expansion, those patients with nodular diabetic glomerulosclerosis present with more severe renal damage, longer diabetic durations, and poorer renal prognosis [28].

GBM thickening can be observed within 2–8 years after the onset of diabetes. It is an early lesion which could be detected and measured by electron microscopy (EM) [29]. GBM width tends to increase linearly according to diabetes duration in type 1 diabetes [30]. Vv(Mes/glom) and GBM width together explain 59% of the AER variability in a group of 125 patients of type 1 diabetes [25].

Although diabetic glomerular lesions have been the focus of the investigation on DN, the extraglomerular lesions are also involved in the progression of the disease. Tubulointerstitial lesions, including tubular atrophy, interstitial inflammation, and tubulointerstitial fibrosis, are closely related to renal function loss in the progression

towards ESRD in patients with preexisting renal insufficiency [31]. Since DN is a kind of diabetic microangiopathy, hyalinosis occurs in both afferent and efferent arterioles. The hyalinosis of the efferent arteriole is a typical lesion by which diabetic nephropathy could be differentiated from hypertensive nephropathy [32].

There is increasing recognition of lesions like glomerular endothelial injury [22], podocyte impairment [33], and glomerulotubular junctions abnormalities in DN [34].

Histopathology of DKD

Mesangial expansion caused by increased matrix secretion and cell enlargement is the first change seen on light microscopy, whereas electron microscopy demonstrates a thickened basement membrane and podocyte effacement [24]. In the vessels, intimal hyaline thickening is present initially and later progresses to arterial hyalinosis of the afferent and efferent arterioles which later leads to glomerular hyperfiltration [35, 36]. Diffuse diabetic glomerulosclerosis and Kimmelstiel-Wilson nodules (nodular glomerulosclerosis) are seen only later in the disease, although the latter is not always seen on biopsy as is classically taught [37]. Ultrastructurally, podocytes suffer hypertrophy and then foot process effacement which leads to functional changes such as

increased albumin excretion [24, 35]. It should be noted that, in patients with type 2 diabetes, GFR loss can occur independently of albuminuria [36, 38, 39] and it has been demonstrated that microalbuminuria is observed in only 45% of this population [40]. The histopathologic change of DKD has been attributed to diabetic macroangiopathy as opposed solely to microangiopathy and has also been attributed to aging, atherosclerosis, hypertension, and episodes of acute kidney injury [36, 39, 41].

Pathways of DKD

Previously, the above histopathologic changes were attributed primarily to metabolic and hemodynamic derangements seen in diabetes, the latter referring to the hyperfiltration which occurs as a result of efferent arteriolar vasoconstriction due to an activated renin-angiotensin-aldosterone system (RAAS). However, it has become increasingly evident over the years that hyperglycemia in and of itself is not the sole cause of DKD, although inarguably, it plays a major role. Several pathophysiologic pathways are involved in the development of DKD, and this review will attempt to elucidate those pathways and hopefully shed some light on therapeutic options that may one day play a role in quelling the epidemic of DKD and suppressing progression to ESRD.

Hemodynamic Pathways of DKD

Activation of the RAS leads to increased angiotensin II levels which subsequently cause efferent arteriolar vasoconstriction. Elevated levels of angiotensin II are associated with increased albuminuria and nephropathy in both humans and mice [35, 42, 43]. ACEIs and ARBs have a long track record in reducing the doubling rate of creatinine, albuminuria, and progression to nephropathy, ESRD, and death [28, 30, 31, 44]. Another potent vasoconstrictor of the efferent arteriole is endothelin-1 (ET-1). ET-1 has various physiologic functions in the kidney that mimic RAS including mediating vasoconstriction and hence playing a role in hypertension, endothelial dysfunction, inflammation, and fibrosis [45]. Additionally, increased ET-1 expression activates a signaling cascade which leads to mesangial cell hypertrophy and proliferation as well as extracellular matrix (ECM) production. It is also thought to activate receptors that directly increase glomerular permeability, hence leading to worsening albuminuria and progression of DKD [45].

This pathway was first detailed by Brownlee in Nature in 2001 [46]. He helped clarify that hyperglycemia leads to increased glycolysis which then upregulates four distinct entities: the polyol pathway, hexosamine pathway, production of advanced glycation end products (AGEs), and activation of protein kinase C (PKC). Before going into the details of each of the above pathways, a review of glycolysis is worthwhile. Glycolysis is the biochemical pathway in which glucose is broken down by cells to make energy. Intracellular glucose is first phosphorylated into glucose-6-phosphate and then fructose-6-phosphate. One step later glyceraldehyde-3-phosphate becomes 1,3-diphosphoglycerate with the help of glyceraldehyde-3-phosphate dehydrogenase. This is

important because glyceraldehyde-3-phosphate dehydrogenase is inhibited by excess superoxide produced by the electron-transport chain which occurs in the setting of hyperglycemia [46–48]. Inhibition of glyceraldehyde-3-phosphate dehydrogenase prevents glycolysis from taking place and causes an upregulation of upstream components of glycolysis, specifically glucose, glucose-6-phosphate, and fructose-6-phosphate, hyperglycemia).

The Polyol Pathway

The polyol pathway is upregulated as a result of excess of hyperglycemia. Glucose is first converted to sorbitol via the NADPH-dependent enzyme, aldose reductase; sorbitol is then converted to fructose using NAD+ as a cofactor [46]. The reduction of glucose to sorbitol results in decreased intracellular NADPH levels, a cofactor involved in regenerating the antioxidant, reduced glutathione (GSH). Decreased levels of GSH are thought to contribute to increased intracellular oxidative stress which in turn causes increased cell stress and apoptosis [49]. Additionally, the oxidation of sorbitol to fructose results in an increased intracellular NADH : NAD+ ratio which also inhibits GADPH activity, thus propagating the inhibition of glycolysis. The increased NADH : NAD+ ratio also increases formation of methylglyoxal and diacylglycerol, precursors of the AGE and PKC pathways [46]. Finally, the end product of the polyol pathway, fructose, has also recently emerged as a potential nephrotoxin. In a diabetic murine model, endogenous production of fructose through the polyol pathway led to increased proteinuria, reduced GFR, and increased glomerular and proximal tubular injury when compared to mice with lower levels of endogenous fructose.

The Hexosamine Pathway

The hexosamine pathway stems from the third step of glycolysis, fructose-6-phosphate, which is converted to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT).Glucosamine-6-phosphate is then used as a substrate to increase transcription of inflammatory cytokines tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) [46].

Advanced Glycation End Products

Advanced glycation end products (AGEs) are the result of irreversible glycation of proteins that occurs in the presence of intracellular hyperglycemia [35, 46, 50]. Three pathways are primarily responsible for the production of AGE precursors: oxidation of glucose to make glyoxal, degradation of Amadori products, and aberrant glycolysis which shunts glyceraldehyde-3-phosphate into forming methylglyoxal [51]. Once formed, AGEs damage cells by modifying or impairing the function of both intracellular and extracellular proteins [60]. For example, AGE modifies both laminin and type IV collagen and was shown to increase the permeability of the glomerular basement membrane (GBM) [52–55]. Additionally increased concentrations of AGE are known to dose-dependently increase expression of fibronectin and collagen types I and IV which are thought to lead to increased density and expansion of the extracellular matrix in the kidney [52, 56–60]. AGEs themselves can bind various proinflammatory receptors which then activate downstream production cytokines such as IL-1, IL-6, and TNF-α, growth factors such a TGF-B1, vascular endothelial growth factor (VEGF), platelet-derived growth factor subunit B (PDGF-B), connective tissue growth factor (CTGF), and increased generation of reactive oxygen species (ROS) [36, 52,61, 62]. VEGF is

necessary for survival of endothelial cells, podocytes, and mesangial cells whereas CTGF is a profibrotic agent; both have been implicated in diabetic nephropathy [52, 63, 64].

The PKC Pathway

The PKC pathway, like the AGE pathway, stems from the fourth step in glycolysis. Hyperglycemia drives the conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate (DHAP) and ultimately diacylglycerol (DAG) which is a cofactor for PKC activation [65]. In the presence of hyperglycemia, DAG is chronically upregulated and contributes to sustained PKC activation [66]. PKC is thought to contribute to DKD in various ways. It increases activity levels of prostaglandin E_2 and nitric oxide [67–69] leading to vasodilation of the afferent arteriole and augmentation of angiotensin II's actions on the efferent arteriole [70, 71]; these actions collectively contribute to glomerular hyperfiltration [65]. In the later stages of diabetic nephropathy, there is a state of progressive deficiency in nitric oxide which has been associated with severe proteinuria, declining renal function, and hypertension [72, 73]. PKC also mediates VEGF which, as noted above, is linked to abnormal intrarenal blood flow and capillary permeability and is thought to play a role in the development of microalbuminuria [65, 74]. PKC activation also increases CTGF and TGF-β levels as well as production of fibronectin and type IV collagen and contributes to GBM thickening and ECM accumulation [65].

Inflammatory Pathways of DKD

The inflammatory pathway supports the idea that DKD is not solely a result of uncontrolled hemodynamics and hyperglycemia but is also a consequence of a chronically activated innate immune system and a low-grade inflammatory state in patients with diabetes [75, 76]. Inflammatory-mediated renal injury was reviewed recently and is summarized here [75].

NF-κB is a transcription factor that regulates the expression of multiple genes related to inflammation, immunity, apoptosis, and chemoattractant protein-1, amongst others [77, 78], and localizes to glomerular, interstitial, and tubular epithelial cells in the human kidney. Hyperglycemic conditions are known to increase expression of NF-κB [79]. In DKD [77, 80], NF-κB activation correlates with proteinuria and interstitial cell infiltration [77, 78, 80]. Proteinuria is known to further stimulate NF-κB and contributes to persistent proteinuria in a cyclic fashion [78].

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is a way for chemical signals outside of a cell to be relayed to gene promoters at the DNA level. JAK2 is present in renal and vascular tissue [81]. It is activated by ROS caused by hyperglycemic states and is associated with hypertrophy of mesangial cells [75]. Berthier et al. demonstrated that JAK2 mRNA levels inversely correlated with estimated glomerular filtration rate (eGFR) in patients with diabetic nephropathy [82].

Inflammatory cytokines such as TNF-α and interleukins 1, 6, and 18 (IL-1, IL-6, and IL-18, resp.) are expressed in greater proportions in the kidneys of diabetic models when compared to nondiabetic controls [83, 84]. In diabetic rat models, increased expression of TNF-α and IL-6 was also associated with increased kidney weight and

urine albumin excretion [83]. In patients with DKD, serum IL-18 and TNF-α levels were higher in patients with diabetes than nondiabetic controls. IL-18 and TNF-α levels also correlated positively with the degree of albuminuria in the patients with diabetes [85, 86]. At the cellular level, these cytokines are thought to increase vascular endothelial cell permeability, contribute to glomerular hypercellularity and GBM thickening, induce apoptosis of endothelial cells, and can be directly toxic to renal cells [87–95].

Alternative Pathways of DKD

Autophagy is a highly conserved protective mechanism that allows cells and organisms to maintain homeostasis during periods of cell starvation or oxidative stress [96, 97]. It involves intracellular degradation of cytotoxic proteins and organelles by lysosomes whenever a cell is experiencing stress [97, 98]. Decreased autophagic activity has been demonstrated in both obesity and diabetes [99–101] suggesting that autophagy is hampered in the setting of hypernutrition [102]. Podocytes are known to have a high basal level of autophagy [101]. In vitro studies of podocytes exposed to high glucose conditions demonstrated defective autophagy which resulted in podocyte injury [101]. On renal biopsy of obese patients, autophagic activity was decreased in proximal tubular epithelial cells when compared to nonobese patients suggesting that obese patients with diabetes may be prone to renal injury due to suppressed autophagy [102]. Dietary restriction in rats was shown to improve urinary albumin excretion and creatinine clearance and increase levels of Sirt1, a positive regulator of autophagy [103].

Another conserved evolutionary mechanism is linked to the sodium-glucose transporter 2 (SGLT2) in the proximal tubule. SGLT2 is a low-affinity and high-capacity transporter and is responsible for >90% of glucose reabsorption in the proximal tubule [51, 104, 105]. Animals with a genetic deficiency of SGLT2 lose approximately 60% of their filtered glucose into the urine [104]. In settings of hyperglycemia, there is upregulation of SGLT2 expression which is believed to be of evolutionary benefit as it allows for glucose reabsorption and hence energy conservation for both the body and brain [104, 106]. Unfortunately, in settings of hyperglycemia due to diabetes, this mechanism is counterproductive and further contributes to a hyperglycemic state.

Natural clinical course of diabetic kidney disease

The natural history of DN is divided into five stages (2):

Stage 1: Renal pathology develops at the onset of diabetes. The growth of the kidney increases by several centimetres. By the time of diagnosis, the GFR and urinary albumin excretion (UAE) have been increased. It can be controlled at this level by onset of insulin.

Stage 2: The second stage typically lasts for 5-15 years after diagnosis of diabetes. The characteristics of the second stage include:

1. GFR remains elevated due to hyperfiltration.
2. Kidneys remain hypertrophied and UAE rate stays normal.

Stage 3: The characteristics of stage three are:

1. Microalbuminuria is present. It occurs in 30-50% of patients after diabetes onset, 80% of whom go on to develop overt nephropathy over 10-15 years.
2. GFR remains elevated or returns to normal range
3. Blood pressure starts to rise in 60% of patients

Histological changes-progression is as seen in stage two.

Stage 4: This stage is also known as clinical nephropathy or overt nephropathy. The characteristic histological features of stage four are formation of the Kimmelstiel-Wilson nodule (focal glomerular sclerosis) and macroproteinuria. It can progress to nephrotic in 30% of patients or may decline in 80% depending on deterioration of GFR.

Stage 5: As the GFR continues to decline, ESRD may develop. DN is considered the most common cause of ESRD because of associated autoimmune neuropathy and cardiac disease.

The stages of chronic kidney disease (CKD) are mainly based on measured or estimated GFR. There are five stages but kidney function is normal in stage 1 and minimally reduced in stage 2 (Table 1).

Table 1

Stages of diabetic nephropathy [modified from the renal association]

Stage	GFR (ml/min/1.73 m^2)	Description	Management
1	>90	Normal or increased GFR with another evidence of renal damage	Screening CKD and risk reduction
2	60-89	Slightly decreased GFR with another evidence of renal damage	Diagnosis and treatment: slow progression of CKD; comorbidities and cardiovascular disease; risk reduction
3a	45-59	Moderately decreased GFR without evidence	Evaluate and treat complication

		of renal damage	
3b	34-40	Irreversible renal damage	
4	15-29	Severely decreased GFR without evidence of renal damage	Prepare for renal replacement therapy
5	<15	Established renal failure	Renal replacement if uremic

Diabetic nephropathy is classically defined as a progressive increase in urine albumin excretion, accompanied by rising blood pressure and a relentless decline in glomerular filtration, culminating eventually in end-stage renal failure [107]. In the presence of retinopathy and long duration of diabetes, diabetic glomerulosclerosis is generally accepted to be the cause. Although it was suggested many years ago that nephropathy was one of a triad of complications of diabetes, the other components of which are retinopathy and vascular disease [108], it is only relatively recently that the enormous impact of premature cardiovascular disease on people with any evidence of diabetic nephropathy has been fully recognized. Thus, many diabetic people, even with early nephropathy, and particularly with type 2 diabetes, do not progress to end-stage

renal disease (ESRD), but die prematurely of cardiovascular disease. A modern definition of diabetic nephropathy might thus encompass a state of greatly increased cardiovascular risk, accompanied. If untreated, by a progressive decline in renal function and associated with diabetic retinopathy.

The progressive, continuous increase in urinary albumin excretion is generally considered in categorical stages to help clinical and research interpretation. The divisions have been made partly on the basis of renal risk but are to some extent arbitrary. Classification for research purposes is generally done by estimation of urine albumin excretion rates in timed urine collections, but for clinical purposes, calculation of the albumin: creatinine ratio in an early morning urine sample is sufficient. Microalbuminuria is defined as an increase in urine albumin excretion above normal, but which is not detectable by conventional dip-stick testing, whilst proteinuria represents an increase in urine albumin that is detectable by conventional dip-stick testing.

In type 1 diabetes, abnormalities of renal function, including glomerular hyper filtration (elevated glomerular filtration rate, GFR) [109], increased albumin excretion rate (AER) [110, 111] and increased renal size [109, 110] are apparent at diagnosis. With initiation of insulin treatment, GFR returns to normal in the majority [109, 110] and AER probably in all patients [112-114]. However, in 25-40% of patients, GFR remains above normal [109, 115]. Although it has been suggested that these hyper-filtering patients will progress to nephropathy, most [116, 117], but not all [118], longitudinal studies indicate that they are not at higher risk than patients who do not have persistent hyperfiltration.

In those patients who will never develop nephropathy, albumin excretion remains normal, except during periods of particularly poor glucose control, or during acute intercurrent illness, when a transient increase in AER into the microalbuminuric or even proteinuric range may occur.

In those patients who eventually reach end-stage renal failure, a progressive rise in AER occurs, with microalbuminuria developing within the first 10 years of diabetes. Some studies have suggested that AER remains normal for several years after diagnosis [113, 114, 119], although others suggest that the prevalence of microalbuminuria is 6-19% within 1-5 years' duration of diabetes [120, 121]. The explanation for such marked differences is unclear, and they seem unlikely to be due simply to differences in glycaemic control. The prevalence of microalbuminuria thereafter is related to duration of diabetes, and reaches 40-50% after 30 years [122, 123], although the proportion is falling in more recent reports of cohorts [120, 124]. Approximately 1.5-2.5% per annum of patients with normal AER develop microalbuminuria [125-127], although the incidence can be reduced with extremely good glycaemic control [128]. The factors reported most strongly and consistently to influence the transition from normal albumin excretion to microalbuminuria are baseline AER, blood glucose control, blood pressure and the presence of retinopathy. Smoking and the presence of dyslipidaemias are less consistently associated. Not surprisingly, those patients with an AER in the 'high normal' range are more likely to develop microalbuminuria than those in the lower range: > 7.4 mg/L [129], > 10ug/min [126], and > 11 mg/24 h [125]. Those who do develop microalbuminuria have a faster rate of increase in AER in the 2-5 years before microalbumin-uria is evident than those whose albumin excretion remains normal [130].

There may be a period of 'intermittent' microalbuminuria before microalbuminuria becomes persistent [131], presumably because of the high day-to-day variation in AER [132].

Early studies suggested that approximately 80% of microalbuminuric type 1 diabetic patients would develop proteinuria [133-135]. However, more recent studies suggest that in approximately 30% of micro-albuminuric patients, AER reverts to normal, 50% remain microalbuminuric and 20% progress to pro-teinuria over a 5-9-year period [114, 136, 137]. The risk of developing proteinuria, however, remains much greater in those with microalbuminuria than in those with a normal AER. The reasons for the discrepancy between the older and more recent studies may reflect general improvements in diabetes care: early studies involved follow-up from the 1960s to 1980. Epidemiological studies suggest that those who develop microalbuminuria after a short duration of diabetes are more likely to progress to proteinuria [138], whilst those with microalbuminuria and duration of diabetes > 15 years are less likely to develop proteinuria and have well-preserved renal function [139]. However, with general improvements, particularly in glycaemic and blood pressure control, it is likely that this pattern is altering, and the whole process of development of diabetic nephropathy is being slowed. Thus, microalbuminuria and proteinuria may now develop after longer duration of diabetes.

Blood pressure is particularly important in the rate of progression from microalbuminuria to proteinuria, those with mean arterial pressure > 94 mmHg being more likely to progress, [137]. In those who do progress, the rate of change of AER is very variable from individual to individual, but is on average 20% per annum [140], so

that it takes 10-15 years for proteinuria to appear. As the somewhat arbitrary cut-off between microalbuminuria and proteinuria is reached, patients pass through a phase of intermittent proteinuria, before proteinuria becomes persistent [141]. Thereafter, protein excretion rises continuously, perhaps into the nephrotic range. Again at this stage, the main determinant of proteinuria is blood pressure [142].

In older series, the cumulative incidence of proteinuria after 25 years' duration of type 1 diabetes was approximately 41% in those diagnosed from 1933 to 1942, and 25% in patients diagnosed from 1953 to 1962 [143,144]. In some more recent series, after 15-29 years' duration of diabetes, the cumulative incidence of proteinuria appears to have fallen further—to 26% in 1986 data [145] and 14-17% in the 1990s [120,124,146]. However, in other populations, no change in the incidence of proteinuria by year of diagnosis has been observed [147, 148].

The GFR remains high or normal until AER is in the high-microalbuminuric/low-proteinuric range [125, 149], although in those with hyperfiltration initially, there may be a fall into the normal range. Only whenproteinuria is present does GFR fall below normal, the rate of fall varying from person to person but being relatively constant within the individual. Untreated, the average rate of fall of GFR is 10-12 mL/min/year, so that it takes 8-10 years from the onset of proteinuria to ESRD [150-152]. Again, blood pressure is the most important determinant of the rate of fall of GFR [142, 151]. As renal function deteriorates, fluid overload and pulmonary oedema may occur even in the absence of hypoalbuminaemia, probably because of poor left ventricular function. Hyperkalaemia results from a state of low renin and aldosterone production.

Hypertension has long been recognized as an invariable accompaniment of persistent proteinuria and ESRD. The excess prevalence of hypertension in type 1 diabetes is confined to patients with nephropathy, those individuals who have no evidence of nephropathy having a prevalence of hypertension similar to the non-diabetic population [153]. This suggests that hypertension is an integral part of diabetic nephropathy, perhaps arising from the same under-lying mechanisms. This view is supported by the recognition that blood pressure rises very early in the course of development of nephropathy. Indeed, some studies suggest that patients with an AER in the high normal range. Patients with increased risk of progression to microalbuminuria, also have a higher blood pressure than those with lower albumin excretion (126,154]. However, in other studies, blood pressure does not rise until microalbuminuria is already present [125, 127, 155]. Changes in blood pressure are likely to be very subtle at this stage and may only be documented on 24-h blood pressure monitoring, perhaps as reduced dipping in nocturnal diastolic blood pressure [156].

In most cross-sectional studies, patients with micro-albuminuria have higher blood pressure than those with normal albumin excretion, although the levels may not meet a formal definition of hypertension. Blood pressure rises in parallel with albumin excretion. Once proteinuria is present, blood pressure untreated is > 140/90 mmHg in over 80% of patients, and in ESRD hypertension is almost universal.

In children with type 1 diabetes, the prevalence of microalbuminuria in cross-sectional studies varies from 3% to 25%, even after a short duration of diabetes [157, 158]. Several longitudinal studies have clarified the time course of the development of microalbuminuria [159-161]. In one study of children from diagnosis of diabetes, 13%

developed persistent microalbuminuria after a median duration of 5 years [159]. In those diagnosed after puberty, there was a relatively constant rate of development of microalbuminuria over time, whilst in those diagnosed before puberty, there was a latent period followed by a more rapid onset -of microalbuminuria with time after onset of puberty. Thus, the cumulative probability of developing micro-albuminuria was similar irrespective of the age at diagnosis of diabetes. The effect of puberty on the prevalence of microalbuminuria was independent of glycaemic control, suggesting that other mechanisms, perhaps relating to sex steroids or growth factors, may be important. Microalbuminuria was commoner in girls, in contrast to the adult male preponderance. Although 19.2% were microalbuminuric on at least one occasion, in only 4.5% was microalbuminuria persistent for 2 years or more. In a small but detailed 3-year study of children before, during and after puberty, the increase in AER was significantly greater in pubertal and post pubertal compared to pre pubertal children [162], and the risk of microalbuminuria was twice as high in the pubertal compared to the pre pubertal children.

Thus, transient microalbuminuria is common in adolescents around puberty and may relate to alterations in haemodynamic factors secondary to hormonal and growth factor changes. Its significance with respect to long-term glomerulosclerosis remains to be elucidated.

Recent data suggest that the course of the development of nephropathy in type 2 diabetes is in general very similar to that in type 1 diabetes, although with some important differences. Longitudinal studies in the Pima Indians, including data obtained during normal and impaired glucose tolerance, have been particularly informative.

At presentation of type 2 diabetes, the AER may be normal or high [163-166]. As blood glucose is controlled, albumin excretion falls to within the normal range in the majority [166-168]. However, in 10-48%, microalbuminuria is persistent, presumably reflecting a long period of diabetes before diagnosis and irreversible renal structural damage [166, 167, 169]. Some studies suggest that the blood glucose concentration and glycated haemoglobin percentage at diagnosis are higher in those in whom microalbuminuria persists [167].

In cross-sectional studies in established diabetes, the prevalence of microalbuminuria is 10-42%, depending on population selection and ethnicity [170-175]. Higher prevalences are reported in UK Asians [171], Pima Indians [175], African-Americans [176] and in Maori and Pacific Islanders [173] compared to Europid patients. The relationship of prevalence of microalbuminuria to duration of type 2 diabetes is not as strong as in type 1 diabetes. Many longitudinal studies suggest that the rate of progression from normal albumin excretion to microalbuminuria is approximately 4% per annum—at least as high as, if not higher than, in type 1 diabetes [175,177-180]. Factors most consistently associated with progression include baseline AER, glycated haemoglobin percentage, blood pressure and serum cholesterol [177, 179, 181].

There is less information on progression from microalbuminuria to proteinuria in type 2 diabetes. In Caucasian populations, approximately one-third of microalbuminuric type 2 diabetic patients develop proteinuria in 5 years [182,183] and after 20 years' duration of type 2 diabetes, the cumulative incidence of proteinuria is 27%, similar to that in type 1 diabetes [184]. In the Pima Indians, more than 50% develop proteinuria

within 20 years [181]. In contrast to the decline in the cumulative incidence of proteinuria in type 1 diabetes with onset in the 1970s and 1980s, in Pima Indians the incidence has doubled in the last 40 years [185], despite improvements in glucose and blood pressure control. This suggests a rapidly changing environmental or behavioral effect.

Because of the high prevalence of hypertension in type 2 diabetes, the relationship of hypertension to micro-albuminuria and proteinuria is much less obvious than in type 1 diabetes. However, most people with micro-albuminuria or proteinuria have hypertension, and blood pressure rises as albuminuria increases.

Cross-sectional studies have shown a high [186] or normal [166] GFR at presentation of type 2 diabetes, presumably because of variable times from onset to diagnosis of diabetes, or the concomitant presence of other renal diseases. However, careful longitudinal studies in the Pima Indians show that GFR is generally increased in subjects with impaired glucose tolerance who later progress to frank diabetes [181]. With duration of diabetes < 3 years, GFR is approximately 15% higher than in subjects with normal glucose tolerance. GFR remains high and stable with the onset of micro-albuminuria, only beginning to decline when proteinuria develops [181, 187, 188]. No relationship of hyperfiltration at diagnosis to subsequent nephropathy has been found.

In proteinuria, the rate of decline of GFR is similar to that in type 1 diabetes, 10-12 mL min/ ear [184,189], although there is some suggestion that it may be greater in non-Caucasian subjects [190]. In England, the age-standardized acceptance ratios for renal replacement therapy are reported as 4.2 and 3.7 times higher in African-

Caribbean and lndo-Asian compared to Caucasian diabetic patients [191]. This is in agreement with data from several studies [192-194], including a sub analysis of the Modification of Diet in Renal Disease Study, which showed that despite equivalent achieved mean blood pressure, subjects of African descent still had a sevenfold greater rate of decline in renal function compared to white subjects [194].

Overall, the development and course of nephropathy in Europid type 2 diabetes is remarkably similar to that in type 1, the differences seen probably relating to delayed diagnosis and to the older age and consequent more marked cardiovascular disease of the subjects. In the ethnic minorities, the prevalence of nephropathy is higher, and progression to ESRD may be faster.

Screening and diagnosis

In all new patients with diabetes it is imperative to record the previous history of renal diseases or any particular history of hypertension or cardiovascular disease. Urine analysis and correct recording of history of supine or erect blood pressure must be done.

Patients should be screened for microalbuminuria in the diabetic clinic. Albumin is measured as the earliest clinically detectable evidence of DN. Microalbuminuria is a misnomer for albumin in urine. A 24-urine collection is also useful for measuring total protein excretion and creatinine clearance. It is important to note the transient increase in UAE can be caused by uncontrolled hyperglycaemia; or hypertension, fever, urinary tract infection, congestive heart failure or physical exertion. Therefore, it is suggested that microalbuminuria should be confirmed by repeating the test of the urine sample

over the following 3-6 months. The values have been settled to check the level of risk, which are listed below:

1) Normal: 300 mg, 2) Microalbuminuria: 30-300 mg, 3) Overt proteinuria: >300 mg, 4) Nephrotic syndrome: >3000 mg

The albumin/creatinine ratio (ACR) can be assessed in an early morning demonstration or equally well in random spot urine samples. An ACR of 2.5 is usually taken as the cut-off for microalbuminuria (equivalent to a UAER of >30 mg/24h).

Cyclophilin A (CypA)

Cyclophilin A (CypA) is an 18-kDa protein with ubiquitous characteristics. [195] It is mostly distributed in the cytoplasm and facilitates protein folding and protein trafficking. It also acts as a cellular receptor for cyclosporine A (CsA). The expression of CypA is relatively high in the kidney, [196] where proximal tubular epithelial cells (PTECs) are reported to contain considerably more CypA than other kidney tissues.[197] With respect to kidney diseases, the majority of research has been on the cellular relationship between CypA and CsA, which is used as an immunosuppressant, and leaves behind its secreted form. This secreted CypA (sCypA) was reported to be correlated with cardiovascular disease (CVD), asthma, rheumatoid arthritis (RA), and lung and liver injury.[198] sCypA has been suggested to be a potential biomarker and mediator in CVD.[199]

In addition, sCypA is associated with inflammatory or infectious diseases such as RA, asthma, and periodontitis.[200] Interestingly, sCypA was also detected in diabetic patients' plasma [201] and was shown to be secreted by monocytes in response to

hyperglycemia, [202] indicating that sCypA could be a potential secretory marker in type 2 DM.[202] As a product directly produced by kidney, urine could be best measure for renal injury detection. In this study, the urinary level of Cyclophilin in patients with Diabetic Nephropathy were assayed.

AIMS AND OBJECTIVES

- To estimate the urine level of Cyclophiline-A in patients with diabetic nephropathy.
- To correlate the urine level of Cyclophiline- A with Serum Creatinine, Spot urine Microalbumin, Albumin Creatinine Ratio, & eGFR.
- To evaluate the correlation between urine level of cyclophiline -A and other several known risk factors such as Body mass Index, Blood pressure, Fasting plasma glucose, Postprandial plasma Glucose and blood urea.

MATERIALS AND METHODS

The study was conducted at Thanjavur Medical College, Thanjavur, after getting the approval from the Ethical committee of Thanjavur Medical college, Thanjavur

Hundred subjects were chosen for the study. Both males and females in the age group above 40 years were included and an informed consent was obtained from all of them. The study population included 2 groups.

Control group consist of 50 healthy individuals (males &females) and study group consists of 50 patients (males &females) with Diabetic Nephropathy.

INCLUSION CRITERIA

- Patients with type-2 diabetes
- Patients older than 18 years.

EXCLUSION CRITERIA

Patients with

- Primary tubular diseases,
- Recent or concurrent administration of potentially nephrotoxic drugs,
- Acute kidney injury ,
- Terminal kidney failure requiring dialysis,
- Known Neurological disease.

ESTIMATION OF UREA

PRINCIPLE

The enzyme methodology employed in this reagent is based on the reaction first described by Talke and Schubert. To shorten and simplify the assay, the calculations are based on the discovery of Tiffany et al. that urea concentration is proportional to absorbance change over a fixed time interval.

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

$$NH3 + \alpha\text{-KG} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-Glutamate} + \text{NAD}$$

1. Urea is hydrolysed in the presence of water and Urease to produce ammonia and carbon dioxide.

2. In the presence of Glutamate Dehydrogenase (GLDH) and reduced Nicotinamide Adenine Dinucleotide (NADH), ammonia combines with α-ketoglutarte (α-KG) to produce L-Glutamate.

3. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm as NADH is converted to NAD.

REAGENT COMPOSITION

Reagent 1 :

Tris Buffer	100 mmol/l
α-Ketoglutarate	5.49 mmol/l
Urease (Jack Bean)	≥ 10 kU/l
GLDH (Microorganism)	≥ 3.8 kU/l

Reagent 2 :

NADH	1.66 mmol/l

Also contains Non-reactive fillers and stabilizers.

R3 standard See bottle label

REAGENT PREPARATION Reagents are liquid, ready to use.

STABILITY AND STORAGE

The unopened reagents are stable till the expiry date stated on the bottle and kit label when stored at 2–8°C.

Two reagents method – substrate start

Reagents are ready to use. After opening, reagents are stable until expiry date at 2–8°C if stored at appropriate conditions, closed carefully and without any contamination.

	Reagent blank	Standard (Cal.)	Sample
Working reagent	1.000 ml	1.000 ml	1.000 ml
Sample	-	-	0.010 ml
Standard (Cal.)	-	0.010 ml	-
Distilled water	0.010 ml	-	-

Mixed and measured the initial absorbance after 30 sec (A1), start timer simultaneously and read again exactly after 1 min (A_2). Measured against reagent blank. Calculated absorbance change $\Delta A_{sam} = (A_2 - A_1)$/min.

CALCULATION

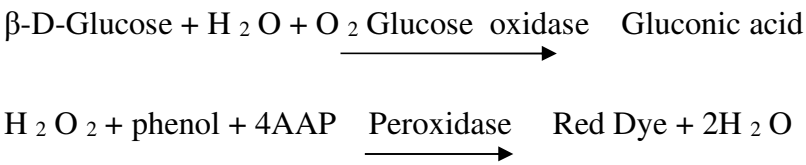
$$\text{Urea (mg/dl)} = \frac{\Delta A_{sam} - \Delta A_{bl}}{\Delta A_{cal} - \Delta A_{bl}} \times C_{cal}$$

C_{cal} = calibrator (standard) concentration

ESTIMATION OF GLUCOSE

PRINCIPLE

Trinder's method Glucose in the sample is oxidised to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a coloured quinonemine complex, with absorbance proportional to the concentration of glucose in sample.



REAGENT COMPOSITION

Reagent 1:

Phosphate buffer	250 mmol/l
Glucose oxidase	>25 U/ml
Peroxidase	>2 U/ml
Phenol	5 mmol/l
4-aminoantipyrine	0.5 mmol/l

R2 standard	See bottle label

REAGENT PREPARATION

Reagents are liquid, ready to use.

STABILITY AND STORAGE

The unopened reagents are stable till the expiry date stated on the bottle and kit label when stored at 2–8 °C.

SPECIMEN COLLECTION AND HANDLING

Use unheamolytic serum, plasma (heparin, EDTA) or urine. It is recommended to follow NCCLS procedures (or similar standardized conditions). Stability after addition of a glycolytic inhibitor (Fluoride, monoiodoacetate, mannose): 4

2 days	at 20–25°C
7 days	at 4–8°C

Stability in serum (separated from cellular contents, hemolysis free) without adding a glycolytic inhibitor: 2,5 8

8 hours	at 25°C
72 hours	at 4°C

Stability of glucose in urine:

24 hours	at 4–8°C

For the determination in urine diluted the sample using redistilled water in 1 + 10 ratio. Discarded contaminated specimens.

CALIBRATION

Calibration with the standard included in the kit or the calibrator XL MULTICAL, Cat. No. XSYS0034 is recommended.

QUALITY CONTROL

For quality control ERBA NORM, Cat. No. BLT00080 and ERBA PATH, Cat. No. BLT00081 were recommended. QUALITY CONTROL For quality control ERBA NORM, Cat. No. BLT00080 and ERBA PATH, Cat. No. BLT00081 were recommended.

UNIT CONVERSION mg/dl x 0.056 = mmol/l

ESTIMATION OF CREATININE ENZYMATIC

PRINCIPLE In the first reaction, creatinase and sarcosine oxidase are used in the enzymatic hydrolysis of endogenous creatine to produce hydrogen peroxide, that is eliminated by catalase.

Creatininase and 4-aminoantipyrine are added, and only the creatine generated from creatinine by creatininase is hydrolysed sequentially by creatinase and sarcosine oxidase to produce hydrogen peroxide. This newly formed hydrogen peroxide is measured in a coupled reaction catalysed by peroxidase, with N- -ethyl-N-sulphopropyl-m-toluidine

(ESPMT) as a chromogen. The absorbance of the produced complex at 546 nm is proportional to the creatinine concentration in the sample.

REAGENT COMPOSITION

Reagent 1 :

Good's buffer	pH 7.5 25 mmol/l
Creatinase	12 kU/l
Sarcosine oxidase	8 kU/l
Ascorbate oxidase	2 kU/l
Catalase	200 kU/l
ESPMT	0.47 mmol/l
Detergent	< 1 %
Gentamicin	< 0.1 %

Reagent 2 :

Good's buffer	pH 7.5 100 mmol/l
Creatininase	300 kU/l
Peroxidase	20 kU/l
4-aminoantipyrine	2.95 mmol/l
Detergent	< 0.5 %

Sodium azide < 0.1 %

REAGENT PREPARATION

Reagents R1 and R2 are liquid, ready for the use.

STABILITY AND STORAGE

The unopened reagents are stable till the expiry date stated on the bottle and kit label when stored at 2–8°C.

After first opening, reagents are stable for 30 days at 2–8°C if it is stored at appropriate conditions, closed carefully and without any contamination. On board stability: min. 30 days if it is refrigerated (2–10°C) and not contaminated.

SPECIMEN COLLECTION & HANDLING

Use serum, plasma (heparin, EDTA) ,urine. It is recommended to follow NCCLS procedures (or similar standardized conditions).

Stability in serum / plasma:

7 days	at 4–25°C
at least 3 months	at -20°C

in urine:

2 days	at 20–25°C
6 days	at 4–8°C

6 months at -20°C

For the determination in urine use 24 hours specimen. It is important to exactly measure the volume of collected urine. Dilute urine samples in 1+19 ratio with distilled water and multiplied the results by 20. Discarded the contaminated specimens.

CALIBRATION

Calibration with calibrator XL MULTICAL, Cat. No. XSYS0034 is recommended.

Calibration frequency: it has been recommended to do a calibration

- after reagent lot change
- as required by internal quality control procedures

Traceability:

This calibrator has been standardized to ID-MS.

QUALITY CONTROL

For quality control ERBA NORM, Cat. No. BLT00080 and ERBA PATH, Cat. No. BLT00081 were recommended.

CALCULATION

Results were calculated automatically by the instrument.

UNIT CONVERSION

mg/dl x 88.4 = µmol/l

Human CYPA (Cyclophilin A) ELISA Kit

Catalogue No.: EH2918

Size: 48T/96T

Reactivity: Human

Range: 1.25-80ng/ml

Sensitivity: < 0.75ng/ml

Application: For quantitative detection of CYPA in serum, plasma, tissue homogenates and other biological fluids.

Storage: 4°C for 6 months

NOTE: FOR RESEARCH USE ONLY.

Kit Components

Item	Specifications(48T/96T)	Storage
Micro ELISA Plate(Dismountable)	8 ×6 or 8×12	4°C/-20°C
Lyophilized Standard	1 vial or 2 vial	4°C/-20°C
Sample / Standard dilution buffer	10ml/20ml	4°C
Biotin- detection antibody (Concentrated)	60ul/120ul	4°C
Antibody dilution buffer	5ml/10ml	4°C
HRP-Streptavidin	60ul/120ul	4°C(shading light)

Conjugate(SABC)		
SABC dilution buffer	5ml/10ml	4°C
TMB substrate	5ml/10ml	4°C(shading light)
Stop solution	5ml/10ml	4°C
Wash buffer (25X)	15ml/30ml	4°C
Plate Sealer	3/5pieces	
Product Description	1 copy	

Principle of the Assay

This kit is based on sandwich enzyme-linked immune-sorbent assay technology. Anti-CYPA antibody is pre-coated onto 96-well plates. And the biotin conjugated anti-CYPA antibody has been used to detect antibodies. The standards, test samples and biotin conjugated detection antibody are added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin is added and unbound conjugates are washed away with wash buffer. TMB substrates has been used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the CYPA amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of CYPA will be calculated.

Precautions for Use

1. For the inspection and the validity of experiment operation , the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples are recommended.
2. The plate should be kept dry, after opening and before using the same.
3. Before using the Kit, the tubes were spun.
4. All the components were brought to the bottom of tubes.
5. TMB reagents were stored in a dark avoiding light.
6. Washing process is very important; if it is fully washed it will easily cause a false positive.
7. Duplication of well assay was recommended for both standard and sample testing.
8. Micro plate were never allowed to fully dry at the assay, as it will inactivate active components on plate.
9. Used tips and tubes were discarded to avoid cross contamination.
10. Reagents from single batch were used at a time

Manual Washing

Discarded the solution in the plate without touching the side walls. Clapped the plate on absorbent filter papers or other absorbent material. Filled each well completely with 350ul wash buffer and soaked for 1 to 2 minutes, then aspirated contents from the plate, and clapped the plate on absorbent filter papers or other absorbent material. Repeated this procedure two more times for a total of THREE washes.

Automated Washing

Aspirated all wells, then washed plate THREE times with 350ul wash buffer. After the final washing, inverted the plate, and clapped the plate on absorbent filter papers or other absorbent material. It was recommended that the washer be set for a soaking time of 1 minute.

Sample Collection and Storage

Isolated the test samples soon after the collection, then, analyzed immediately (within 2 hours).

Serum: Allowed samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collected the supernatant and carried out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

- Plasma: Collected plasma using EDTA-Na2 as an anticoagulant. Centrifuged samples for 15 minutes at 1000×g at 2 - 8°C within 30 minutes of collection. Collected the supernatant and carried out the assay immediately.

Note:

Samples to be used within 5 days may be stored at 4°C, otherwise the samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid the loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this assay.

Sample Dilution Guideline

End user should estimate the concentration of the targeted protein in the test sample first, and then select a proper dilution factor to make the diluted target protein concentration that falls the optimal detection range of the kit. Diluted the sample with the provided dilution buffer, and several trials may be practiced when it is necessary. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be made in pre-experiment.

- High target protein concentration (800-8000ng/ml): Dilution: 1:100. (i.e. Add 1µl of sample into 99 µl of Sample / Standard dilution buffer.)
- Medium target protein concentration (80-800ng/ml): Dilution: 1:10.(i.e. Add 10 µl of sample into 90 µl of Sample / Standard dilution buffer.)
- Low target protein concentration (1.25-80ng/ml): Dilution: 1:2.(i.e. Add 50 µl of sample into 50 µl of Sample / Standard dilution buffer.)
- Very low target protein concentration (≤1.25ng/ml): Unnecessary to dilute, or dilute at 1:2.

Reagent Preparation and Storage

All the reagents should be brought to the room temperature before use.

1, Wash Buffer:

Diluted the 30mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4°C.

2, Standard:

1) 80ng/ml of standard solution was prepared by adding 1 ml of Sample / Standard dilution buffer into one Standard tube, and keeping the tube at the room temperature for 10 min and allowing it to mix thoroughly.
2) 6 Eppendorf tubes were labeled with 40ng/ml, 20ng/ml,10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, respectively.
3) Aliquot 0.3 ml of the Sample / Standard dilution buffer into each tube.
4) 0.3 ml of the above 80ng/ml standard solution was transferred into 1st tube and mixed thoroughly.
5) Then 0.3 ml from 1st tube was transferred to 2nd tube and mixed thoroughly.
6) Later 0.3ml from 2nd tube was transferred to 3rd tube and mixed thoroughly, and so on.

Note:

The standard solutions are best used within 2 hours. The standard solution should be at 4°C for up to12 hours. Or store at -20 °C for up to 48 hours. Avoid the repetition of freeze-thaw cycles.

3, Preparation of Biotin- detection Antibody working solution

Prepared within 1 hour before the experiment.

1) Calculated the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)

2) Diluted the Biotin- detection antibody with Antibody dilution buffer at 1:100 and mixed thoroughly. (i.e. Add 1 μl of Biotin- detection antibody into 99 μl of Antibody dilution buffer.)

4, Preparation of HRP-Streptavidin Conjugate (SABC) working solution:

Prepared within 30min before the experiment.

1) Calculated the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2) Diluted the SABC with SABC dilution buffer at 1:100 and mix thoroughly. (i.e. Add 1 μl of SABC into 99 μl of SABC dilution buffer.)

Assay Procedure

Before adding to wells, equilibrated the SABC working solution and TMB substrate for at least 30 min at the room temperature (37 °C). When diluting samples and reagents, they must be mixed completely and evenly. It is recommend to plot a standard curve for each test.

1. Set standard, test sample and controlled the (zero) wells on the pre-coated plate respectively, and then, recorded their positions. It is recommend to measure the each standard and sample in duplicate. Washed plate 2 times before adding standard, sample and controlled the (zero) wells
2. Aliquot 0.1ml of 80ng/ml, 40ng/ml, 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, standard solutions into the standard wells.
3. 0.1 ml of Sample / Standard dilution buffer added into the control (zero) well.

4. 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) added into test sample wells.
5. Sealed the plate with a cover and incubated at 37 °C for 90 min.
6. Removed the cover and discarded the plate content, clapped the plate on the absorbent filter Papers or other absorbent material.
7. 0.1 ml of Biotin- detection antibody working solution is added into the above wells (standard, test sample & zero wells).
8. Sealed the plate with a cover and incubated at 37°C for 60 min.
9. Removed the cover, and plate was washed 3 times with Wash buffer.
10. 0.1 ml of SABC working solution was added into each well covered the plate and incubated at 37°C for 30 min.
11. Removed the cover and plate was washed 5 times with the Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.
12. 90 µl of TMB substrate into each well, covered the plate and incubate at 37°C in dark within 15-30 min. (Note: This incubation time is for reference use only, the optimal time should be determined by end user.) And the shades of blue can be seen in the first 3-4 wells (with most concentrated CYPA standard solutions), the other wells showed no obvious colour.
13. Added 50 µl of Stop solution into each well and mixed thoroughly. The colour changed into yellow immediately.
14. Read the O.D. absorbance at 450 nm in a micro plate reader immediately after adding the stop solution.

For the calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well).The standard curve was plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The CYPA concentration of the samples can be interpolated from the standard curve.

Note: If the samples measured were diluted, multiplied the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Summary:

1. Washed plate 2 times before adding standard, sample and controlled the (zero) wells
2. Added 100µL standard or sample to each well for 90 minutes at 37°C
3. added 100µL Biotin- detection antibody working solution to each well for 60 minutes at 37°C
4. Aspirated and washed 3 times
5. Added 100µL SABC working solution to each well. Incubated for 30 minutes at 37°C
6. Aspirated and washed 5 times
7. Added 90µL TMB substrate. Incubated 15 -30 minutes at 37°C
8. Added 50µL Stop Solution. Read at 450nm immediately

9. Calculation of results

Typical Data & Standard Curve

Results of a typical standard run of a CYPA ELISA Kit are shown below. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment. (N/A=not applicable)

X	ng/ml	0	1.25	2.5	5	10	20	40	80
Y	OD450	0.035	0.097	0.158	0.352	0.611	1.112	1.729	2.298

Specificity

This assay has high sensitivity and excellent specificity for detection of CYPA. No significant cross-reactivity or interference between CYPA and analogues were observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between the CYPA and all the analogues, therefore, cross reaction may still exist.

Recovery

Matrices listed below were spiked with certain level of CYPA and the recovery rates were calculated by comparing the measured value to the expected amount of CYPA in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	85-99	91
EDTA plasma(n=5)	88-101	94
heparin plasma(n=5)	85-104	92

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of CYPA and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16
serum(n=5)	87-102%	85-105%	87-98%	88-104%
EDTA plasma(n=5)	82-95%	84-100%	83-101%	86-94%
heparin plasma(n=5)	82-93%	81-97%	80-98%	82-100%

Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level CYPA were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level CYPA were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean X 100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Stability

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard(n=5)	37°C for 1 months	4°C for 6 months
Average (%)	80	95-100

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

RESULTS AND STATISTICS

GENDER MATCHED ANALYSIS

BETWEEN CONTROL AND STUDY GROUP

Chi-square test

Sex	Control Group		Study Group		Total		Statistical inference
	n	***%***	***n***	***%***	***n***	***%***	
Male	24	48.0%	28	56.0%	52	52.0%	X^2=0.641 Df=1 .423>0.05 Not Significant
Female	26	52.0%	22	44.0%	48	48.0%	
Total	**50**	**100.0%**	**50**	**100.0%**	**100**	**100.0%**	

AGE MATCHED ANALYSIS

BETWEEN CONTROL AND STUDY GROUP

Chi-square test

Age (years)	Control Group		Study Group		Total		Statistical inference
	n	**%**	***n***	**%**	***n***	**%**	
Below 40yrs	3	6.0%	0	.0%	3	3.0%	X^2=59.267
41 to 50yrs	47	94.0%	13	26.0%	60	60.0%	Df=3
51 to 60yrs	0	.0%	22	44.0%	22	22.0%	.000<0.05
Above 61yrs	0	.0%	15	30.0%	15	15.0%	Significant
Total	**50**	**100.0%**	**50**	**100.0%**	**100**	**100.0%**	

COMPARISON OF PARAMETERS

BETWEEN CONTROL AND STUDY GROUP

T-Test

	n	Mean	S.D	t	Df	Statistical inference
Age (years)						
Control	50	45.16	3.165	11.688	98	.000<0.05 Significant
Study	50	56.18	5.868			
Duration (years)						
Control	50	.00	.000	18.360	98	.000<0.05 Significant
Study	50	12.58	4.845			
Fasting (mg/dl)						
Control	50	90.56	8.894	8.526	98	.000<0.05 Significant
Study	50	127.38	29.212			
Creatinine (mg)						
Control	50	.7570	.13256	10.196	98	.000<0.05 Significant
Study	50	1.3240	.37019			
eGFR (ml/min)						
Control	50	111.64	6.586	4.275	98	.000<0.05 Significant
Study	50	98.68	20.400			
ACR (mg/g)						
Control	50	11.92	2.039	5.317	98	.000<0.05

Study	50	248.62	314.790			Significant
CYP A (ng/ml)						
Control	50	.7896	.12144	6.930	98	.000<0.05
Study	50	1.8800	1.10589			Significant
BP (mmHg) -1						
Control	50	114.40	5.014	9.130	98	.000<0.05
Study	50	141.60	20.460			Significant
BP (mmHg) - 2						
Control	50	74.40	5.014	9.319	98	.000<0.05
Study	50	90.68	11.289			Significant
Urea (mg/dl)						
Control	50	24.92	3.510	5.940	98	.000<0.05
Study	50	43.98	22.416			Significant

DESCRIPTIVE STATISTICS

Variables	Control Group (n=50)				Study Group (n=50)			
	Min.	***Max.***	***Mean***	***S.D***	***Min.***	***Max.***	***Mean***	***S.D***
Age (years)	40	50	45.16	3.165	45	65	56.18	5.868
Duration (years)	0	0	.00	.000	5	20	12.58	4.845
Fasting (mg/dl)	78	110	90.56	8.894	91	190	127.38	29.212
Creatinine (mg)	.50	.90	.7570	.13256	.90	2.40	1.3240	.37019
eGFR (ml/min)	98	126	111.64	6.586	70	130	98.68	20.400
ACR (mg/g)	8	16	11.92	2.039	15	945	248.62	314.790
CYP A (ng/ml)	.50	.90	.7896	.12144	.90	5.00	1.8800	1.10589
BP (mmHg) -1	110	120	114.40	5.014	110	170	141.60	20.460
BP (mmHg) - 2	70	80	74.40	5.014	70	104	90.68	11.289
Urea (mg/dl)	20	30	24.92	3.510	20	90	43.98	22.416

PEARSONS CORRELATION

Control Group (n=50)

cyp A (ng/ml)	Correlation value	Statistical inference
AGE (YEARS)	.119	.412>0.05 NS
DURATION (YEARS)	-	-
FASTING (mg/dl)	.101	.483>0.05 NS
CREATININE (mg)	.640(**)	.000<0.01 S
eGFR (ml/min)	.395(**)	.004<0.01 S
ACR (mg/g)	.235	.101>0.05 NS
BP (mmHg) -1	-.004	.979>0.05 NS
BP (mmHg) - 2	-.004	.979>0.05 NS
UREA (mg/dl)	-.026	.856>0.05 NS
N	50	

** Correlation is significant at the 0.01 level

Study Group (n=50)

cyp A (ng/ml)	Correlation value	Statistical inference
AGE (YEARS)	.780(**)	.000<0.01 S
DURATION (YEARS)	.808(**)	.000<0.01 S
FASTING (mg/dl)	-.187	.194>0.05 NS
CREATININE (mg)	.844(**)	.000<0.01 S
eGFR (ml/min)	-.826(**)	.000<0.01 S

ACR (mg/g)	.902(**)	.000<0.01 S
BP (mmHg) -1	.842(**)	.000<0.01 S
BP (mmHg) - 2	.768(**)	.000<0.01 S
UREA (mg/dl)	.883(**)	.000<0.01 S
N	50	

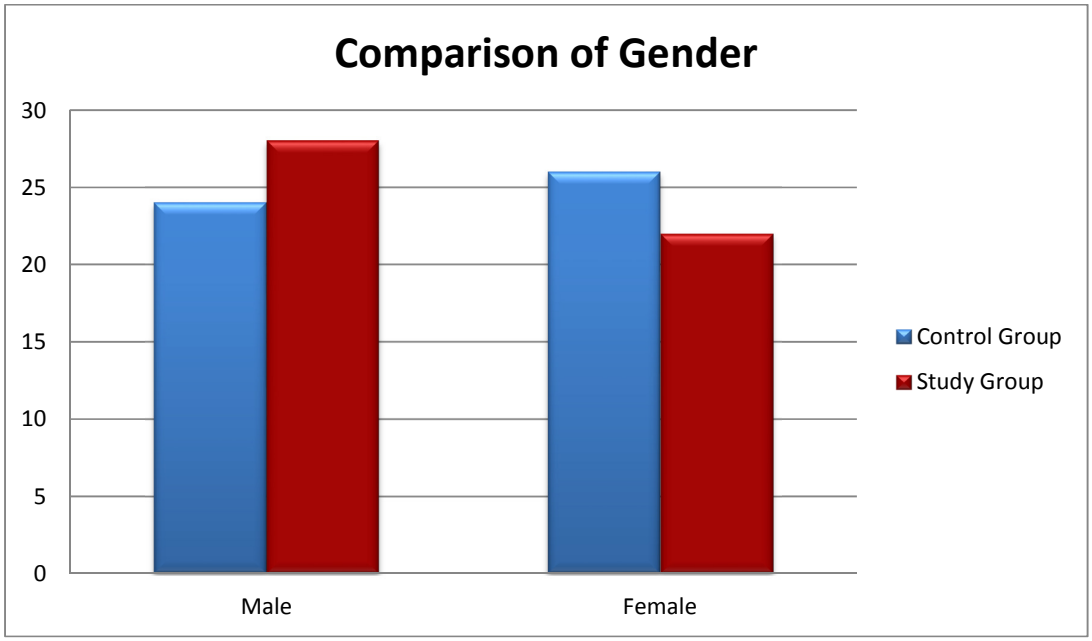
** Correlation is significant at the 0.01 level

COMPARISON OF GENDER

BETWEEN CONTROL AND STUDY GROUP

sex	Control Group	Study Group

Male	24	28
Female	26	22

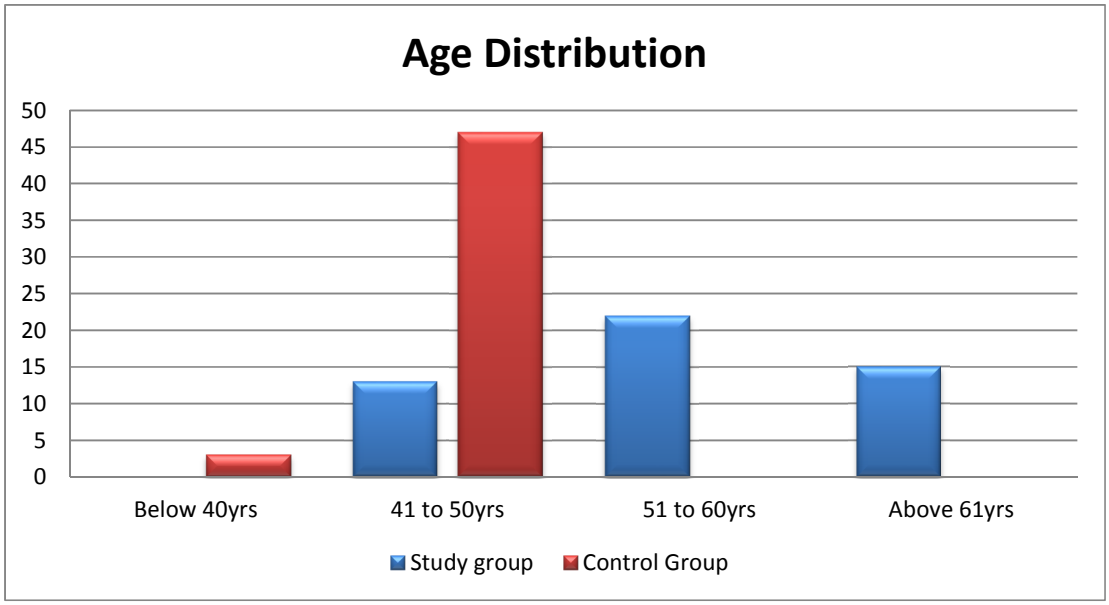


COMPARISON OF AGE DISTRIBUTION

BETWEEN CONTROL AND STUDY GROUP

Age wise distribution	Study group	Control Group

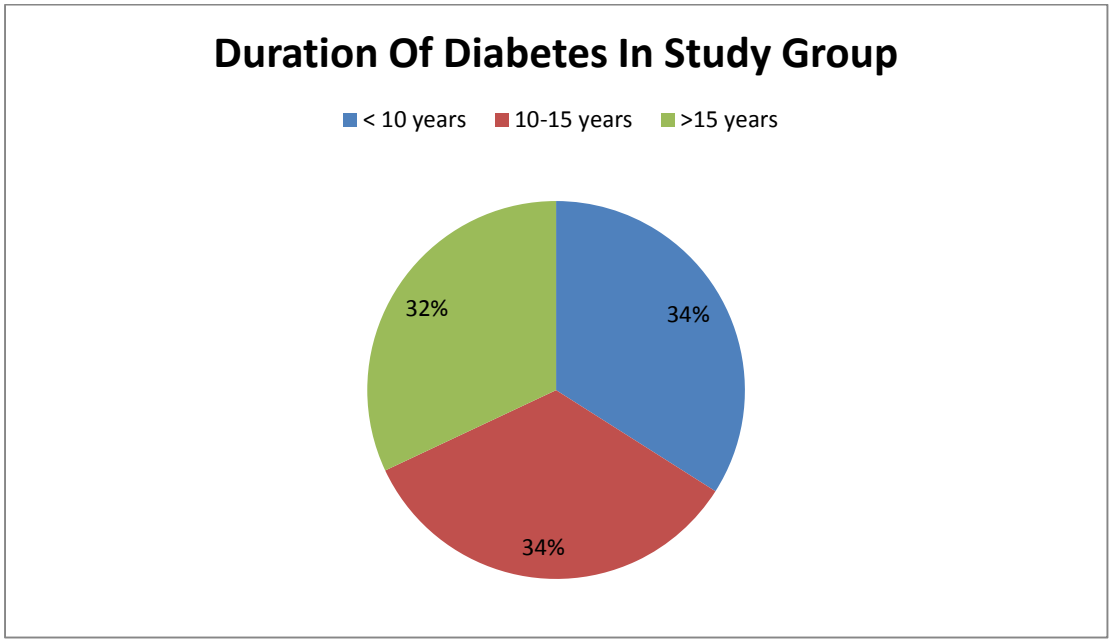
Below 40yrs	0	3
41 to 50yrs	13	47
51 to 60yrs	22	0
Above 61yrs	15	0



DURATION OF DIABETES IN STUDY GROUP

Duration of disease	study group	Control Group
< 10 years	17	0
10-15 years	17	0

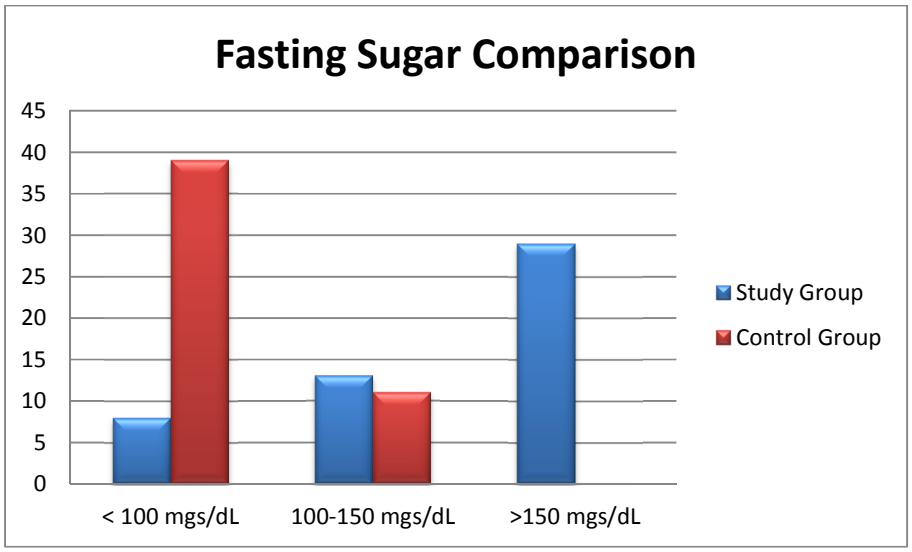
>15 years	16	0



COMPARISON OF FASTING BLOOD SUGAR

BETWEEN CONTROL AND STUDY GROUP

Fasting Sugar	Control Group	Study Group
< 100 mgs/dL	39	8
100-150 mgs/dL	11	13
>150 mgs/dL	0	29

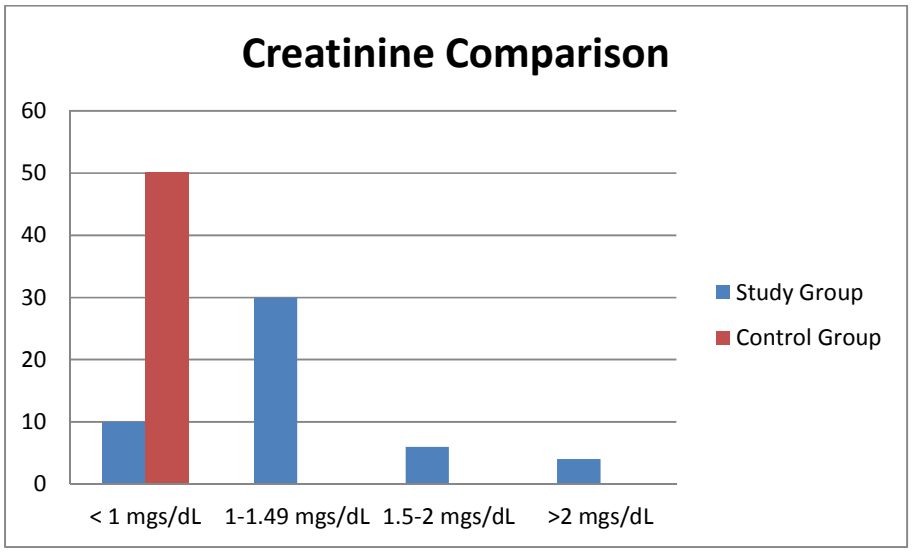


COMPARISON OF CREATININE LEVEL

BETWEEN CONTROL AND STUDY GROUP

Creatinine

Creatinine level	Study Group	Control Group
< 1 mgs/dL	10	50
1-1.49 mgs/dL	30	0
1.5-2 mgs/dL	6	0
>2 mgs/dL	4	0

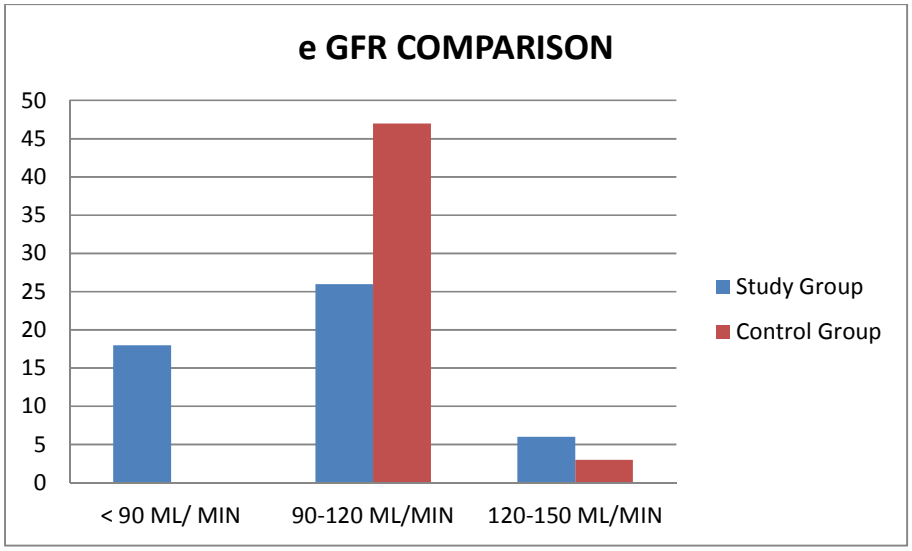


COMPARISON OF eGFR

BETWEEN CONTROL AND STUDY GROUP

eGFR	Study Group	Control Group
< 90 ML/ MIN	18	0

90-120 ML/MIN	26	47
120-150 ML/MIN	6	3

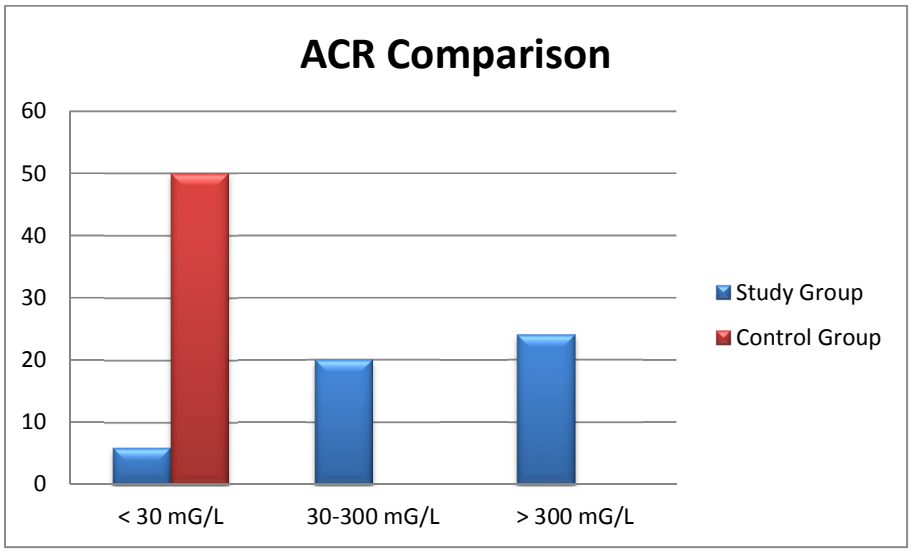


COMPARISON OF ALBUMIN CREATININE RATIO

BETWEEN CONTROL AND STUDY GROUP

ACR	Study Group	Control Group
< 30 mG/L	6	50

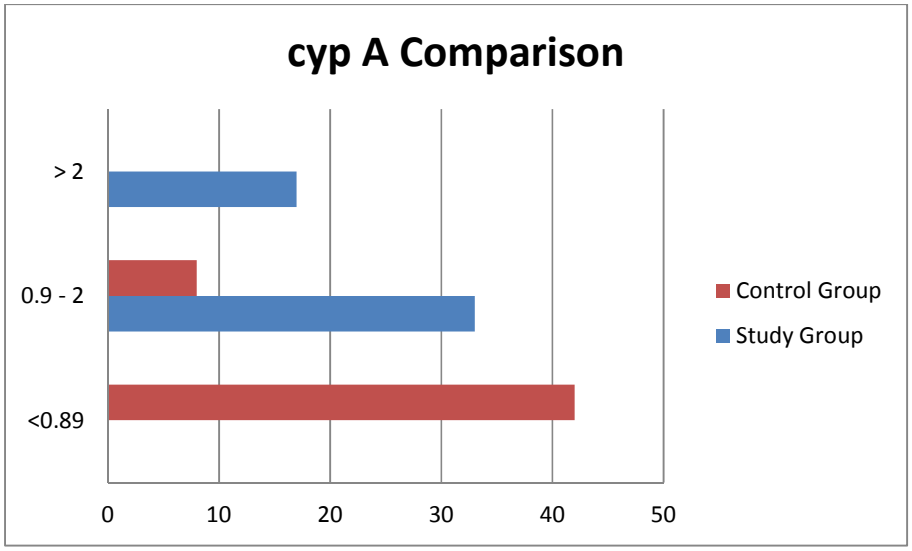
30-300 mG/L	20	0
> 300 mG/L	24	0



COMPARISON OF URINARY CYCLOPHILIN A LEVEL

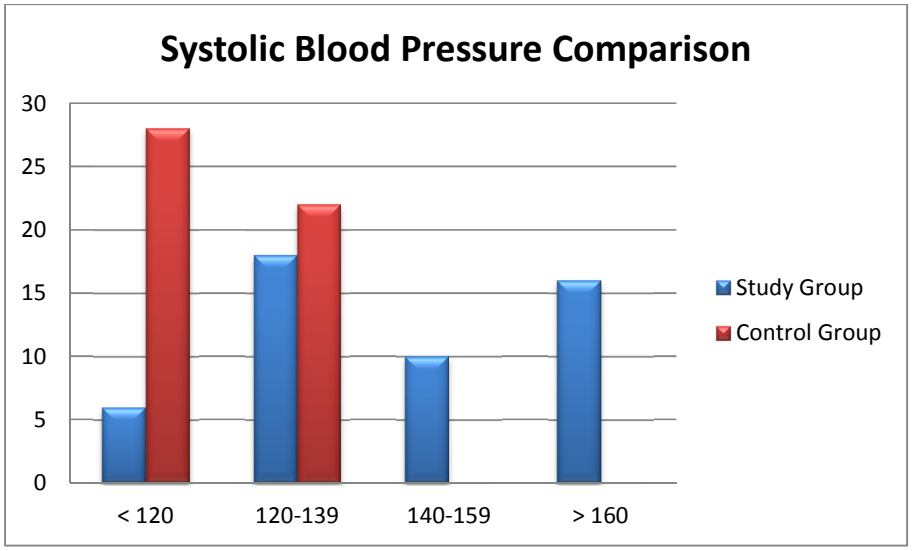
BETWEEN CONTROL AND STUDY GROUP

cyp A LEVELS	Study Group	Control Group
<0.89	0	42
0.9 - 2	33	8
> 2	17	0



COMPARISON OF SYSTOLIC BLOOD PRESSURE BETWEEN CONTROL AND STUDY GROUP

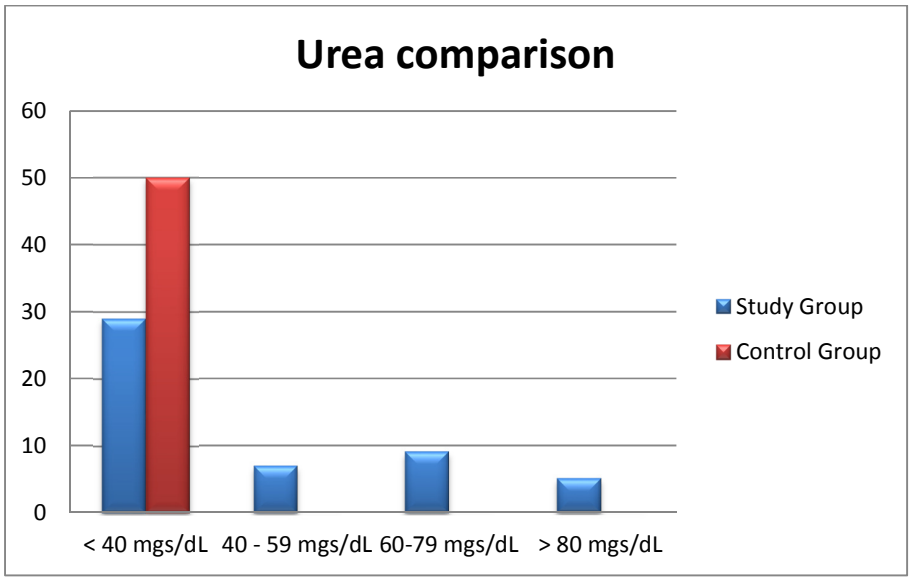
Systolic Blood Pressure	Study Group	Control Group
< 120	6	28
120-139	18	22
140-159	10	0
> 160	16	0



COMPARISON OF UREA BETWEEN

CONTROL AND STUDY GROUP

Urea	Study Group	Control Group
< 40 mgs/dL	29	50
40 - 59 mgs/dL	7	0
60-79 mgs/dL	9	0
> 80 mgs/dL	5	0



DISCUSSION

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease. Albuminuria is the most commonly used marker to predict onset of diabetic nephropathy (DN) without enough sensitivity and specificity to detect early DN. This is the first study to identify urinary cyclophilin A (CypA) as a new biomarker for early DN.

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD). ESRD in almost half of patients is due to diabetic nephropathy (DN), and these cases have the worst outcome compared to patients with other causes of ESRD. Although there are many novel drugs for DM, there are no specific curative treatments yet for DN. Reasons for poor outcome include inadequate markers and the complicated mechanisms of DN. Currently, the stage of severity is determined

according to the levels of albuminuria. Albuminuria is the most commonly used marker to predict onset and progression of DN clinically. However, this traditional marker for DN lacks both sensitivity and specificity to detect early stage of DN. Furthermore, some DN patients with ESRD do not present with significant albuminuria. The lack of a strong association between glomerular filtration rate (GFR) and albuminuria suggests that an alternative to this albuminuria-based staging system is needed. Some studies have noted the existence of pathological change before microalbuminuria. Therefore, even if microalbuminuria can be regarded as the earliest manifestation of DN, it is possible that a new biomarker for DN exists. Recently, different markers of DN were reviewed including fibroblast growth factor 23, tubular markers (kidney injury molecule 1, neutrophil gelatinase-associated lipocalin, and liver-type fatty acid-binding protein [L-FABP]), inflammatory markers (interleukin 6 [IL-6], IL-8, monocyte chemoattractant protein 1, and interferonγ–inducible protein), urinary 8-hydroxy-20-deoxyguanosine, serum cystatin C, and so on. Among these, genetic susceptibility almost always leads to irreversible DN, and detection of the clinical markers mostly occurs too late to diagnose and monitor the progression of DN. As such, it is crucial to find an earlier and reliable marker for DN. Earlier diagnosis and intervention may provide an opportunity to stop the permanent damage caused by DN.

The concentration of urinary CypA correlated well with the progression of renal function. Urinary CypA is a good biomarker for early DN detection in humans and it can be released from either mesangial or tubular cells.

In the study of Urinary Cyclophiline A were found to be significantly increased in patients with Diabetic nephropathy mean (0 .7896 ± 0.12144) when compared with control group mean (1.8800 ± 1.10589).

ACR level were progressively increased in cases than control and shows positive correlation with Urinary Cyclophilin A (0.235). 20% of the patients of the study group having normal ACR value with increased urinay Cyclophiline level. So we can diagnose Diabetic Nephropathy prior to appearance of albuminuria. The concentration of urinary CypA was more than 0.7250 ng/mL, we could diagnose the silent stage of DN.

The concentration of urinary CypA correlated well with duration of Diabetes mellitus (0.808).

The concentration of urinary CypA correlated well with Serum creatinine (0.844).

The concentration of urinary CypA correlated well with Blood Urea (0.833).

The concentration of urinary CypA negatively correlated with e GFR.

When patients in different stages of Diabetic nephropathy were compared Urinary Cyclophiline A level were found to be progressively increased from stage 2 to 5. This observation shows that Urinary Cyclophiline increases as renal function declines and inversely correlates with e GFR (0.395).

Serum creatinine and blood urea were progressively increased in cases then control and shows positive correlation with urinary Cyclophilin A.

Fasting blood sugar were significantly higher in cases (127.38±29.212) than in controls (90.56±8.894).

Blood pressure were significantly higher in cases (141.60±20.460) than in controls (114.40±5.014).

CONCLUSION

The present study demonstrated Urinary Cyclophiline A concentrations are significantly increased in patients with Diabetic Nephropathy. Urinary Cyclophiline A correlated well with other markers of Nephropathy. Urinary Cyclophiline A significantly increased even in early stages of Diabetic Nephropathy.

Urinary Cyclophiline A estimation can be used for the early diagnosis of renal damage due to long standing diabetes mellitus.

LIMITATIONS OF THE STUDY

- The sample size was small.
- Calculation of e GFR is based on serum creatinine which is a crude marker for e GFR estimation.

FUTURE SCOPE OF THE STUDY

- Urinary Cyclophilin A level may be used as alternative biomarker for microalbumin.
- Urinary Cyclophilin A level may be used as alternative biomarker for vascular diseases and coronary artery diseases.
- Urinary Cyclophilin A monoclonal antibodies may be developed and used for treatment of diabetic nephropathy.

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CONSENT FORM

Dr. C.ANBUMANI, Post Graduate Student in the Department of Biochemistry, Thanjavur Medical college, Thanjavur is doing a work on "A STUDY OF URINARY CYCLOPHILIN A". The procedure has been explained to me clearly.

The procedure has been explained to me clearly. I understand that there is no risk involved in the above procedures. I hereby give my consent to participate in this study. The data obtained here may be used for research and publication.

Signature

Name:

Place:

STUDY OF URINARY CYCLOPHILIN A LEVEL IN DIABETIC NEPHROPATHY

PROFORMA

NAME OF THE PATIENT: O.P NO/I.P NO:

AGE :

OCCUPATION :

ADDRESS :

COMPLAINTS :

PAST HISTORY:

PERSONAL HISTORY:

FAMILY HISTORY:

DRUG HISTORY:

GENERAL EXAMINATION:

HT: WT: BMI: PR: BP:

SYSTEMIC EXAMINATION:

CVS: RS:

ABD: CNS:

INVESTIGATIONS :

BLOOD

1. BLOOD SUGAR: FBS: PPBS:
2. SERUM CREATININE

URINE

1. URINE LEVEL OF CYCLOPHILINE- A
2. URINE ALBUMIN
3. ALBUMIN CREATININE RATIO
4. eGFR

ABBREVIATIONS

CKD	Chronic Kidney Disease
ESRD	End Stage Renal Disease
GFR	Glomerular Filteration Rate
Cyp A	Cyclophilin A
DN	Diabetic Nephropathy
ACR	AlbuminCreatinineRatio
CVD	Cardio VascularDisease
DKD	Diabetic Kidney Disease
AER	Albumin ExcretionRate

MASTER CHART I - CONTROL GROUP										
SL.NO	SEX	AGE (YEARS)	DURATION (YEARS)	FASTING (mg/dl)	CREATININE (mg)	eGFR (ml/min)	ACR (mg/g)	CYP A (ng/ml)	BP (mmHg)	UREA (mg/dl)
1	M	47	0	85	0.73	117	12	0.74	110/70	22
2	F	43	0	79	0.69	103	10	0.69	120/80	25
3	M	40	0	88	0.81	111	13	0.58	120/80	30
4	F	42	0	80	0.52	113	12	0.55	110/70	21
5	M	45	0	92	0.54	109	11	0.66	120/80	26
6	M	43	0	100	0.61	106	10	0.71	110/70	20
7	F	41	0	90	0.50	102	9	0.90	110/70	27
8	F	46	0	97	0.59	104	11	0.64	120/80	23
9	M	49	0	82	0.64	107	10	0.50	110/70	30
10	M	44	0	78	0.57	105	15	0.90	120/80	24
11	F	48	0	93	0.70	110	12	0.60	110/70	20
12	F	40	0	110	0.53	108	11	0.50	110/70	25
13	M	47	0	102	0.60	111	8	0.54	120/80	23
14	F	49	0	79	0.57	98	14	0.57	110/70	27
15	M	41	0	81	0.59	101	9	0.79	120/80	29

16	F	44	0	78	0.64	103	16	0.82	120/80	20
17	M	48	0	92	0.56	114	10	0.85	110/70	30
18	M	50	0	105	0.79	117	14	0.77	110/70	21
19	F	43	0	99	0.80	120	12	0.81	120/80	29
20	F	41	0	80	0.65	113	15	0.75	120/80	22
21	M	44	0	91	0.77	108	11	0.78	110/70	25
22	M	45	0	86	0.69	105	8	0.69	120/80	30
23	F	43	0	84	0.57	101	9	0.71	110/70	24
24	M	49	0	108	0.90	120	16	0.90	120/80	20
25	F	50	0	80	0.89	118	14	0.87	110/70	23
26	F	45	0	89	0.74	111	10	0.81	110/70	28
27	M	47	0	78	0.80	119	15	0.76	120/80	21
28	F	46	0	90	0.84	108	12	0.85	110/70	29
29	M	41	0	102	0.79	126	11	0.90	110/70	25
30	M	43	0	79	0.90	112	14	0.79	110/70	30
31	F	40	0	88	0.84	119	13	0.82	120/80	26
32	M	43	0	85	0.87	110	11	0.86	110/70	22
33	F	48	0	94	0.89	107	10	0.89	110/70	20

34	F	50	0	92	0.90	109	12	0.9	120/80	24
35	M	49	0	88	0.86	107	10	0.87	110/70	27
36	F	47	0	90	0.78	105	13	0.83	120/80	30
37	M	41	0	85	0.87	109	12	0.85	120/80	21
38	M	45	0	88	0.84	112	14	0.89	110/70	28
39	F	43	0	100	0.90	120	13	0.90	120/80	24
40	F	47	0	102	0.88	124	10	0.86	110/70	20
41	M	50	0	105	0.90	119	12	0.90	110/70	23
42	F	49	0	99	0.89	110	11	0.88	120/80	30
43	F	45	0	79	0.79	113	14	0.87	110/70	25
44	F	42	0	84	0.80	115	12	0.89	110/70	21
45	M	47	0	89	0.85	117	10	0.90	120/80	29
46	M	50	0	101	0.90	120	15	0.89	110/70	27
47	F	43	0	97	0.90	110	13	0.86	120/80	30
48	F	48	0	90	0.88	119	11	0.90	110/70	24
49	M	41	0	95	0.90	121	14	0.90	110/70	20
50	F	46	0	100	0.89	116	12	0.89	120/80	26

MASTER CHART II - STUDY GROUP										
SL.NO	SEX	AGE (YEARS)	DURATION (YEARS)	FASTING (mg/dl)	CREATININE (mg)	eGFR (ml/min)	ACR (mg/g)	CYP A (ng/ml)	BP (mmHg)	UREA (mg/dl)
1	M	49	6	92	0.93	128	15	0.91	120/80	20
2	M	49	6	92	0.93	128	15	0.91	120/80	20
3	F	47	5	99	0.95	127	17	1.00	110/70	27
4	F	50	6	107	0.97	129	20	0.97	110/70	21
5	M	52	9	123	1.11	115	45	1.10	130/86	24
6	M	54	10	135	1.19	117	52	1.00	120/80	22
7	M	53	8	127	1.17	111	48	1.10	130/86	28
8	F	50	9	138	1.1	118	37	1.00	120/80	25
9	M	58	15	170	1.2	90	70	1.30	146/96	30
10	F	56	14	177	1.27	94	75	1.25	140/90	35
11	M	57	13	184	1.22	96	82	1.50	146/96	40
12	M	55	14	171	1.23	92	84	1.60	140/90	31
13	M	60	15	112	1.29	98	175	2.00	160/100	66
14	F	63	16	125	1.35	84	212	2.10	156/100	53
15	M	62	15	110	1.38	86	317	2.30	160/100	64

16	M	61	17	117	1.32	81	188	2.22	160/100	49
17	F	65	20	115	1.5	70	780	4.00	170/104	85
18	F	63	18	120	1.6	73	890	2.50	160/100	77
19	M	62	17	105	2.28	71	756	3.30	170/104	81
20	F	60	19	114	2.3	74	945	4.50	170/104	73
21	M	45	6	95	0.91	128	18	0.92	110/70	22
22	M	48	5	92	0.94	127	19	0.98	120/80	27
23	F	46	6	97	0.99	130	22	0.90	110/70	20
24	F	49	5	91	0.96	121	25	0.97	110/70	25
25	F	50	8	125	1.13	112	55	1.00	130/86	30
26	M	52	9	139	1.2	110	43	1.10	130/86	21
27	M	54	8	128	1.19	115	62	1.00	120/80	29
28	F	53	10	136	1.1	119	59	1.10	120/80	26
29	M	56	13	176	1.3	91	73	1.20	146/96	39
30	F	58	15	188	1.22	98	79	1.40	140/90	32
31	M	57	13	172	1.29	99	81	1.60	146/96	34
32	F	55	14	190	1.3	90	87	1.80	140/90	38
33	M	60	16	113	1.3	80	104	2.00	160/100	69

34	F	62	15	129	1.4	90	277	2.40	156/100	55
35	M	61	17	120	1.36	85	314	2.30	160/100	43
36	F	63	16	126	1.33	84	294	2.20	156/100	48
37	M	65	19	102	1.8	72	826	4.00	170/104	74
38	F	63	17	108	1.6	73	890	2.50	160/100	85
39	F	60	20	115	2	71	756	3.30	170/104	88
40	M	62	18	100	2.4	70	932	5.00	170/104	90
41	F	49	6	94	0.99	126	20	0.97	120/80	23
42	M	47	5	109	0.9	129	24	1.00	110/70	20
43	M	54	8	140	1.2	117	64	1.10	120/80	28
44	F	50	10	138	1.1	112	38	1.00	130/86	30
45	M	57	14	181	1.3	99	76	1.80	146/96	33
46	M	55	13	176	1.2	92	88	1.50	146/96	39
47	F	60	17	110	1.4	80	340	2.10	160/100	61
48	M	63	16	126	1.3	87	275	2.30	156/100	46
49	M	65	20	100	2.3	73	898	3.00	160/100	79
50	F	64	18	120	1.5	72	769	5.00	170/104	74