EVALUATION OF URINARY TUBULAR ENZYMES AS SCREENING MARKERS OF RENAL DYSFUNCTION IN PATIENTS SUFFERING FROM DIABETES MELLITUS TYPE 2

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April 2010

DECLARATION

I declare that the work presented in this thesis is my original work and has not been presented for a degree in any other university or any other award.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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This work is dedicated to my lovely wife, Stella Kimani and our son Maxwell Gatua Kimani and our parents who have been a source of inspiration.

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Last but not the least, I thank God for enabling me complete this study and contribute something to science.

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ABBREVIATIONS AND ACRONYMS

AAP.....Alanine Aminopeptidase.

ACE.....angiotensin converting enzyme

AGES.....Advanced Glycosylated Products

ALP .....Alkaline Phosphatase

AMP amino-2-methyl-1-propanol

ARF .....acute renal failure

BUN .....Blood urea nitrogen

CKD .....chronic kidney disease

CRF ..... chronic renal failure

DKA .....Diabetic Ketoacidosis

$FADH_2$.....Reduced flavin Adenine Dinucleotide

G6P.....Glucose-6-Phosphate

GFR .....Glomerular Filtration Rate

GFR .....glomerular filtration rate

GGT.....Gamma glutamyltransferase

GLDH.....Glutamate Dehydrogenase

GST .....glutathione-s-transferase

KNH .....Kenyatta National Hospital

KNH-ERC .....KNH-Ethics and Research Committee

LAP .....Leucine Aminopeptidase

LDH.....Lactate Dehydrogenase

NADH .....Reduced Nicotinamide Adenine Dinucleotide

NADP .....Nicotinamide Adenine Diphosphate

NAG .....N-acetyl-β-D-glucosaminidase

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NADP .....Nicotinamide Adenine Diphosphate

NAG .....N-acetyl-β-D-glucosaminidase

NKUDIC.....National Kidney and Urologic Diseases Information

NSAIDS.....Non-steroidal anti-inflammatory drugs

PDGF-B.....Platelet Derived Growth Factor-B

PNP.....Para-Nitrophenol

PNPP.....Para-Nitrophenyl phosphate

RBP.....retinol binding protein

SLE.....systemic lupus erythematosus

TCA.....Tricarboxylic Acid Cycle

U. γ-GT.....Urinary gamma glutamyltransferase

U.ALP.....Urinary alkaline phosphatase

U.LDH.....Urinary lactate dehydrogenase

U.NAG.....Urinary N-acetyl-β-D-glucosaminidase

U.RBP.....Urinary retinol binding protein

U.TP.....Urinary total protein

U/L.....units per litre

U/mmolcr.....Units per millimole creatinine

U.α_1m.....Urinary α_1-microglobulin

WHO.....World Health Organization

B_2-m.....B_2-microglobulin

µmol/l.....micromole per litre

Mmol/l.....millimole per litre

Mg/l.....milligramme per litre

Mg/mmolcr.....milligramme per millimole creatinine

ABSTRACT

Diabetes mellitus is a global disorder and complications resulting from the disease are the third leading cause of death in the world. A survey by the National Diabetes Data Group estimates the prevalence of diabetes in the world population at 6.6%. One of the principle complications of diabetes mellitus is diabetic nephropathy and renal function tests are important indicators in diabetic patients needed to identify the early structural and functional changes in diabetic nephropathy. Diabetes mellitus type 2 patients show elevated levels of albumin in urine and assessment of renal injury based on the concentrations of blood urea nitrogen (BUN), serum creatinine (S.Cr) or urinary micro protein (U.MP) that are commonly used are usually insensitive since these parameters could be within normal ranges despite considerable impairment of the renal function because of the great reserve capacity of the kidney. More sensitive urinary biomarkers, which could be used to detect nephrotoxicity at early stages on various parts of the nephron, are being investigated. Animal studies have identified enzymes as potential urinary biomarkers of renal injury. These biomarkers include the high molecular weight albumin for evaluating glomerular integrity, the brush border enzymes alkaline phosphatase (U.ALP) and gamma glutamyl transferase (U.γ-GT), lysosomal enzyme N-acetyl-β-D-glucosaminidase (U.NAG), and cytoplasmic enzyme lactate dehydrogenase (U.LDH) for indicating proximal tubular injury. The present study investigated early signs of renal injury due to diabetes mellitus type 2 by measuring urinary indicators of nephrotoxicity. The study subjects comprised 251 patients with diabetes mellitus type 2 (mean age 54.2yrs) and 73 healthy normal individuals recruited as control group (mean age 40.9 yrs). The diabetic group was further subdivided into those with normoproteinuria, microproteinuria and diabetics with renal failure. Glomerular function was studied by determining the urinary levels of micro protein (U.MP), serum urea and creatinine while proximal tubular structural integrity was studied by determining the activities of the enzymes U.ALP, U.NAG, U.γ-GT, and U.LDH. Compared with normal healthy individuals, diabetic patients with normoproteinuria excreted significantly high levels of U.ALP, U.LDH, U.γ-GT, and U.NAG ($p<0.05$). Patients with renal impairment excreted high levels of the enzymes and urinary micro protein compared to healthy individuals and diabetic patients without renal failure. In conclusion, the present study confirms that diabetes mellitus leads to nephrotoxicity; that urinary excretion of U.ALP, U.LDH, U. γ-GT, and U.NAG could be useful biomarkers for proximal tubular injury. These results suggest that site-specific urinary biochemical markers provide valuable information about early renal proximal tubular insult that ultimately may precede glomerular permeability in subjects with diabetes mellitus.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli, characterized by nodular glomerulosclerosis due to long-standing diabetes mellitus. Research has led to understanding of the complex pathophysiology in diabetic nephropathy which is attributed to increased glomerular capillary pressure associated with hyper filtration at the glomerulus.

Diabetic nephropathy is characterised structurally both by glomerular lesions and changes to the tubulo-interstitial compartment of the kidney, and functionally by increasing severity of microproteinuria and altered glomerular filtration rate (GFR), the later usually being assessed in the laboratory by measurements of serum or plasma creatinine concentrations (Panchapakesan *et al.,* 2005). Furthermore, end-stage renal disease (ESRD) in diabetics is increasing and now accounts for approximately 40% of treated ESRD by either transplantation or dialysis (Mogensen *et al.,* 1993).

Although incipient diabetic kidney disease is usually characterised functionally by the presence of microproteinuria, serum creatinine is of limited value in the early detection of renal dysfunction due to its poor sensitivity to early nephron damage and renal dysfunction (Friedman *et al.,* 2007). It is now well established that chronic hyperglycemia, and consequently effective glycemic control, is the main metabolic determinant associated with irreversible kidney damage in diabetes mellitus type 2 (Turner, 1998). Although microproteinuria has been used for many years as a predictor of incipient diabetic nephropathy, reflecting the loss of glomerular

selectivity, estimation of renal tubular function and integrity may provide an early indication of renal dysfunction and thus identify those at risk of developing kidney failure (Whiting *et al.,* 2001). It has been shown that early renal tubular damage is characterised by increased proximal tubular enzymuria (Uslu *et al.,* 2005).

Renal dysfunction in general and glomerular dysfunction in particular are observed only rarely in early diabetic nephropathy, and the identification of early renal damage and those at risk of developing renal dysfunction is important so that corrective therapies can be applied at an early stage. The aim of this study was therefore to investigate the association between the urinary enzyme activities of N-acetyl-β-D-glucosaminidase (NAG), Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP), and Gamma Glutamyl Transferase (γ-GT) in subjects with type 2 diabetes and the severity of microproteinuria.

Most cytosol and brush-border enzymes leak from the cells as a result of increased tissue damage. These enzymes are normally degraded through the normal metabolism but most of them are excreted from the body unchanged through the kidney. Increased excretion of urinary enzymes is often used to detect nephrotoxicity and renal disease (Jung *et al.,* 1993). Such measurements of enzyme activity in urine provide a sensitive assessment for renal tubular cell damage, thereby enabling detection of sub-clinical tubular injury (Amico *et al.,* 2003).

These enzymes have been commonly used in various studies as sensitive markers of renal damage but NAG is more specific to the kidney damage and hence more reliable (Ikenage *et al.,* 1993). The assay of enzyme activity in urine can therefore be used to monitor renal pathology. It has been shown to be more sensitive than routine tests of renal function e.g. microalbuminuria for the detection of tubular damage (Donadio *et al.,* 1996). Variations in enzyme activity due to the fluctuations of urine flow rate have been shown to be almost eliminated by expressing the values as the ratio of the enzyme activity to urinary creatinine concentration (Hsu *et al.,* 1989). Determination of proteinuria has been used to demonstrate and monitor kidney and urinary tract diseases for many years (Rosenberg *et al.,* 1992). The determination of urinary micro protein however, does not differentiate between glomerular and tubular proteinuria.

The metabolic organization of the nephron has been assessed (Dubach *et al.,* 1968) and shown to be highly heterogeneous morphologically, and about 12 segments distinguished according to their enzymatic activities (Guder, 1986). Brush-border, lysosomal, and cytosolic enzymes are excreted in the urine of healthy persons and enzymuria rise as a consequence of cell breakdown, necrosis, and increased cellular turn-over (Mutti, 1989). Therefore the type of enzymuria reflects the site of damage to proximal tubules. Of these urinary enzymes, the brush-border membrane enzyme, Alkaline Phosphatase (ALP) and the lysosomal N-acetyl-β-D-glucosaminidase (NAG) (Carr *et al.,* 1994), and cytosol enzymes such as Lactate Dehydrogenase (LDH) and Gamma Glutamyltransferase (γ-GT) have been investigated as markers for structural integrity of renal proximal tubules (Bedir, 1986). However there is need to investigate the same enzymes in our local population with an intent of determining their sensitivity and specificity as well as reference ranges in the local population.

Present clinical and diagnostic screening tests for assessing the functional integrity of the kidney include serum creatinine, urea and urinalysis with dipstick. However, these tests are less sensitive for detecting early renal changes due to diabetes mellitus type 2 (Lauwerys and Benard 1989; Ivandic *et al.,* 2000). Renal urinary biomarkers that are most useful in defining defects on various parts of the nephron include the following: high molecular weight-protein, albumin for evaluating glomerular integrity; low molecular-weight protein; retinol binding protein (RBP) for assessing tubular protein reabsorption, and cytoplasmic enzyme glutathione-S- transferase (GST) in addition to the lysosomal enzyme NAG. Other urinary renal enzymes including γ-GT, and ALP, which are found on the epithelial cells of the proximal tubule and the LDH, located at distal tubule cells respectively have been used to detect early kidney damage in rats (Melo, 2006) and in humans (Mwangi *et al.,* 2009).

According to Otieno *et al.* (2002), the prevalence of albuminuria in patients with uncontrolled diabetes mellitus type 2 at KNH is about 26%. Studies on the reliability and sensitivity of NAG to detect early kidney damage have not been evaluated widely despite the fact that diabetic nephropathy is a common problem in the country (WHO, 1999). This study aimed to investigate whether urinary activities of NAG, ALP, LDH and γ-GT can be used as renal dysfunction screening markers in patients suffering from diabetes mellitus type 2. Against the above background, this study investigated the effects of diabetes mellitus type 2 on the kidney by measuring urinary excretion levels of NAG, γ-GT, ALP, LDH and other proteins among patients suffering from diabetes mellitus type 2.

1.2 Kidney structure and functions

The kidney is a paired organ whose functions include removing waste products from the blood and regulating the amount of fluid in the body. The basic units of the kidneys are microscopically thin structures called nephrons, which filter the blood and remove wastes in the form of urine. Together with the bladder, two ureters, and the single urethra, the kidneys make up the body's urinary system. Human beings, as well as members of all other vertebrate species, typically have two kidneys. Like kidney beans, the body's kidneys are dark red in color and have a shape in which one side is convex, or rounded, and the other is concave, or indented (Figure 1). The kidneys of adult humans are about 10 to 13 cm (4 to 5 in) long and about 5 to 7.5 cm (2 to 3 in) wide—about the size of a computer mouse.

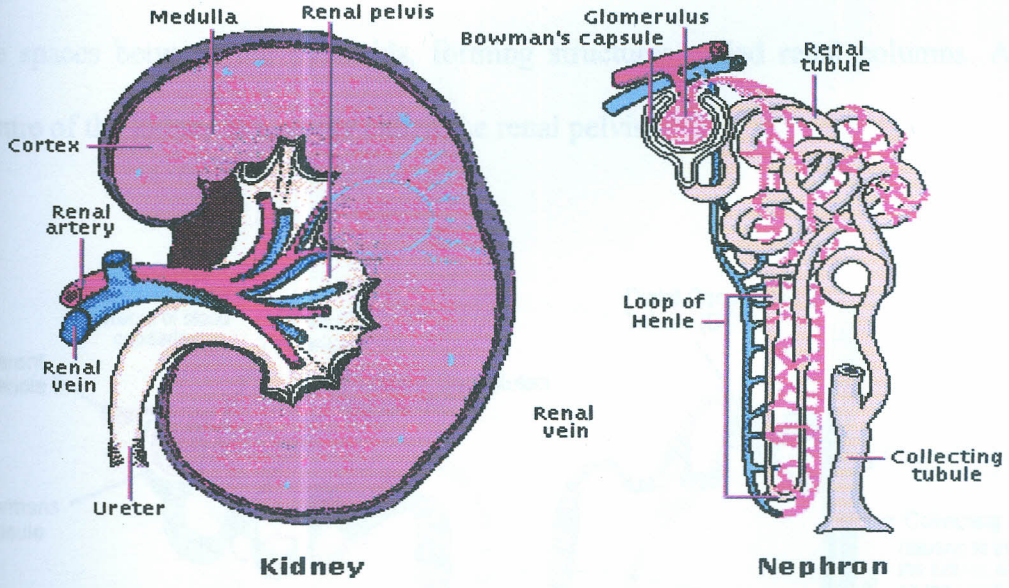

Figure 1: Structure of the kidney and nephron

The kidneys lie against the rear wall of the abdomen, on either side of the spine. They are situated below the middle of the back, beneath the liver on the right and the spleen on the left. Each kidney is encased in a transparent, fibrous membrane called a renal capsule, which helps protect it against trauma and infection. The concave part of the

kidney attaches to two of the body's crucial blood vessels—the renal artery and the renal vein—and the ureter, a tube like structure that carry urine to the bladder.

The primary function of kidneys is the removal of poisonous wastes from the blood. Chief among these wastes are the nitrogen-containing compounds urea and uric acid, which result from the breakdown of proteins and nucleic acids. Life-threatening illnesses occur when too many of these waste products accumulate in the bloodstream. Fortunately, a healthy kidney can easily rid the body of these substances. The outermost layer of the kidney is the cortex, beneath which lies the medulla, an area that contains between 8 and 18 cone-shaped sections known as pyramids, which are formed almost entirely of bundles of microscopic tubules. The tips of these pyramids point toward the centre of the kidney. The cortex extends into the spaces between the pyramids, forming structures called renal columns. At the centre of the kidney is a cavity called the renal pelvis.

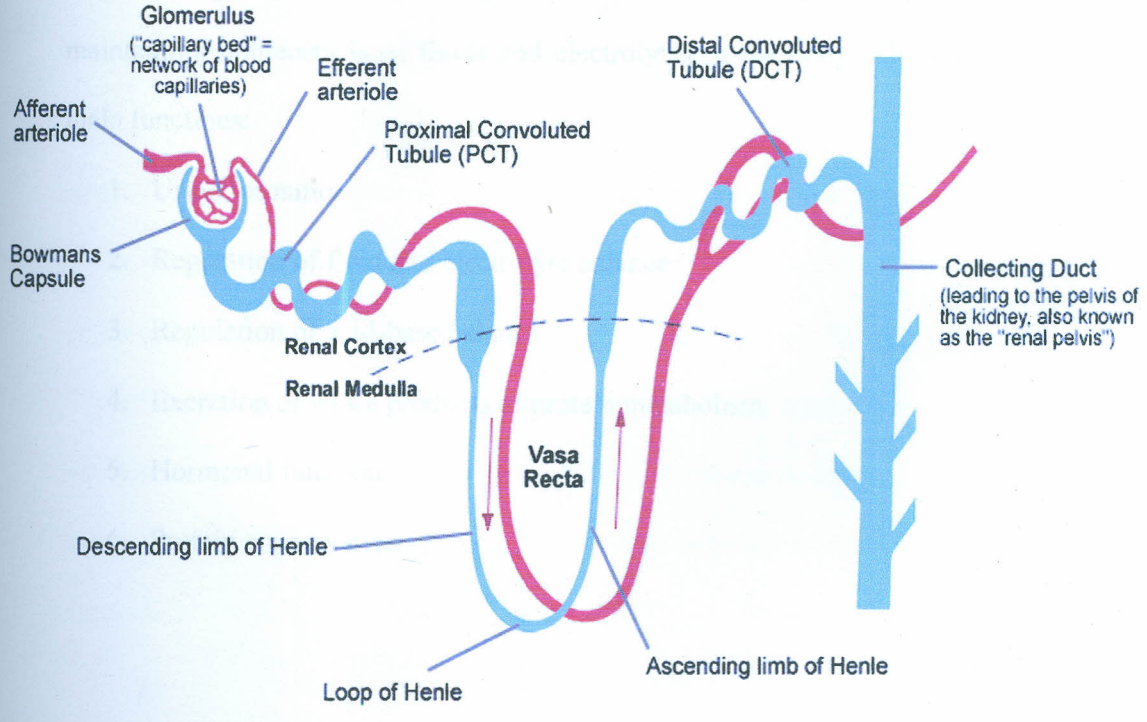

Figure 2: Diagram showing the structure of a nephron

The task of cleaning, or filtering, the blood is performed by millions of nephrons, remarkable structures that extend between the cortex and the medulla. Under magnification, nephrons look like tangles of tiny vessels or tubules, but each nephron actually has an orderly arrangement that makes possible filtration of wastes from the blood. The primary structure in this filtering system is the glomerulus. The glomerulus is contained in a cuplike structure called the Bowman's capsule, from which extends a narrow vessel, called the renal tubule. This tube twists and turns until it drains into a collecting tubule that carries urine toward the renal pelvis. Part of the renal tubule, called Henle's loop, becomes extremely narrow, extending down away from Bowman's capsule and then back up again in a U shape. Surrounding Henle's loop and the other parts of the renal tubule is a network of capillaries, which are formed from a small blood vessel that branches out from the glomerulus (Figure 2).

1.3 Renal physiology

The kidney is the chief regulator of all body fluids and is primarily responsible for maintaining homeostasis of fluids and electrolytes in the body. The kidney has six main functions:

1. Urine formation
2. Regulation of fluid and electrolyte balance
3. Regulation of acid-base balance
4. Excretion of waste products of protein metabolism
5. Hormonal function
6. Protein conservation

The kidney is able to carry out these complex functions because approximately 25% of the volume of blood pumped by the heart into the systemic circulation is circulated through the kidneys (Kaplan, 1996).

1.3.1 Urine formation

The removal of potentially toxic waste products is a major function of the kidneys and is accomplished through the formation of urine. The basic processes involved in the formation of urine are filtration, reabsorption and secretion. The kidneys filter large volumes of plasma, reabsorb most of what is filtered, and leave behind for elimination from the body a concentrated solution of metabolic wastes called urine.

1.3.2 Glomerular filtration

Each minute 1000-1500 millilitre (ml) of blood pass through the kidneys. The glomerulus has a semi-permeable basement membrane that allows free passage of water and electrolytes but is relatively impermeable to large molecules. In glomerular capillaries the hydrostatic pressure is approximately three times greater than the pressure in other capillaries. As a result of this high pressure, substances are filtered through the semi-permeable membrane into Bowman's capsule at a rate of approximately 130ml/min and this is known as the glomerular filtration rate (GFR). Cells and the large-molecular size plasma proteins are unable to pass through the semi- permeable membrane (Kaplan, 1996).

Therefore the glomerular filtrate is essentially plasma without the proteins. In the average healthy person, more than 187,000ml of filtrate is formed per day. Normal urine output is about 1500ml per day, which is only about 1% of the amount of filtrate formed.

1.3.3 Proximal tubule

The proximal tubular cells perform a variety of physiological tasks. Approximately 80% of salt and water are reabsorbed from the glomerular filtrate in the proximal tubule. All the filtered glucose and most of the amino acids are normally reabsorbed here. Low-molecular weight proteins, urea, uric acid, bicarbonate, phosphate, chloride, potassium, magnesium and calcium are reabsorbed to varying extents. A variety of organic acids and bases, as well as hydrogen ions and ammonia, are secreted into the tubular fluid by tubular cells. Tubular secretion, which transports substances into the tubular lumen, may be an active or passive process. Substances that are transported from the blood to the tubules and excreted in the urine include potassium, hydrogen ions, ammonia, and uric acid.

1.4 Nitrogenous waste excretion

One of the major functions of the kidney is the elimination of nitrogenous products of protein catabolism. The enormous reserves of the kidney for excretion of the products are indicated by the fact that the blood concentrations of these products are not elevated in renal failure until renal function is reduced to less than one-half of normal (First *et al.,* 1982).

1.4.1 Urea

Ammonia is produced following deamination of amino acids. The development of toxic levels of ammonia in the blood is prevented by the conversion of ammonia to urea in the liver. Urea in the blood is reported as the blood urea nitrogen (BUN). Urea production and the BUN are increased when more amino acids are metabolized in the liver (Walser *et al.,* 1990). This can occur with a high protein diet, tissue breakdown,

or decreased protein synthesis. In contrast, urea production and the BUN are reduced in the presence of a low-protein intake and severe liver disease (Ware *et al.,* 1981). Urea is readily filtered, but approximately 40-50% of the filtered urea is normally reabsorbed by the proximal tubules. Since many factors as indicated above may influence the BUN level while the GFR remains constant, BUN is a less specific indicator of renal function and should not be relied on for that purpose (First *et al.,* 1981).

1.4.2 Creatinine

Creatinine is derived from the non-enzymatic dehydration of creatine in skeletal muscle. Serum creatinine levels and urinary creatinine excretion are a function of muscle mass in normal persons and shows little response to dietary changes (Perrone *et al.*, 1992). The amount of creatine per unit of muscle mass is constant, and thus the rate of spontaneous breakdown of creatine is also constant. As a result, the plasma creatinine concentration is very stable, varying less than 10% per day in serial observations in normal subjects (Perrone *et al.,* 1992). Since the serum creatinine concentration is a direct reflection of muscle mass, the serum level is higher in males than in females. Creatinine is freely filtered at the glomerulus and is not reabsorbed by the tubules but is excreted.

1.4.3 Uric acid

Uric acid is derived from the oxidation of purine bases. Plasma levels of uric acid are quite variable and are higher in males than in females. Plasma urates are completely filterable, and both proximal tubular resorption and distal tubular secretion occur.

With advanced chronic renal failure there is a progressive increase in the plasma uric acid level.

1.5 Kidney diseases and their etiology

Many diseases affect kidney function by attacking the glomeruli. Glomerular disease fall into two major categories namely glomerulonephritis and glomerulosclerosis, which can lead to renal failure. The former is characterised by inflammation of the membrane tissue in the kidney that serves as filters separating waste and extra-cellular fluid from the blood while the later describes the scarring or hardening of the blood vessels within the kidney. The signs and symptoms of glomerular diseases include proteinuria, hematuria, reduced glomerular filtration rate (GFR), hypoproteinuria and oedema. One or more of these symptoms can be the first sign of kidney disease (NKUDIC, 2006).

Depending on the form of glomerular disease, renal function may be lost in a matter of days or weeks or may deteriorate slowly and gradually over the course of decades leading to either acute renal failure (ARF) or chronic renal failure (CRF). End-stage renal disease (ESRD) indicates permanent loss of kidney function. The ARF can be life threatening if it is not treated but kidney function returns immediately after the cause of this condition is treated. In many patients ARF is not associated with any permanent damage. However, some patients who recover from ARF may subsequently develop chronic kidney disease (CKD). In many cases, CKD leads to a total kidney failure (Firth & Wineearls 1996; Nahas & Wineearls 1996; NKUDIC 2006). Some forms of CKD can be controlled or slowed down. For example, by delaying diabetic nephropathy (DN) which is associated with CKD through better

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control of blood sugar levels, and using angiotensin converting enzyme inhibitors (ACE inhibitors) and angiotensin receptor blockers (ARBs) to reduce proteinuria and control of blood pressure. Other strategies for managing CKD include either dialysis or renal transplantation (NKUDIC, 2006). According to NKUDIC (2006) glomerular diseases are attributed to a number of etiologies. These are direct microbial infections, autoimmune diseases, and other systemic conditions such as diabetes and toxic substances.

1.5.1 Microbial infections

Glomerular disease sometimes develops rapidly after bacterial infections. Acute post-streptococcal glomerulonephritis (PSGN) can occur after an episode of *Streptococcus* throat or skin infection. The Streptococcus bacteria do not attack the kidney directly, but an infection may stimulate the immune system to over produce antibodies, which are circulated in the blood and deposited in the glomeruli, causing damage. Post-streptococcal glomerulonephritis (PSGN) can bring on sudden symptoms of oedema, oliguria, and haematuria. Laboratory tests show large amounts of proteins in the urine and elevated levels of creatinine and urea nitrogen in the blood, indicating reduced kidney function. High blood pressure frequently accompanies reduced kidney function in this disease. Post-streptococcal glomerulonephritis is most common in children between the ages of 3 and 7, (Feldmeire *at al.*, 2005; NKUDIC, 2006).

Bacterial endocarditis, infection of the tissues inside the heart is also associated with subsequent glomerular disease. Researchers are not sure whether the renal lesions that form after a heart infection are caused entirely by the immune response or whether some other disease mechanisms contributes to kidney damage. Endocarditis

sometimes produces CKD (NKUDIC, 2006; Sevinc, 2006). Human Immunodeficiency Virus (HIV), the virus that leads to Acquired Immunodeficiency Syndrome (AIDS), can also cause glomerular disease. Between 5 and 10 percent of people with HIV experience kidney failure, even before developing full brown AIDS. Human Immunodeficiency Virus-associated nephropathy begins with heavy proteinuria and progresses rapidly (within a year of detection) to total kidney failure. Therapies that can slow down or reverse this rapid deterioration of renal function are as yet unknown. Therapeutic solutions involving immunosuppressant are risky because of the patients' already compromised immune system (Sandhu et al., 2004; NKUDIC, 2006).

1.5.2 Autoimmune disease and other systemic conditions

Previous reports have attributed autoimmune conditions to glomerular diseases including Systemic Lupus Erythematosus (SLE), Goodpusture's syndrome, IgA nephropathy, Alport's syndrome (Hereditary nephritis) and other systemic diseases such as diabetic nephropathy, focal segmental glomerulosclerosis, membranous nephropathy and minimal change disease (Bakris, 1993; Adu, 1996; Cunningham, 1997; Kim *et al.*, 1999; Hudson *et al.*, 2003; Oortwijn *et al.*, 2006; NKUDIC, 2006; Crosson, 2007; Olowu *et al.*, 2007).

Systemic lupus erythematosus (SLE) affects many parts of the body: primarily the skin and joints, but also the kidneys. Because women are more likely to develop SLE than men, some researchers believe that a sex-linked genetic factor plays a part in making a person susceptible, although viral infection has also been implicated as a triggering factor. Lupus-nephritis is the name given to the kidney disease caused by

SLE, and it occurs when autoantibodies form or are deposited in the glomeruli, causing inflammation. Ultimately, the inflammation may create scars that stop the kidney from functioning well. Conventional treatments for lupus-nephritis include a combination of two drugs, cyclophosphamide, a cytotoxic agent that suppresses the immune system, and prednisolone, a corticosteroid used to reduce inflammation. A newer immunosuppressant, mychophenolate mofetil (MMF) that is as effective as cyclophosphamide and has milder side effects can be used instead of cyclophosphamide (NKUDIC, 2006; Olowu *et al.*, 2007).

Goodpusture's syndrome (GS) involves an autoantibody that specifically targets the kidneys and the lungs. Often, the first indication that patients have the autoantibody is when they cough up blood. But lung damage in GS is usually superficial compared to the progressive and permanent damage to kidneys. Treatment of GS includes immunosuppressive drugs and a blood cleaning therapy called plasmapheresis that removes the auto antibodies (Hudson *et al.*, 2003; NKUDIC, 2006).

Immunoglobulin A nephropathy (IgAN) is a form of glomerular disease that results when immunoglobulin A (IgA) forms deposits in the glomeruli, where it creates inflammation. The most common symptom of IgAN is blood in the urine, but is often a silent disease and may go undetected for many years. It is reported to be the most common cause of primary glomerulonephritis, glomerular disease not caused by a systemic disease like lupus or diabetes mellitus. It affects more men than women. Although found in all age groups, young people rarely display signs of kidney failure because the disease takes several years to progress to the stage where it causes detectable complications. No treatment is recommended for early or mild cases of

IgAN when the patient has normal blood pressure and less than 1 gram of protein in a 24-hour urine out-put. When proteinuria exceeds 1g/day, treatment is aimed at protecting kidney function by reducing proteinuria and controlling blood pressure. ACE inhibitors or ARBs that block angiotensin are most effective at achieving those two goals simultaneously (Oortwijn *et al.,* 2006; NKUDIC, 2006).

Alport's syndrome (AS) is a family history of chronic glomerular disease; it may also involve hearing or vision impairment. This syndrome affects both men and women, but the former are more likely to experience CKD and sensory loss. Men with AS first show evidence of renal insufficiency while in their twenties and reach total kidney failure by age 40. Women rarely have significant renal impairment, and hearing loss may be so slight that it can be detected only through testing with special equipment. Men can pass the disease to their daughters but women can transmit the disease to their sons or daughters. Treatment focuses on controlling blood pressure to maintain kidney function (Hudson *et al.,* 2003; NKUDIC, 2006).

Focal segmental glomerulosclerosis (FSGS) describes scarring in scattered regions of the kidney, typically limited to one part of the glomerulus and to a minority of glomeruli in the affected region. FSGS may result from a systemic disorder or it may develop as an idiopathic kidney disease. Proteinuria is the most common symptom of FSGS. Proteinuria is associated with several other kidney conditions. Biopsy may confirm the presence of glomerular scarring if the tissue is taken from the affected section of the kidney. Finding the affected section is a matter of chance, especially early in the disease process, when lesions may be scattered. Confirming a diagnosis of FSGS may require repeat kidney biopsies. Arriving at a diagnosis of idiopathic FSGS

requires the identification of focal scarring and the elimination of possible systemic causes such as diabetes or an immune response to infection. Since idiopathic FSGS is of unknown cause, it is difficult to treat. No universal remedy has been found, and most patients with FSGS progress to total kidney failure over 5 to 20 years. Some patients with an aggressive form of FSGS reach total kidney failure in 2 to 3 years. Treatments involving steroids or other immunosuppressive drugs help some patients by decreasing proteinuria and improving kidney function. But these treatments are beneficial to only a minority of those in whom they are tried, and some patients experience even poorer kidney function as a result. ACE inhibitors and ARBs may also be used in FSGS to decrease proteinuria. Treatment should focus on controlling blood pressure and blood cholesterol levels, factors that may contribute to kidney scarring (Kim *et al.*, 1999; Crosson, 2007; NKUDIC, 2006).

Membranous nephropathy (MN) is the second most common cause of the nephrotic syndrome after DN in the US adults. Diagnosis of MN requires a kidney biopsy, which reveals unusual deposits of immunoglobulin G and complement C3, substances created by the body's immune system. 75% of cases are idiopathic, which means that the cause of the disease is unknown. The remaining 25% of cases are the result of other diseases like SLE, hepatitis B or C infection, or some form of cancer. Drug therapies involving penicillamine, gold, or Captopril are also associated with MN. 20 to 40% of patients with MN progress, usually over decades, to total kidney failure, but most patients experience either complete remission or continued symptoms without progressive kidney failure. 20% of patients recover without treatment. ACE inhibitors and ARBs are used to reduce proteinuria. Medication to control high blood pressure and edema is frequently required. Some patients benefit from steroids, but this

treatment does not work for everyone. Immunosuppressive medications are helpful for some patients with progressive disease (Adu, 1996; Cunningham, 1997; NKUDIC, 2006).

Minimal change disease (MCD) is the diagnosis given when a patient has the nephrotic syndrome (NS) but the kidney biopsy reveals little or no change to the structure of glomeruli or surrounding tissues when examined by a light microscope. Tiny drops of lipid may be present, but no scarring has taken place with the kidney. MCD may occur at any age, but it is most common in childhood. A small percentage of patients with idiopathic NS do not respond to steroid therapy. For these patients, a low-sodium diet and a diuretic to control oedema is recommended. Nonsteroidal anti-inflammatory drugs (NSAIDS) are used to reduce proteinuria. ACE inhibitors and ARBs are also used to reduce proteinuria in patients with steroid- resistant MCD. These patients may respond to larger doses of steroids, more prolonged use of steroids, or steroids in combination with immunosuppressant drugs, such as chlorambucil, cyclophosphamide, or cyclosporine (Adu, 1996; Cunningham, 1997; NKUDIC, 2006).

1.5.3 Toxic chemical substances

Individuals can be exposed to nephrotoxic substances in the workplace, such as glycols, heavy metals, and organic solvents, as well as in the environment, such as insecticides, herbicides, and fungicides. The kidney is highly susceptible to chemical injury as a result of its high blood flow and anatomic structure. These chemicals have the potential to accumulate in the tubular fluid due to reabsorption, and transcellular transport system of the kidney. In addition, the kidney is a major metabolic organ

leading to biotransformation of chemicals into toxic derivatives (Price, 1982). Tubular interstitial structures of the kidney are particularly vulnerable to toxic compounds, which can be explained by the high concentration of the toxicants in the medulla as well as medullary hypoxia and renal hypoperfusion. Clinical nephrotoxicity involves toxins of diverse origin. The culprits are often registered and non-registered drugs either prescribed or purchased over the counter. Some of the chemicals that are known to be nephrotoxic to the kidney include some antibiotics, analgesics, cyclosporines, ochratoxins, Chinese herbs, and heavy metals such as Lead, Cadmium, Lithium, Germanium, Silica and other substances such as tobacco.

1.6 Diabetes mellitus and kidney failure

Diabetes has become the most common single cause of End-Stage Renal Disease (ESRD) (Jung *et al.,* 1993). This is due to the fact that, diabetes, particularly type 2 is increasing in prevalence (Ramachandran *et al.,* 1999), diabetes patients now live longer, and patients with diabetes ESRD are now being accepted for treatment in ESRD programs. Recent studies have demonstrated that the onset and course of diabetic nephropathy can be avoided to a very significant degree by several interventions, but these interventions have their greatest impact if instituted at a point very early in the course of the development of this complication. For many years clinicians have relied on the presence of raised levels of albumin in urine to detect nephropathy. Studies have indicated that certain enzymes such as N-acetyl-β-D-glucosaminidase (NAG) have increased activity in urine in diabetic nephropathy even before albumin increases in urine hence making it the best marker for diabetic nephropathy (Dedor *et al.*, 1989).

N-acetyl- β-D-glucosaminidase is an enzyme found in the cytosol of most cells where it catalyses the breakdown of gangliosides in the lysosomes. Gangliosides are continually synthesized and degraded by the sequential removal of their terminal sugars (Glucose or Galactose). The glycosyl hydrolases that catalyze these reactions are highly specific. Ganglioside breakdown occurs inside lysosomes (Stryer, 1975). These organelles contain many types of degrading enzymes and are specialized for the orderly destruction of cellular components. The kidneys contain a substantial amount of NAG.

Lactate dehydrogenase is an enzyme found in the cytosol of all human cells where it catalyses the reduction of pyruvate to lactate using Nicotinamide Adenine Dinucleotide (NAD) (Kaplan *et al.,* 1996). The enzyme is found predominantly in the heart and red blood cells and to a lesser extent in the kidney where it catalyzes the conversion of pyruvate to lactate as shown below:

Pyruvate + NADH + H^+ = L-Lactate + NAD^+.

Alkaline phosphatase is an enzyme found on the cell membranes of most cells. It is a member of a group of enzymes that hydrolyze monophosphate esters at an alkaline pH. The natural substrate for ALP is not known, however, many nonphysiological phosphate esters can serve as substrates. The enzyme has been identified in most body tissues including the kidney. ALP activity is highest in the liver, bone, intestine, kidney, and placenta (Kaplan, 1996). Gamma glutamyl transferase is a membrane-localized enzyme that plays a major role in glutathione metabolism and resorption of amino acids from the glomerular filtrate and from the intestinal lumen. Glutathione in the presence of γ-GT and an amino acid or peptide transfers glutamate to the amino

acid forming a peptide bond on the y-carboxylic acid, thereby forming cysteinylglycine and the corresponding y-glutamyl peptide.

Urea is synthesized in the liver as a result of deamination of amino acids. It is excreted in the kidney through the urine. Creatinine is found in the muscles in the form of phospho-creatine where it's used to generate AMP by reacting with Adenosine Diphosphate (ADP) in the presence of the enzyme creatine phospho kinase to produce creatinine, which is excreted in the kidney. Albumin is synthesized in the liver from amino acids. It's very useful in the maintenance of oncotic pressure in the extra cellular fluid. It is also used in the transportation of various molecules eg hormones in the blood stream where they are transported in a bound form. Albumin is a high molecular weight substance that is not easily filtered in the glomerulus of the kidney. Only undetectable small amounts of albumin pass through the glomerulus in healthy individuals. However, in individuals with nephropathy, the glomerulus filtration capacity is altered and increased amounts of albumin are excreted in urine through the kidney. The amount excreted in this condition is still little and hence the name microalbumin. Diabetic nephropathy is caused by progressive damage of the glomerulus capillaries due to persistent and enhanced hyperglycemia. Increased levels of microalbumin are found in the urine of patients suffering from diabetes mellitus due to glomerulus damage. Normal urine contains less than 200 mg of protein per day (Fioletto *et al.*, 1998).

1.6.1 Glucose metabolism and diabetes mellitus type 2

Diabetes mellitus type 2 is a disease characterized by persistent hyperglycemia (high blood sugar). The disease is a manageable but chronic condition, and the main risk

to health are its characteristic long-term complications e.g. chronic renal failure (CRF). Insulin enables the body cells to absorb glucose from the blood for use as fuel, for conversion to other needed molecules, or for storage. Insulin is also the principal control signal for the conversion of glucose to glycogen for internal storage in the liver and muscle cells. The principal biochemical function of glucose is to provide energy for life processes. Glucose oxidation by the glycolytic and tricarboxylic acid pathways is the primary source of energy for the biosynthesis of AMP (Kaplan, 1996).

Type 2 diabetes mellitus is due to a combination of defective insulin secretion and defective responsiveness to insulin (reduced insulin sensitivity). In early stages, the predominant abnormality is reduced insulin sensitivity, characterized by elevated levels of insulin in the blood. During this stage, hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver, but as the disease progresses the impairment of insulin secretion worsens, and therapeutic replacement of insulin often becomes necessary. Type 2 diabetes is quite common, comprising 90% (Zimmet *et al.*, 2001) or more of cases of diabetes, especially in the developed world. There is a strong, but not exclusive association with obesity, aging, and family history, although in the last decade it has increasingly been seen to affect children and adolescents (Mokdad *et al.*, 2000).

1.6.2 Clinical signs, symptoms and diagnostic criteria

These include polyuria, polydipsia, and blurred vision. Thirst develops because of osmotic effects. Sufficiently high glucose in the blood is excreted by the kidneys, but

this requires water to carry it and causes increased fluid loss, which must be replaced. A rarer but equally severe presentation is hyperosmolar non-ketotic state, which is more common in type 2 diabetes. Diabetes mellitus is characterized by recurrent or persistent hyperglycemia, and is diagnosed by demonstrating any one of the following (WHO, 1999)

i. Fasting plasma glucose level at or above 7.0mmol/l.
ii. Plasma glucose at or above 11.1 mmol/l two hours after glucose load in a glucose tolerance test.
iii. Random plasma glucose at or above 11.1 mmol/l.

1.6.3 Pathophysiology of diabetic nephropathy

Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nodular glomerulosclerosis due to long-standing diabetes mellitus. Ongoing research has led to further understanding of the complex pathophysiology in diabetic nephropathy. Increased glomerular capillary pressure occurs early in diabetes and is associated with hyperfiltration at the glomerulus. The glomerular mesangium expands, by cell proliferation and then by cell hypertrophy. Increased mesangial stretch and pressure can stimulate this expansion, as can high glucose levels.

Mediators of proliferation and expansion include platelet-derived growth factor and transforming growth factor-B (TGF-B) (Fioletto *et al.*, 1998). These factors are known to increase fibrosis. Glucose can also bind irreversibly to proteins in the kidney and circulation to form so-called advanced glycosylation end products. Advanced glycosylated end products can form complex crosslink over years of

hyperglycemia and can contribute to renal damage by stimulation of growth and fibrotic factors via receptors for it. The other factor is Angiotensin II, which is typically high in diabetes (Fioletto *et al.*, 1989). Angiotensin II preferentially constricts the efferent arteriole in the glomerulus, leading to higher glomerular capillary pressures. It also stimulates renal growth and fibrosis. Diabetic nephropathy is also an inflammatory process with microphage infiltration in glomeruli with early diabetic sclerosis (UK Prospective Diabetes Study Group, 1998).

Tubulointerstitial fibrosis is seen in early stages of diabetic nephropathy and is a better predictor of renal failure than glomerular sclerosis. There is an epithelial-mesenchymal transition that takes place in the tubules, with proximal tubular cell conversion to fibroblast-like cells (Diabetes Control and Complication Trial Research Group, 1993) Inflammatory cells cause cellular damage and scarring through release of cytokines and oxygen radicals, and ultimately renal cells themselves may transform into fibroblasts and cause tubulointerstitial fibrosis (Uslu *et al.*, 2005). Increased urinary enzyme excretion supposedly indicates tubular dysfunction. Detection is useful for assessing the preclinical stage of diabetic nephropathy (Rosenthal *et al.*, 2004). Oxidative stress has been considered a common pathogenic factor in diabetes mellitus and its complications, including nephropathy (Baynes 1991). Hyperglycemia leads to enhanced reactive oxygen species production, and tubular cell damage and abnormal urinary enzyme excretion develop according to Ishii *et al.* (2001).

Nonenzymatic protein glycosylation commonly occurs in red blood cells, glomeruli, and nerve cells as well as in other tissues. The extent of the glycosylation is proportional to extracellular glucose concentration. Such glycosylation occurs by the

mechanism shown for the glycosylation of haemoglobin. The carbonyl functional groups of glucose and other sugars react with free amino groups of proteins to form intermediates called Schiff bases, or aldimine. The aldimine subsequently rearranges to form a ketamine. This rearrangement is called the Amadori rearrangement. The aldimine is labile; it can readily hydrolyze to re-form a free amino group and a carbonyl group. The ketamine is relatively stable, and its formation is not reversible. Glycosylation of the basement membrane of blood vessels is known to cause basement membrane thickening similar to that found in most if not all diabetics (Makita *et al.*, 1991; Kaneshige, 1987).

1.6.4 Diagnosis of diabetic nephropathy

For a long time microalbuminuria has been used as the earliest marker for diabetic nephropathy. Frequently the question has been raised as to whether proteinuria is from diabetes or from a primary renal disease. Suspicion may arise in patients with significant proteinuria without a long history of diabetes, or without other signs of end organ damage such as retinopathy or neuropathy. Transient elevation of microalbuminuria can be caused by exercise, urinary tract infection, hyperglycemia, febrile illness, severe hypertension, or heart failure (Gall *et al.*, 1997). The measurement of activities of urinary enzymes is considered to be a useful non-invasive test in renal function deterioration in the early stages (Price *et al.*, 1992). The increase in urinary levels of some enzymes such NAG, γ-GT, LDH, and ALP can reflect tubular injury (Hong *et al.*, 1998; Flynn *et al.*, 1990). N-acetyl-β-D-glucosaminidase is the most widely assayed enzyme for renal disease detection and forms part of my study. It originates from proximal tubular cell lysosomes. Urinary NAG activities are normally higher in normoalbuminurics diabetic patients than in

normal subjects (Uslu *et al.,* 2005). Hyperglycemia leads to enhance reactive oxygen species production, and as a result tubular cell damage and abnormal urinary enzyme excretion develop (Yagoo *et al.,* 1993). Detection is useful for assessing the preclinical stage of diabetic nephropathy (Rosenthal *et al.*, 2004). Jung *et al.* (1993) and Morta *et al.* (1991) have shown that NAG reflects lysosomal dysfunction of both glomerular and proximal tubular epithelium (Morta *et al.*, 1991). The diagnostic validity of urinary NAG has indicated a sensitivity of 78.6% and a specificity of 100% (Uslu *et al.,* 2005).

1.7 Biomarkers for early renal diseases

It has been suggested that biological markers offer accurate, rapid and cheap ways for assessing kidney damage (Taylor *et al.,* 1997). Various studies are underway to aid in the development of biological screening tools, which can with good sensitivity and specificity, identify early kidney damage (nephrotoxicity). These include assaying for a specific protein or an enzyme activity in a sample of urine. Since urine flow varies, it is best to correct random (the second of the day is preferred) urine samples and factoring by creatinine to allow for correction in the variation of urine flow (Barratt & Topham, 2007). Care in collection of samples is essential and different buffers may be incorporated, depending on which test is to be performed (Taylor *et al.,* 1997). In most cases, unless the sample is to be assayed immediately, it should be frozen into smaller aliquots until they are required (Price, 2002).

1.7.1 Application of renal biomarkers

Depending on their origin in the kidney, biomarkers can be site specific or reflect regional function. For example, the ratio of high to low molecular weight proteins

(such as albumin, IgG and transferrin) in the urine can be used to distinguish glomerular from tubular damage. Microproteinuria is regarded as an indicator of glomerular dysfunction whereas transferrin excretion indicates more severe damage. There are a number of low-molecular weight proteins, which reflect tubular damage including β_2-microglobulin, lysozyme, retinol binding protein, ribonuclease and α_1-microglobulin. Lysosomal enzymes, (such as NAG, lysozyme, β-galactosidase, α-glucosidase, β-glucoronidase, arylsulphatase, glutathione-s-transferase) and brush-border enzymes (such as Alanine Aminopeptidase, γ-GT, Leucine Aminopeptidase, intestinal ALP and total non-specific ALP) are highly sensitive indicators of proximal tubular damage.

Isoenzymes can also be useful in distinguishing regional effects of toxins, and α-GST reflects proximal tubular damage, whereas GST reflects distal tubular damage (Branten *et al.,* 2000). Isoenzymes of NAG (Price, 1992) can be used to distinguish glomerular and tubular damage. Tubular antigens are also variable, and structural proteins (such as fibronectin, laminin fragments) are useful in monitoring the later stages of the cascade. Functional markers such as the prostaglandins are useful at the early stage, particularly in children who are vulnerable to nephrotoxins and especially those living in areas of high industrial pollution with clear signs of renal damage (Fels *et al.,* 1998; Price, 2002).

1.8 Justification

It is well established that chronic hyperglycemia, and consequently effective glycemic control, is the main metabolic determinant associated with irreversible kidney damage in diabetes mellitus. Although microprotein has been used for many years as a

predictor of incipient diabetic nephropathy, reflecting the loss of glomerular selectivity, estimation of renal tubular function and integrity may yet provide an early indication of renal insult and thus identify those at risk of developing kidney dysfunction. Consequently, early renal tubular insult is characterised by increased proximal tubular enzymes. However, renal dysfunction in general and glomerular dysfunction in particular are observed only rarely in early diabetic nephropathy, and the identification of early renal insult and those at risk of developing renal dysfunction is important so that corrective therapies can be applied at an early stage. Consequently, the aim of this study is to investigate the association between the urinary enzyme activities of NAG, LDH, ALP, and γ-GT in subjects with type 2 diabetes and the severity of microproteinuria, the standard laboratory indicator of the onset of nephropathy.

1.8.1 Research questions

i. Is urinary micro protein level a good marker for early renal dysfunction in diabetes mellitus?

ii. Are serum and serum creatinine levels good markers for early kidney damage?

iii. What is the reference range for urinary micro protein and enzymes in healthy Kenyan population?

iv. What is the correlation between urinary micro protein and urinary enzymes?

1.8.2 Hypothesis

(i) Null-hypothesis

There are no significant differences in the levels of measured Urinary NAG, LDH, ALP and γ-GT enzymes between normal healthy individuals and normoproteinuric diabetic patients.

1.9 Objectives

1.9.1 General objective

The broad objective was to determine the relationship between sub clinical nephrotoxicity caused by diabetes mellitus type 2 and the levels of urinary enzymes such as N-acetyl-β-D-glucosaminidase (U.NAG), lactate dehydrogenase (U.LDH), alkaline phosphatase (U.ALP) and gamma glutamyl transferase (U. γ-GT) in relation to urinary concentration of micro protein, serum urea and creatinine.

1.9.1 Specific objectives

i. To determine the urinary reference range of the urinary micro protein and enzymes in healthy adult Kenyan population
ii. To determine the sensitivity and specificity of the urinary micro protein and enzymes using NAG as the gold standard
iii. To determine levels of urinary and serum renal parameters in normal health individuals, normoproteinuric diabetic patients, microproteinuric diabetic patients, and individuals with renal disease
iv. To determine the correlation between the measured serum and urinary renal parameters in the healthy and diabetic individuals

1.10 Significance and the anticipated output

The study aimed to demonstrate the nephrotoxicity caused by diabetes mellitus type 2. Incipient diabetic nephropathy is characterized by structural, functional and biochemical changes associated with glomerular and tubular elements of the kidney. The results of the present study demonstrated clearly that increased severity of microproteinuria, the standard clinical predictor of incipient diabetic nephropathy, and increased glomerular permeability, are associated with early changes in urinary parameters (such as NAG, LDH, ALP and γ-GT), indicating renal tubular dysfunction in a cohort of type 2 diabetic subjects. Furthermore, urinary NAG, LDH, ALP and γ-GT activities were elevated in those diabetic subjects with microprotein levels within the normal range.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study area

The study subjects were recruited from the Kenyatta National Hospital Medical Out-Patient Clinic (Diabetic Clinic No. 17), while the control population were derived from Kenyatta National Hospital workers and healthy blood donors. Diabetic clinic is an out- patient clinic in Kenyatta National Hospital that deals with both new and known diabetic patients. All diabetic cases are monitored, investigated and managed from this particular clinic. The clinic is normally held on Fridays where patients are seen by consultants Diabetologist and other health personnel. About 100 patients are normally seen every Friday. Over 90% of these patients suffer from diabetes mellitus type 2 and most of them are either on oral hypoglycaemic drugs or insulin or both. In order to monitor the status of the kidney, doctors usually request for urinary microprotein, serum urea and creatinine.

2.2 Ethical considerations

Ethical approve was sought and granted by the Kenyatta National Hospital Ethics and Research Committee (KNH-ERC, appendix 4). The participants were reassured of confidentiality in the handling of information and procedures involved in this study. Informed consent was sought from the participants and they were also requested to append their signature on the consent form in order to acknowledge their voluntary approval of participation.

2.3 Study design and population size

This was a randomized, prospective, cross-sectional, and comparative study and the sample size was calculated using the formular given by Lwanga *et al.* (1986), used to calculate the adequacy of sample size in health studies as:

$$N = Z^2 \ P \ (1\text{-}P)/d^2,$$

Where N= minimum sample size;

Z= Standard normal deviation value corresponding to 95% confidence interval (=1.96)

P= Estimated prevalence of diabetic nephropathy is 26%, (Otieno *et al.,* 1992)

d= degree of precision (set=5%), Therefore $N=1.96^2 x\ 0.26(1-0.26)/0.05^2 =265$

The study adjusted the number to 325 in order to complement for any error due to chance variations. In the final data analysis, 177 type 2 diabetic patients with no renal failure, 75 diabetic patients with renal failure and 73 healthy normal subjects were used.

2.3.1 Inclusion and exclusion criteria

All the individuals were asked to participate in the study on a voluntary basis after a brief explanation of the aims and purpose of the study. Those who accepted to participate were subjected to an interview using a questionnaire. All those patients suffering from diabetes mellitus type 2 above the age of 18 years and normal healthy individuals within the same age group were included in the study. All those subjects with renal failure, kidney transplant, urinary tract infections, smoking habits, history of alcohol intake and those on antibiotics were excluded from the study.

For the referent group, in addition to the above criteria, the study subjects were excluded from the study if they had regularly consumed drugs with potential

nephrotoxicity (such as analgesics/anti-inflammatory agents, and aminoglycosides). They were also excluded from the study if they tested positive for HIV, hepatitis B, hepatitis C and syphilis. Urinalysis was done on the urine samples provided to screen for various medical conditions that could consequently affect the excretion of urinary parameters. The parameters checked in the urinalysis-included glucose, proteins, leucocytes, blood, nitrates, Bilirubin, Urobilinogen, pH, S.G., and ketones. Those with positive results were excluded from the study. Eligible subjects were then requested to sign a consent form provided.

After application of the mentioned criteria, only 325 study subjects were selected to participate in the present study . They were grouped as follows: 1) Non-diabetic control group (referent), consisting of 88 subjects (52 males and 36 females) working in the clinical areas of Kenyatta National Hospital. Age range was 21-65 years, with 41.11 ± 12.15 years as mean ± standard deviation (SD); 2) Normoproteinuric diabetic patients consisting of 159 subjects (73 males and 86 females) attending out-patient diabetic clinic at Kenyatta National Hospital. Age range was 24-80 years, with 49.97 ± 13.28 years as mean ± SD; 3) Microproteinuric diabetic patients consisting of 43 subjects (25 males and 18 females) attending out-patient diabetic clinic at Kenyatta National Hospital. Age range was 22-77 years, with 56.29 ± 15.32 years as mean ± SD and; 4) Renal diabetic patients consisting of 82 subjects (45 males and 37 females) attending renal out-patient clinic at Kenyatta National Hospital. Age range was 34-81 years, with 62.01 ± 10.69 years as mean ± SD.

2.4 Sample collection and analysis

A random single voided, second morning urine sample in a closed container was collected from each participant. The urine was centrifuged at 3000 rpm for 5 minutes. The supernatant was distributed in vials of 1.5ml each and biochemical analysis was done within four hours. The remaining sample was kept frozen at -20°C. A sample of blood (about 5.0ml) was also collected from each participant and put in a vacutainer. The containers were labelled with the study number of the participant and the date of birth was also marked to tally into all the required demographic information. This was clearly matched with the demographic information on the questionnaire form to avoid any risk of mix-ups or incorrect identification of samples. The study number was also marked correctly on the bottle and the questionnaire. Spot urine was used because it has been shown that urinary protein/creatinine ratio (Woolerton *et al.,* 1987), as well as dividing urinary enzyme activity by the urine creatinine concentration (Jung, 1991) in random sample, correlates with 24-hour urinary excretion and eliminates variations due to changing rates of urine out-put and provides a measurement of concentration. The collected urine samples were analyzed within the same day while the blood samples were centrifuged and the serum kept in the deep freezer until the time of analysis.

2.5 Equipments used

The machines that were used for the sample analysis were the Olympus AU640 analyzer (Figure 3), (Japan, Mishima Olympus Optical Company Limited) for the enzymes, urea and creatinine and the Technicon Bayer RA 1000 (Ireland Technicon Limited) for urinary micro protein. The urine samples were analyzed for the following analytes: The spectrophotometric analysis of the brush border enzymes alkaline

phosphatase and gamma glutamyl transferase together with the cytosolic enzyme lactate dehydrogenase and lysosomal enzyme N-acetyl-β-D-glucosaminidase which were all used to assess the tubular integrity. Urinary micro protein was determined by turbidimetric method to assess the functional integrity of the glomerulus. In addition the urinary and serum concentrations of creatinine was measured by Jaffe reaction and urea in serum was measured by kinetic method (Vasiliades, 1976; Tiez *et al.,* 1983; Frings, 1989; Honhadel, 1989; Murray, 1989; Giampietro *et al.,* 1992; Roberts *et al.,* 1998; Schumann *et al.,* 2002a; Schumann *et al.*, 2002b; Schumann *et al.,* 2002c; Schumann *et al.,* 2002d; Lamb *et al.,* 2006; Barratt & Tophan, 2007).

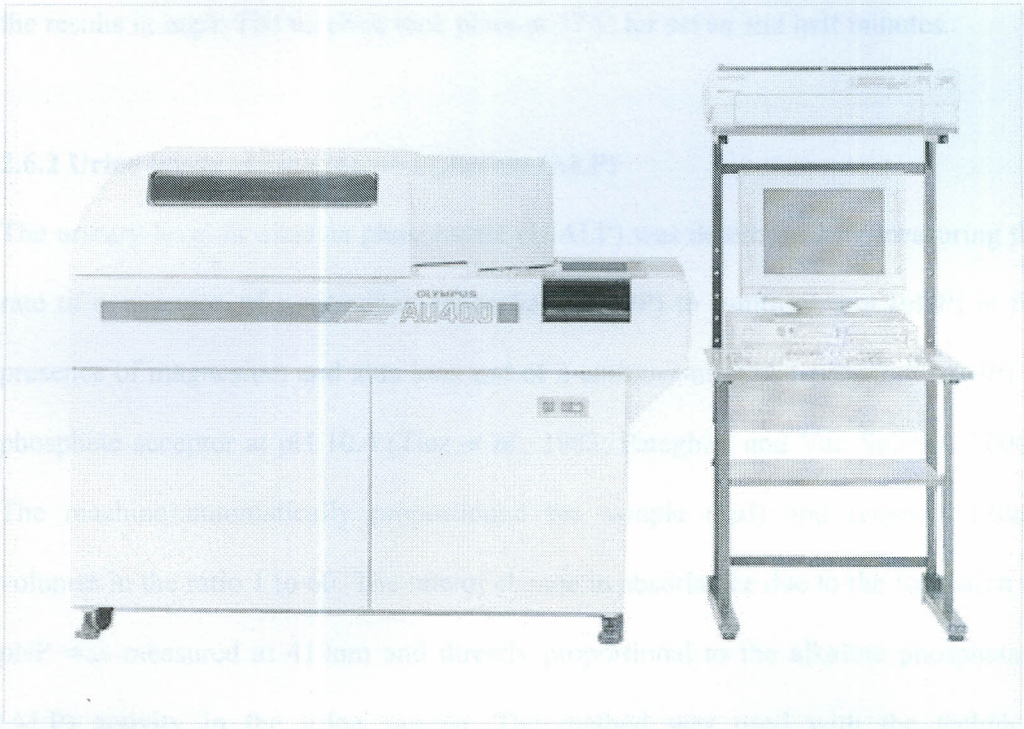

Figure 1: Olympus (AU400) Chemistry Auto analyzer

2.6 Biochemical analysis

2.6.1 Urine levels of Micro protein

Determination of urinary micro protein (U.MP) in urine was done by dye binding method (Honhadel, 1989; Barratt and Tophan, 2007). The turbidimetric procedure in which benzethonium chloride, which is the protein-denaturing agent, was used. Proteins present in the urine were denatured by benzethonium chloride resulting in the formation of fine suspension, which were quantified turbidimetrically at 405 nm. The machine automatically proportioned the sample (8µl) and reagent (360µl) volumes in the ratio 1 to 45. The method was used with the technical application for the fully automated RA 1000. The Technicon RA 1000 automatically calculated and expressed the results in mg/l. The reaction took place at 37°C for seven and half minutes.

2.6.2 Urine levels of alkaline phosphatase (ALP)

The urinary level of alkaline phosphatase (U.ALP) was determined by measuring the rate of conversion of p-nitrophenylphosphate (pNPP) to p-nitrophenol (pNP) in the presence of magnesium and zinc ions and of 2-amino-2-methyl-1-propanol (AMP) as phosphate acceptor at pH 10.4 (Tiez *et al.,* 1983; Pateghini and Van Solinge, 2006). The machine automatically proportioned the sample (3µl) and reagent (120µl) volumes in the ratio 1 to 60. The rate of change in absorbance due to the formation of pNP was measured at 410nm and directly proportional to the alkaline phosphatase (ALP) activity in the urine sample. The method was used with the technical application of the fully automated Olympus AU Clinical Chemistry Analyzer. The machine automatically computed the ALP activity of the urine sample in U/L which is then converted to U/mmolcr by dividing the value by urine creatinine (µmol/l) in

order to control for fluctuations in urine concentrations. The principle of the reaction is:

$$pNPP + AMP \xrightarrow[Mg^{2+}]{ALP} pNP + AMP - PO_4$$

2.6.3 Urine levels of lactate dehydrogenase (LDH)

The lactate dehydrogenase (LDH) reagent was used to measure urinary lactate dehydrogenase (U.LDH) activity by an enzymatic rate method (Schumann *et al.*, 2002; Pateghini Van Solinge, 2006). This applied the method of LDH catalyzing the oxidation of lactate to pyruvate coupled with reduction of NAD^+ to NADH. The ratio of the sample to reagent was 1: 60 (3µl sample to 120µl reagent). The increase of NADH was measured at 340nm and directly proportional to the enzyme activity in the urine sample. The method was used with the technical application for the fully automated Olympus 640 Clinical Chemistry Analyzer. The machine automatically computed the LDH activity of the urine sample in U/L. The principle of the reaction is

$$Lactate + NAD^+ \xrightarrow{LDH} Pyruvate + NADH + H^+$$

2.6.4 Gamma glutamyl transferase (γ-GT)

The gamma-glutamyl transferase (γ-GT) reagent was used to measure urinary gamma glutamyl transferase (U. γ-GT) activity by an enzymatic rate method (Schumann *et al.*, 2002d; Pateghini and Van Solinge, 2006). This was measured by the method of γ-GT catalyzing the transfer of gamma glutamyl group from the substrate gamma-glutamyl-3-carboxy-4-nitroanalide, to glycylglycine, yielding 5-amino-2-nitrobenzoate. The ratio of the sample to reagent was 1:32 (5µl sample to 160µl reagent). The change in absorbance at 410/480nm occurred due to the formation of 5-amino-2-benzoate and was directly proportional to the γ-GT activity in the urine

sample. The method was used with the technical application of the fully automated Olympus 640 Clinical Chemistry Analyzer. The machine automatically computed the γ-GT activity of the urine sample in U/L.

2.6.5 Urine levels of N-acetyl-β-D-glucosaminidase (NAG)

The substrate of choice is 2-methoxy-4- (2-nitrovinyl) phenyl-glucosaminide, which reacts with NAG to yield a red colored chromophoric phenol, which has a high extinction coefficient at 505nm in alkaline solution (Amico *et al.,* 2003). Olympus Chemistry Auto-analyzer was programmed for the determination of NAG in U/L. The principle of the test is:

2-methoxy-4- (2-nitrovinyl) phenyl-glucosaminide is hydrolysed by N-acetyl-β-D-glucosaminidase (NAG) with the release of 2-methoxy-4-(2-nitrophenol), which on addition of alkaline buffer, produces a colour which is measured at 505nm.

2.6.6 Serum and urine levels of creatinine

The determination of serum and urinary creatinine was based on Jaffe reaction (Vasiliades, 1976; Murray, 1989; Lamb *et al.,* 2006). The reaction occurs between creatinine and picrate ion in an alkaline medium; a red-orange colour complex whose intensity is measured at 510nm develops. The machine automatically proportioned the sample (10µl and reagent (100µl) volumes in the ratio 1 to 10. The method was used with the technical application of the fully automated Olympus 640 Clinical Chemistry Analyzer. The units were micromoles/litre (µmol/l).

2.6.7 Serum levels of urea

The determination of urea was based on Coupled enzymes (Unease/glutamate Dehydrogenase [GLDH]), which is a quantitative, end-point, and kinetic type of analysis. The concentration of urea was determined by the use of Olympus Chemistry Auto-analyzer. Plasma was used and the Olympus machine programmed for urea determination in millimoles/litre (mmol/l).

2.7 Quality control assessment

Quality control assessment (QCA)) was regularly done to ensure that the results obtained were reliable, accurate and precise. This involved the total check on the personnel, equipment, reagent, specimen and analytical methodology. To carry out this QCA, a quality control material was always run everyday before sample analysis to validate the results obtained. Through the internal quality control (IQC), the quality of the results was verified. This helped in the detection and rectification of various procedural processes that play a part in error creation. The quality control material normally has predetermined mean and an expected range that lies between 2 standard deviations (SD) of the mean. The standard deviation index (SDI) was calculated everyday from the QC values obtained and plotted against the date of analysis. This helped in the drawing of the Levey-Jennings charts.

2.8 Data analysis

Statistical analysis was done using SPSS program version11.0. The Kolmogrov-Smirnov test was used to test the data for normality. The data obtained was parametric in nature and was tabulated as mean and standard deviation. Means differences between investigated groups (referents, normoproteinuric diabetic, microproteinuric

diabetic and renal patients) were assessed by Anova and post Anova statistical analysis. Pearson's correlation coefficients (r) were calculated to determine relationship between studied urinary markers. Results were considered statistically significant at $p < 0.05$ and all analyses were two-tailed.

The reference ranges for the various renal markers were calculated using the normal healthy individuals (referent group). The sensitivity and specificity of the various renal markers were determined using urinary N-acetyl-β-D-glucosaminidase as the gold standard.

CHAPTER THREE

RESULTS

3.1: Quality control parameters for the studied renal markers

To ensure that the reference ranges for the urinary renal markers were reliable and precise, internal quality control (QC) was included throughout the analytical period of the study. Internal quality control for each analyte was considered independently as shown in Tables 1 and 2. The control value results and the standard deviation (SD) from the target control value (central point value of the quality control range provided by QC supplier) were noted daily. The quality control material used for N-acetyl-β-D-glucosaminidase was Nag Control Kit (Level 2) from PPR Diagnostics, which had a range of 9.4-21.4U/L, mean of 15.4U/L and SD of 3.0. The QC used for all the other parameters such as gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase, micro protein, urea and creatinine was Data Trol N. The range, mean and SD for the respective analytes are indicated in Table 1.

Table 1: Quality control parameters for the studied renal markers

Analyte	QC Material	Range	Mean ± SD
NAG (U/L)	Nag Control Kit	9.4-21.4	15.4 ± 3.0
γ-GT "	Data Trol N	8-32	20 ± 6.0
ALP "	"	92-200	146 ± 27.0
LDH "	"	239-407	323 ± 42.0
MP (mg/l)	"	90-210	150 ± 30.0
UREA (mmol/l)	"	2.9-8.1	5.5 ± 1.3
CREAT (μmol/l)	"	55-115	85 ± 15

3.2: Quality control assessment for the studied renal markers

The values obtained from the quality control materials for each parameter were recorded every day during sample analysis (Table 2). The standard deviation index (SDI) was calculated using the value obtained, the standard deviation and the mean of the corresponding parameter using the formular; the value obtained minus the mean divided by the standard deviation. The standard deviation indexes (SDI) values were used to plot the Levey-Jennings charts shown in figures 1-7. The reliability of the reference range for the studied analytes was confirmed by quality control results, which were within the acceptable quality control range of ± 2 SD. The standard deviation index is either positive or negative and the mean value is usually on the zero mark as indicated in figures 1-7. The Levey-Jennings charts reflected a normal trend and therefore the results obtained were perceived to be accurate. Preanalytical and analytical errors were reduced to the minimal by applying standard laboratory procedures.

Table 2: Quality control values for the studied renal markers

Date	Parameter	NAG	ALP	LDH	γ-GT	MP	UREA	CREAT
01/06/08	Value	10.8	122	362	11	112	3.2	60
	SDI	-1.5	-0.9	0.9	-1.5	-1.3	-1.8	-1.7
02/06/08	Value	11.8	143	350	15	115	4.7	77
	SDI	-1.2	-0.1	0.6	-0.8	-1.2	-0.6	-0.5
03/06/08	Value	9.8	98	340	21	125	5.8	89
	SDI	-1.9	-1.8	0.4	0.2	-0.8	0.2	0.3
04/06/08	Value	14.7	174	378	29	204	3.1	100
	SDI	-0.2	1.0	1.3	1.5	1.8	-1.8	1.0
05/06/08	Value	16.2	150	255	19	200	3.9	65
	SDI	0.3	0.1	-1.6	-0.2	1.7	-1.2	-1.3
06/06/08	Value	19.7	143	400	18	195	6.7	89
	SDI	1.4	-0.1	1.83	-0.3	1.5	0.9	0.3
07/06/08	Value	20.0	158	290	13	98	7.1	112
	SDI	1.5	0.4	-0.8	-1.2	-1.7	1.2	1.8
08/06/08	Value	17.2	178	257	22	145	7.9	61
	SD	0.6	1.2	-1.6	0.3	-0.2	1.8	-1.6
09/06/08	Value	16.7	129	351	28	150	5.9	59
	SDI	0.4	-0.6	0.7	1.3	0.0	0.3	-1.7
10/06/08	Value	18.8	133	380	30	165	6.4	65
	SDI	1.1	-0.5	1.4	1.7	0.5	0.7	-1.3
11/06/08	Value	19.1	108	268	24	198	6.5	78
	SDI	1.2	-1.4	-1.3	0.7	1.6	0.8	-0.5
12/06/08	Value	10.8	168	389	20	150	4.5	96
	SDI	-1.5	0.8	1.6	0.0	0.0	-0.8	0.7
13/06/08	Value	16.8	166	299	12	163	6.6	91
	SDI	0.5	0.7	-0.6	-1.3	0.4	0.8	0.4
14/06/08	Value	15.4	170	379	17	189	7.8	100
	SDI	0.0	0.9	1.3	-0.5	1.3	1.8	1.0
15/06/08	Value	17.9	156	258	27	204	4.2	102
	SDI	0.8	0.4	-1.5	1.2	1.8	1.0	1.1
16/06/08	Value	12.6	188	345	26	157	3.0	77
	SDI	-0.9	1.6	0.5	1.0	0.2	-1.9	-0.5
17/06/08	Value	13.6	145	388	23	100	4.2	88
	SDI	-0.6	-0.04	1.5	0.5	-1.7	1.0	0.2
18/06/08	Value	15.9	150	267	24	140	5.5	72
	SDI	0.2	0.2	-1.3	0.7	-0.3	0.0	-0.9
19/06/08	Value	16.6	144	358	25	160	6.3	62
	SDI	0.4	-0.07	0.8	0.8	0.3	0.6	-1.5
20/06/08	Value	19.8	169	269	21	182	5.8	111
	SDI	1.5	0.9	-1.3	0.2	1.1	0.2	1.7
21/06/08	Value	20.5	178	372	19	190	5.1	109
	SDI	1.7	1.2	1.2	-0.2	1.3	-0.3	1.6

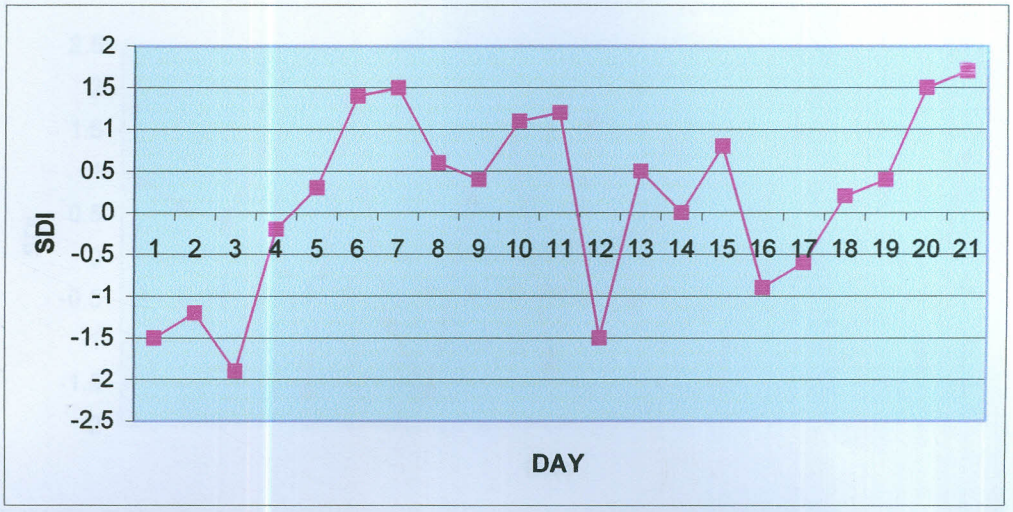

Figure 4: NAG levey-jennings control chart

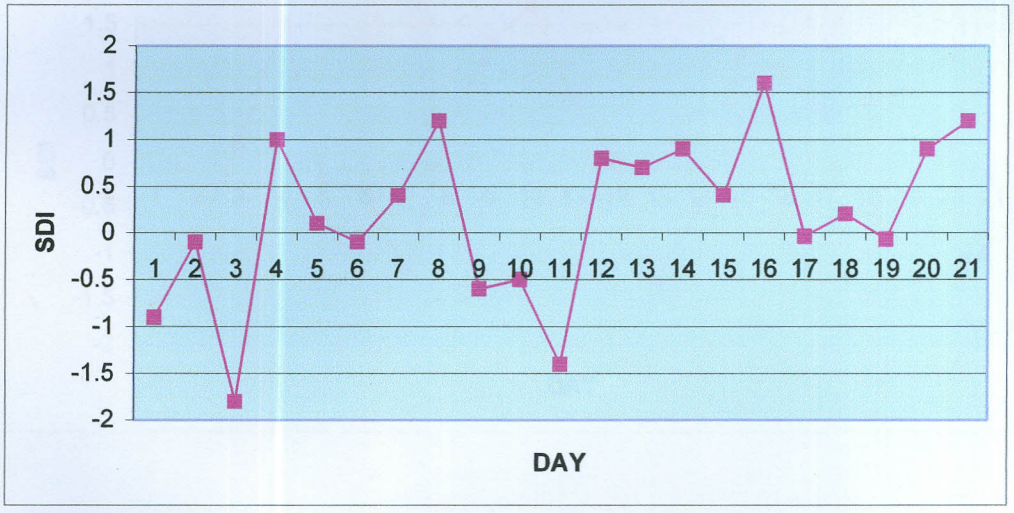

Figure 5: ALP levey-jennings control chart

Figure 6: LDH levey-jennings control chart

Figure 7: γ-GT levey-jennings control chart

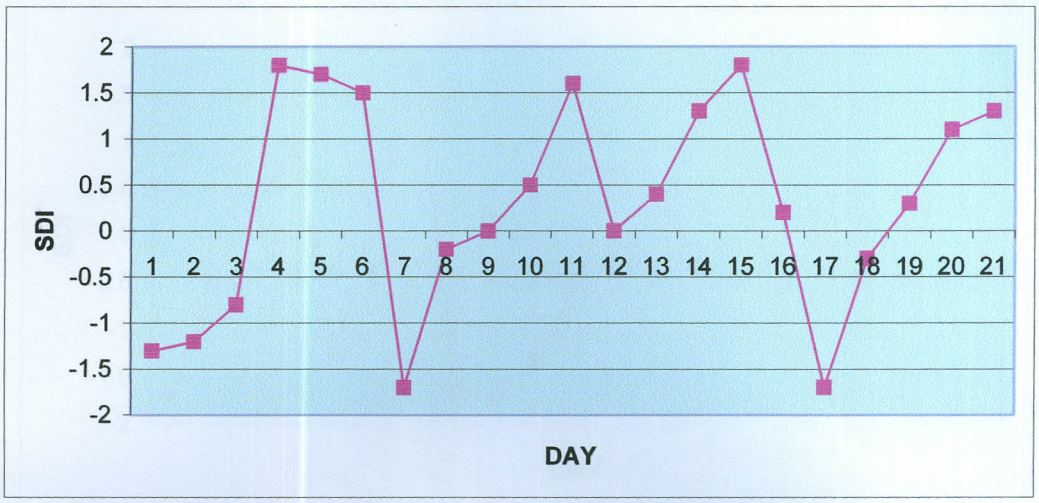

Figure 8: MP levey-jennings control chart

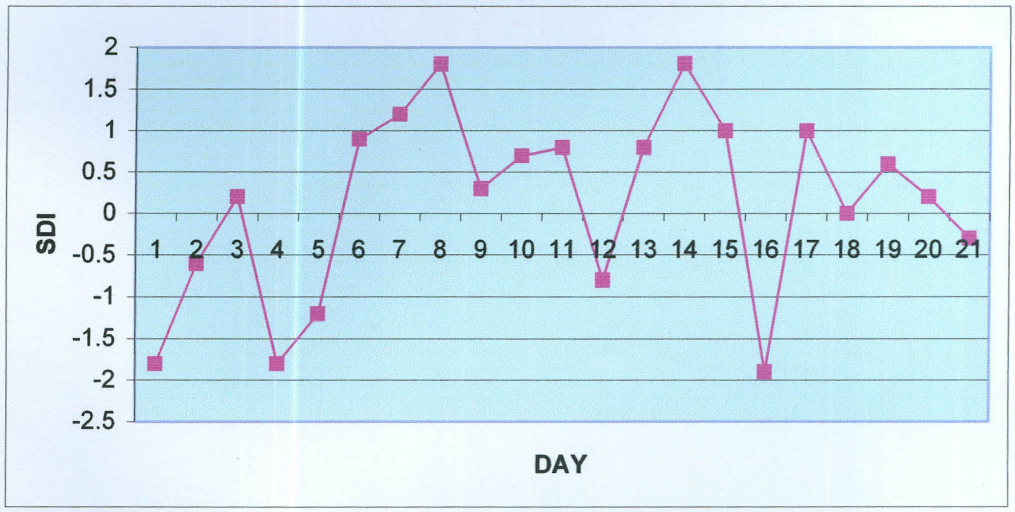

Figure 9: Urea levey-jennings control chart

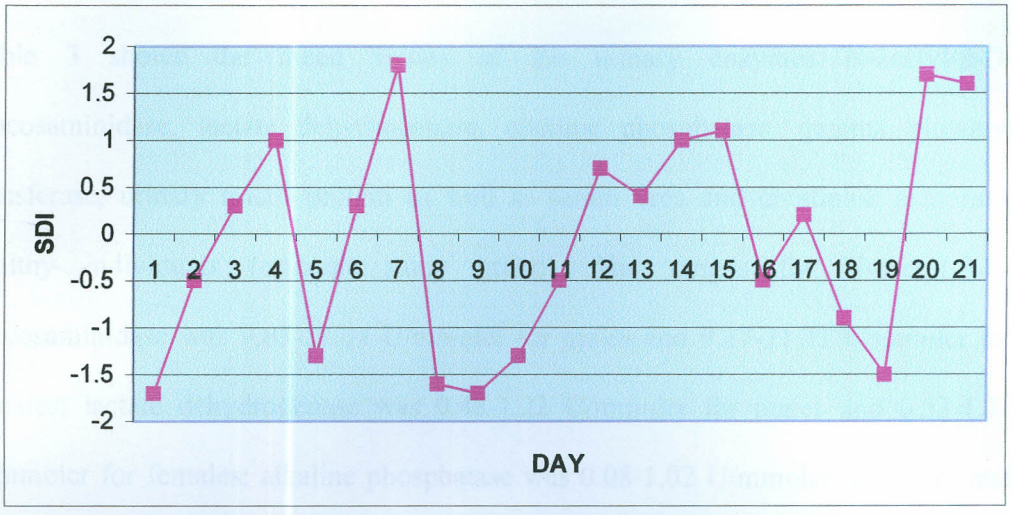

Figure 10: Creatinine levey-jennings control chart

3.3: Established reference ranges for urine and serum renal markers in males and females

Table 3 shows the mean values of the urinary enzymes N-acetyl-β-D-glucosaminidase, lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase, urinary micro protein as well as serum urea and creatinine in normal healthy individuals (referent study group). The ranges for N-acetyl-β-D-glucosaminidase was 9.03-22.04 U/mmolcr for males and 9.27-21.31 U/mmolcr for females; lactate dehydrogenase was 0.48-1.22 U/mmolcr for males and 0.53-1.31 U/mmolcr for females; alkaline phosphatase was 0.08-1.02 U/mmolcr for males and 0.02-1.00 U/mmolcr for females; gamma glutamyl transferase was 1.77-3.61 U/mmolcr for males and 1.74-3.52 U/mmolcr for females while for urinary micro protein it was 0.89-9.63mg/mmolcr for males and 1.54-8.0mg/mmolcr for females.

The reference ranges for urea were 1.60-7.04 mmol/l for males while for the females and 1.60-6.64mmol/l. The male reference ranges for serum creatinine was 63-121μmol/l and that of females was 48-108μmol/l. Since no significant differences were observed in the values of the urinary enzymes, urinary micro protein, and serum urea between males and females, ($p>0.05$) these parameters were determined using combined data for both sexes. Results show a significant difference in the serum concentrations of creatinine between the males and the females ($p<0.05$) and therefore different reference ranges for males and females were developed.

Table 3 also shows the reference values (combined males and females) for the urinary enzymes, urinary micro protein and serum urea and creatinine. The lower and upper limits of the reference range for urinary N-acetyl-β-D-glucosaminidase were 9.17 and 21.71U/mmolcr respectively; gamma glutamyl transferase were 1.69 and

3.57U/mmolcr; urinary alkaline phosphatase 0.06 and 1.00U/mmolcr and urinary lactate dehydrogenase 0.48 and 1.27 U/mmolcr respectively. The lower and upper limits of the reference range for urinary micro protein were 1.21 and 9.00 mg/mmolcr and that for serum urea 1.6 mmol/l and 6.7 mmol/l and serum creatinine reference range were 63µmol/l and 121µmol/l while that for the female was 48µmol/l and 108µmol/l, respectively.

Table 3: The established reference ranges for urinary and serum renal markers in males and females

Analyte	Sex	N	Mean± SD	Reference Range	p-value
U.NAG (U/mmolcr)	M	43	15.54±3.32	9.03-22.04	0.741
	F	30	15.29±3.07	9.27-21.31	
	M/F	73	15.44±3.20	9.17-21.71	
U.ALP (U/mmolcr)	M	43	0.55±0.24	0.08-1.02	0.481
	F	30	0.51±0.25	0.02-1.00	
	M/F	73	0.53±0.24	0.06-1.00	
U.LDH (U/mmolcr)	M	43	0.85±0.19	0.48-1.22	0.176
	F	30	0.92±0.20	0.53-1.31	
	M/F	73	0.88±0.20	0.48-1.27	
U.γ-GT (U/mmolcr)	M	43	2.69±0.47	1.77-3.61	0.196
	F	30	2.54±0.50	1.74-3.52	
	M/F	73	2.63±0.48	1.69-3.57	
U.MP (mg/mmolcr)	M	43	5.26±2.23	0.89-9.63	0.342
	F	30	4.77±1.65	1.54-8.00	
	M/F	73	5.03±1.94	1.23-8.83	
S.Urea (mmol/l)	M	43	4.3±1.4	1.60-7.04	0.742
	F	30	4.1±1.3	1.60-6.64	
	M/F	73	4.1±1.3	1.60-6.70	
S.Creat (µmol/l)	M	43	92±15.0	62.6-121.4	0.001
	F	30	78±15.4	47.8-108.2	

3.4: Association between NAG and other renal biomarkers

Using urinary N-acetyl-β-D-glucosaminidase as a gold standard, kappa statistics were used to establish the degree of association or agreement between urinary N-acetyl-β-D-glucosaminidase and the various urinary biomarkers of renal injury (Tables 4-7). Kappa values range from 0 to 1 and values closer to one (1.0) indicate a high degree of agreement while values close to zero (0) indicate a low degree of agreement. Urinary gamma glutamyl transferase had the highest kappa value (0.8) followed by lactate dehydrogenase (0.7), alkaline phosphatase (0.6) and finally urinary micro protein (0.5). The relationships between the enzyme N-acetyl-β-D-glucosaminidase and the other studied urinary markers were significantly different ($p<0.05$).

Table 4: The sensitivity and specificity of urinary LDH using NAG as the gold standard

NAG 21.7			
LDH	**True State (Gold Standard Test)**		
	Positive (%)	**Negative (%)**	**Total**
Positive	70 (39.5)	9 (5.1)	79 (44.6)
Negative	14 (7.9)	84 (47.4)	98 (55.4)
Total	84 (47.4)	93 (52.5)	177 (100.0)

$p < 0.05$, kappa=0.7

Table 5: The sensitivity and specificity of urinary γ-GT using NAG as the gold standard

	NAG 21.7		
γ-GT	**True State (Gold Standard Test)**		
	Positive (%)	**Negative (%)**	**Total**
Positive	75 (42.4)	8 (4.5)	83 (37.2)
Negative	9 (5.1)	85 (48.0)	94 (53.1)
Total	84 (47.5)	93 (52.5)	177 (100.0)

$p < 0.05$, kappa=0.8

Table 6: The sensitivity and specificity of urinary ALP using NAG as the gold standard

	NAG 21.7		
ALP	**True State (Gold Standard Test)**		
	Positive (%)	**Negative (%)**	**Total**
Positive	59 (33.3)	7 (3.9)	66 (37.2)
Negative	25 (14.1)	86 (48.6)	111 (62.7)
Total	84 (47.4)	93 (52.5)	177 (100.0)

$p < 0.05$, kappa=0.6

Table 7: The sensitivity and specificity of urinary MP using NAG as the gold standard

	NAG 21.7		
MP	**True State (Gold Standard Test)**		
	Positive (%)	**Negative (%)**	**Total**
Positive	39 (22.0)	1 (0.6)	40 (22.6)
Negative	45 (25.4)	92 (52.0)	137 (77.4)
Total	84 (47.4)	93 (52.5)	177 (100.0)

$p < 0.05$, kappa= 0.5

3.8: Sensitivity and specificity of the various renal injury biomarkers

Table 8 shows the sensitivity, specificity, positive and negative predictive values, false positive and negative rates as well as the accuracy of the various urinary renal markers using N-acetyl-β-D-glucosaminidase as the gold standard. Urinary lactate dehydrogenase had a sensitivity of 83%, specificity of 90%, positive predictive value of 89%, negative predictive value of 86% and an accuracy of 88% while alkaline phosphatase had a sensitivity of 70%, specificity of 92%, positive predictive value of 89%, negative predictive value of 77% and an accuracy of 82%. Urinary gamma glutamyl transferase had a sensitivity of 89%, specificity of 91%, positive predictive value of 90%, negative predictive value of 90% and an accuracy of 90%, and finally urinary micro protein indicated a sensitivity of 46%, specificity of 98%, positive predictive value of 98%, negative predictive value of 67% and an accuracy of 74%.

Table 8: Sensitivity and specificity (%) of the various renal injury biomarkers

	γ-GT	ALP	LDH	MP
Sensitivity	89	70	83	46
Specificity	91	92	90	98
False positive rate	9	8	9	1
False negative rate	11	30	17	53
Positive predictive value	90	89	89	98
Negative predictive value	90	77	86	67
Accuracy of test	90	82	88	74

3.9: Levels of urinary and serum biomarkers of renal injury in various stages of diabetic subjects/patients

Table 9 and figures (11-17) show the measured levels of urinary and serum markers among the normal healthy individuals (referent), the normoproteinuric diabetic group, the microproteinuric diabetic group and the confirmed renal failure patients (renal group). The values of urinary N-acetyl-β-D-glucosaminidase for the renal group were two and half times higher than the referent group, two times higher than the microproteinuric group and one and half times higher than the normoproteinuric. The mean levels of the various groups were significantly different ($p<0.05$). The levels of urinary alkaline phosphatase in the confirmed renal failure patients were higher than the referent group by eight times, five times than the normoproteinuric group and three times higher than the microproteinuric group and the means were significantly different ($p<0.05$).

However, there was no significant difference between the mean values of urinary micro protein of the referent and the normoproteinuric diabetic groups ($p >0.05$). On the other hand, there were significant differences between values of the microproteinuric and the normoproteinuric groups as well as the renal and the microproteinuric groups ($p<0.05$). Levels of urinary lactate dehydrogenase indicated a progressive increase from the referent group through the renal group ($p<0.05$). The same trend was demonstrated by urinary gamma glutamyl transferase with the mean levels in the renal group being twice as high as that of the microproteinuric, four times higher that of the normoproteinuric group and six times that of the referent group.

The values of serum urea and serum creatinine were the same for the referent, normoproteinuric and microproteinuric ($p>0.05$) but different between the

microproteinuric and the renal groups ($p<0.05$). The levels of N-acetyl-β-D-glucosaminidase, alkaline phosphatase, lactate dehydrogenase and gamma glutamyl transferase were about one and half times higher in normoproteinuric diabetic patients than in normal health individuals. In microproteinuric diabetic patients, the levels of N-acetyl-β-D-glucosaminidase and gamma glutamyl transferase were twice higher than in normal health individuals while alkaline phosphatase, lactate dehydrogenase and micro protein they were about four times higher.

Table 9: Levels of urinary and serum biomarkers of renal injury in various stages of diabetic subjects/patients

Parameter	Healthy Individuals N=73	Normoproteinuric Diabetic Group N=143	Microproteinuric Diabetic Group N=34	Renal Individuals N=75
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
U.NAG (U/mmolcr)	15.44±3.20	20.01±3.54[a]	33.00±6.41[a, b]	41.0±14.8[a,b,c]
U.ALP (U/mmolcr)	0.53±0.24	1.13±0.28[a]	1.99±0.39[a,b]	4.35±3.1 [a,b,c]
U.LDH (U/mmolcr)	0.88±0.20	1.85±0.79 [a]	4.89±1.86 [a,b]	6.87±2.63 [a,b,c]
U.γ-GT (U/mmolcr)	2.63±0.48	4.39±0.98 [a]	6.05±1.74 [a, b]	13.1±6.7 [a,b,c]
U.MP (mg/mmolcr)	5.03±1.95	5.40±2.10	29.1±10.1 [a,b]	48.2±27.6 [a,b,c]
S.Urea (Mmol/l)	4.2±1.3	4.2±1.8	4.8±1.5	14.2±5.9[a,b,c]
S.Creat (μmol/l)	86±16.4	86±18.9	92±16.3	214±96.1[a,b,c]

Results are expressed as means ± SD. Values with superscript *a* are different from the referents; values with superscript *b* are different from the normoproteinuric diabetes group; values with superscript *c* are different from the microproteinuric diabetic group.

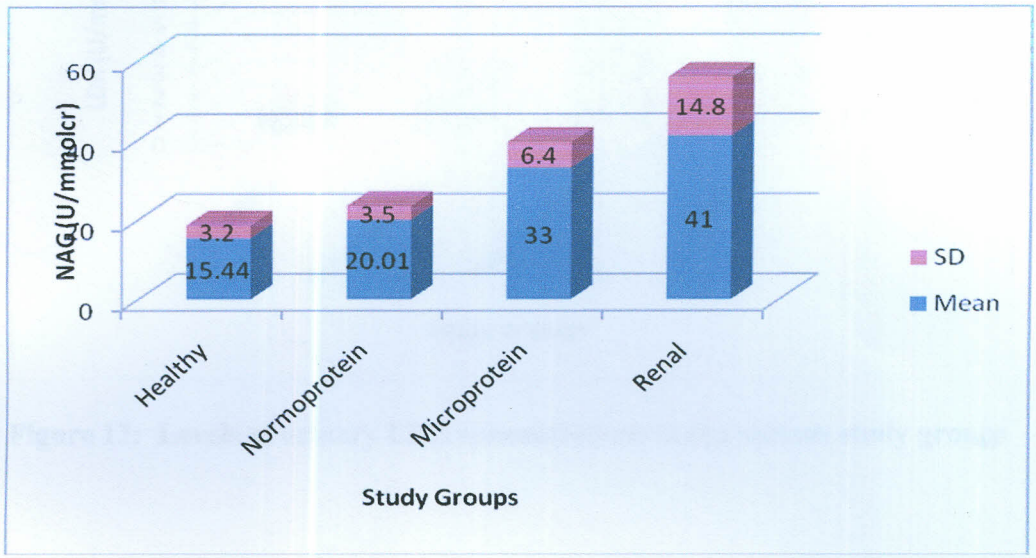

Figure 11: Levels of urinary NAG concentrations in the various study groups

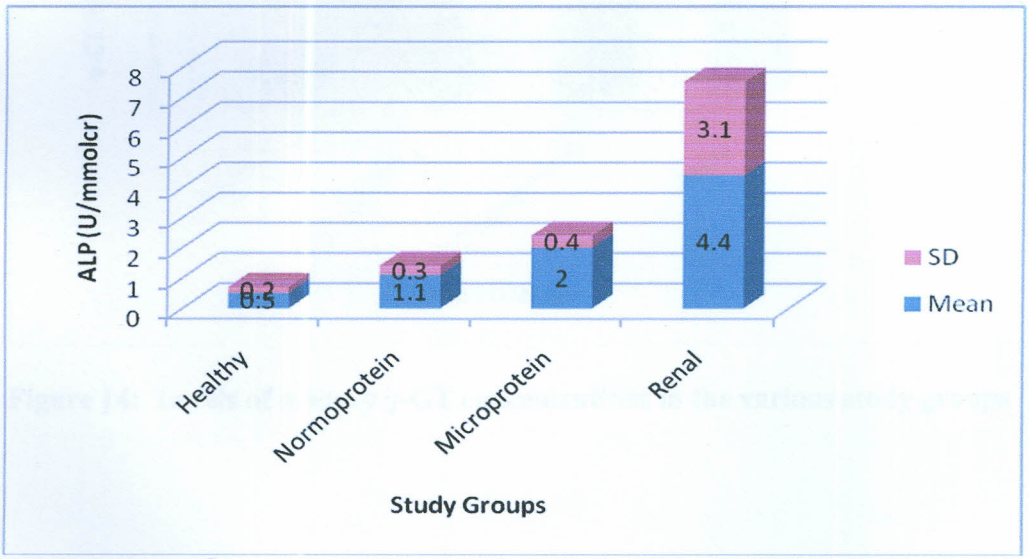

Figure 12: Levels of urinary ALP concentrations in the various study groups

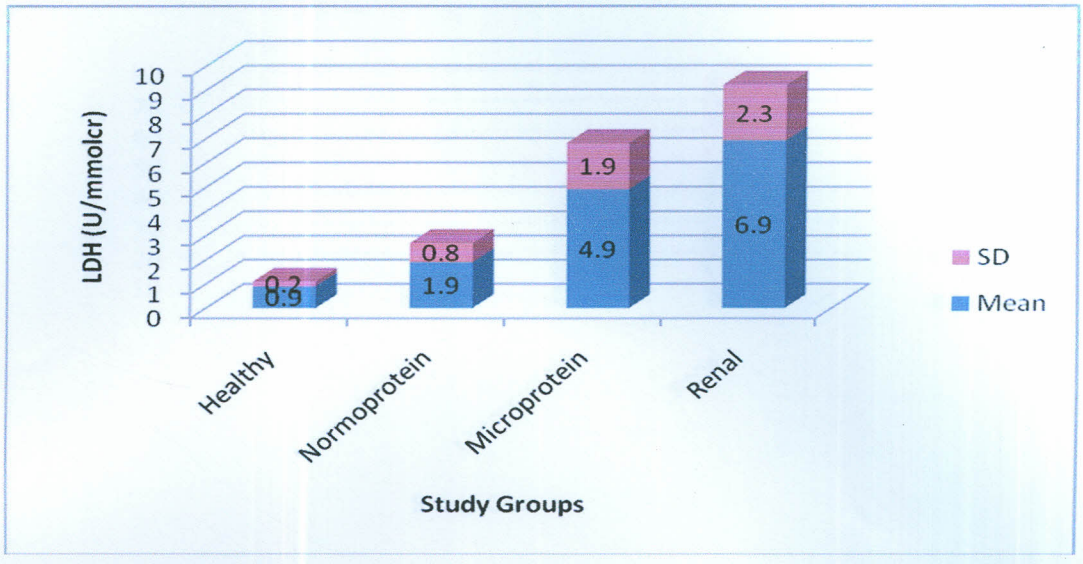

Figure 13: Levels of urinary LDH concentrations in the various study groups

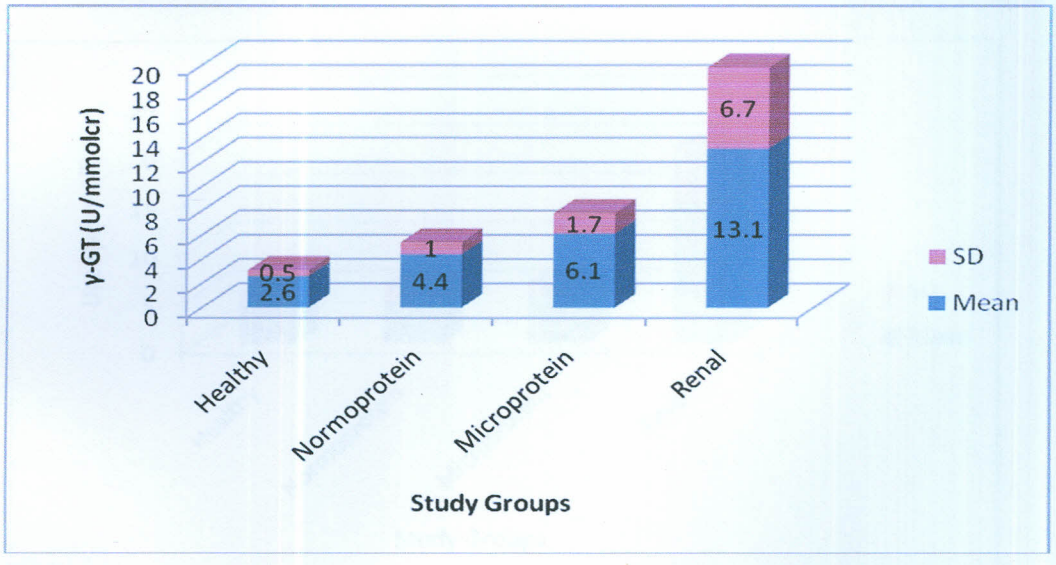

Figure 14: Levels of urinary γ-GT concentrations in the various study groups

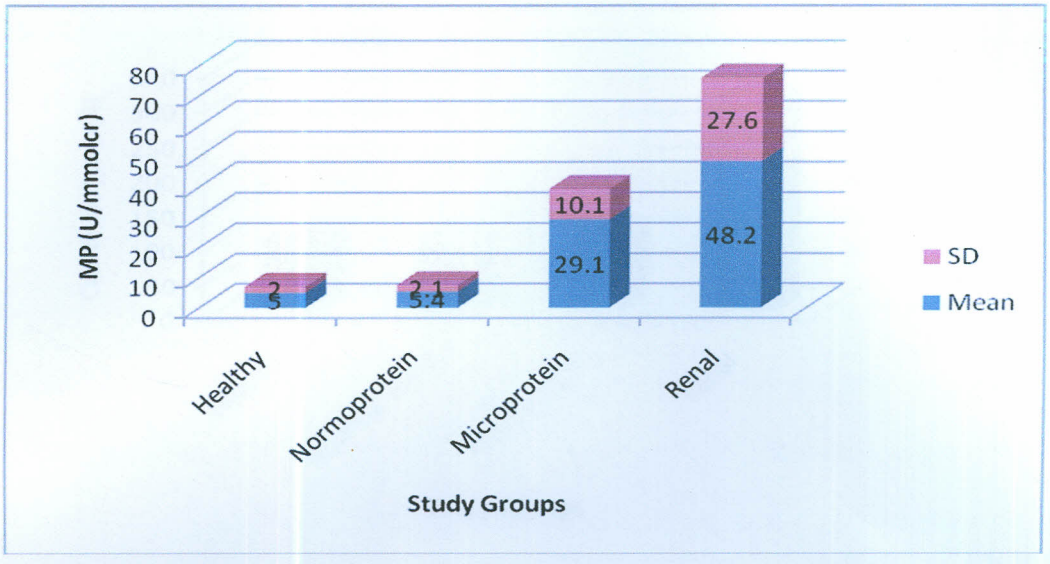

Figure 15: Levels of urinary MP concentrations in the various study groups

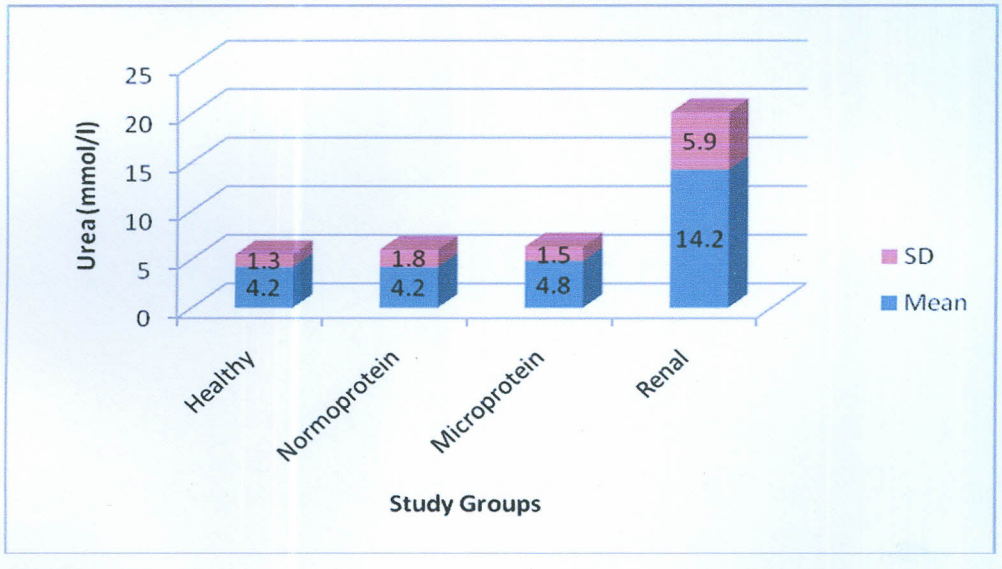

Figure 16: Levels of serum Urea concentrations in the various study groups

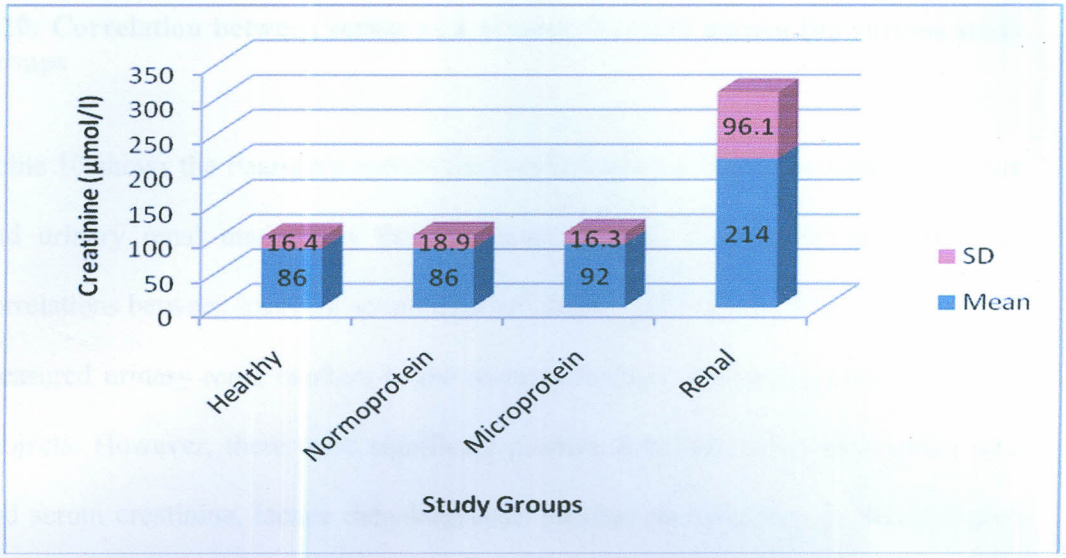

Figure 17: Levels of serum Creatinine concentrations in the various study groups

3.10: Correlation between serum and urinary markers among the various study groups

Table 10 shows the Pearson's correlation coefficient (r) between the measured serum and urinary renal markers in the four study groups. There were no significant correlations between levels of serum urea and serum creatinine as well as all the other measured urinary renal markers in the normoproteinuric and microproteinuric study subjects. However, there were significant positive correlations between serum urea and serum creatinine, lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase, N-acetyl-β-D-glucosaminidase and micro protein in the renal disease subjects ($p<0.05$). The same trend was observed between serum creatinine and all the other urinary renal markers in the same study group. There were significant positive correlations between urinary lactate dehydrogenase and alkaline phosphatase, gamma glutamyl transferase and N-acetyl-β-D-glucosaminidase in normoproteinuric, microproteinuric and renal disease subjects but not in the referent subjects. Urinary micro protein positively correlated with urinary lactate dehydrogenase only in the microproteinuric and renal subjects.

The study also demonstrated significant positive correlations between urinary alkaline phosphatase, gamma glutamyl transferase, and N-acetyl-β-D-glucosaminidase in all the three study groups ($p<0.05$) indicated in Table 10. Urinary micro protein was only positively correlated with alkaline phosphatase in the microproteinuric and renal groups. Likewise, the results of the present study demonstrated that urinary gamma glutamyl transferase positively correlated with urinary N-acetyl-β-D-glucosaminidase ($p<0.05$) but there was no significant correlation with urinary micro protein except in the microproteinuric and renal

groups. Lastly, there was a significant positive correlation between urinary N-acetyl-β-D-glucosaminidase and urinary micro protein in the microproteinuric and renal groups ($p < 0.05$) but not in the normoproteinuric group.

Table 10: Correlations between serum and urinary renal markers among the various study groups

Parameter	Parameter	Normoproteinuric subjects	Microproteinuric Subjects	Renal Disease subjects
S.Urea	S.Cr			r=0.787 p<.001
	U.LDH			r=0.623 p<.001
	U.ALP			r=0.512 p=0.000
	U.γ-GT			r=0.478 p<.001
	U.NAG			r=0.736 p<.001
	U.MP			r=0.582 p<.001
S.Cr	U.LDH			r=0.553 p<.001
	U.ALP			r=0.570 p<.001
	U.γ-GT			r=0.596 p<.001
	U.NAG			r=0.749 p<.001
	U.MP			r=0.610 p<.001
U.LDH	U.ALP	r=0.436 p<.001	r=0.570 p<.001	r=0.534 p<.001
	U.γ-GT	r=0.483 p<.001	r=0.708 p<.001	r=0.358 p=0.002
	U.NAG	r=0.643 p<.001	r=0.856 p<.001	r=0.747 p<.001
	U.MP		r=0.412 p=0.015	r=0.383 p<.001
U.ALP	U.γ-GT	r=0.447 p<.001	r=0.458 p=0.007	r=0.591 p=0.000
	U.NAG	r=0.530 p<.001	r=0.538 p<.001	r=0.666 p<.001
	U.MP		r=0.416 p=0.014	r=0.535 p<.001
U.γ-GT	U.NAG	r=0.617 p<.001	r=0.650 p<.001	r=0.624 p<.001
	U.MP		r=0.566 p<.001	r=0.583 p<.001
U.NAG	U.MP		r=0.475 p=0.005	r=0.621 p<.001

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

In clinical trials, decrease in creatinine clearance, increase in serum creatinine, and especially appearances of microalbuminuria are used as key indicators of diabetic nephropathy (Hong *et al.,* 2000). But these markers are not sensitive, reliable, specific, as there is a time delay between renal injury and detection (Thukral *et al.,* 2005). Thus, if other biomarkers are found with improved specificity and sensitivity, this could reverse or prevent the onset of renal damage. For several years, studies have demonstrated that excreted urinary enzymes may be useful biomarkers for evaluation and diagnosis of tubular dysfunction or injury. These markers include N-acetyl-β-D-glucosaminidase, alkaline phosphatase, lactate dehydrogenase and gamma glutamyl transferase (Uslu *et al.,* 2005), suggesting that tubular damage most likely precedes glomerular damage and therefore reinforcing observations that urinary enzyme excretion can be used as early markers (Uslu *et al.,* 2005).

Prior to establishment of reference values and following portioning of the subjects under study into sex categories, the Gaussian distribution of the data from different analytes was established by visual inspection of histograms obtained from analytical results of each analyte. Data sets deviating from normal distribution (outliers) were removed by visual inspection of the histograms. The remaining results were consequently used to construct the reference ranges for the five urinary markers, which were: - lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase, N-acetyl-β-D-glucosaminidase and micro protein. Emphasis was laid on the internal control, which was undertaken throughout the study period in order to

ensure that the analytical results were accurate, precise and reliable. Most of the quality control results revolved around the target values of the analytes and quite often registered values similar to the target values of the analyte under study. All the quality control results throughout the study period were properly documented, and this made it easier to assess the performance of the analytical work. Preanalytical and analytical errors were reduced to the minimal by applying standard laboratory procedures. Quality control materials were always analyzed in parallel with the samples to confirm the reliability of the results obtained.

There were no significant differences in the established reference ranges between the males and the females and therefore they were combined together to give a common reference range. The reference ranges for urinary micro protein and urinary N-acetyl-β-D-glucosaminidase that were obtained in the present study for the local population differ from the ranges given by the kit manufacturers. In a study by Uslu *et al.* (2005), the control group had a range of 1.0-9.5mg/mmolcr and 7.0-28.0 U/mmolcr for urinary micro protein and urinary N-acetyl-β-D-glucosaminidase respectively. In the present study the reference ranges for both parameters were slightly lower than the values given by Uslu *et al.* (2005). Another study by Sahira (2004), the mean for urinary micro protein and N-acetyl-β-D-glucosaminidase in normal healthy subjects were 7.0 ± 0.6 and 12.5 ± 0.6, respectively. In the study by Sahira (2004), the mean levels of N-acetyl-β-D-glucosaminidase in normal health individuals were much lower than the present study and this could be attributed to the use of different kits and therefore different principles of the test. The reference ranges studied differed significantly from a study done by Uslu *et al.* (2005), which indicated a reference range of 0.2-0.9 U/mmolcr for alkaline phosphatase, 0.6-2.3 U/mmolcr for lactate

dehydrogenase and 1.9-4.0 U/mmolcr for gamma glutamyl transferase. These differences can be explained by genetic and geographical differences and consequently different physiological activities, and therefore the need to develop local reference ranges as opposed to using ranges given by the manufacturers.

The similarities in reference range of urinary N-acetyl-β-D-glucosaminidase, lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase and micro protein as well as serum urea in both males and females can be explained by the fact that there are no sex differences in the metabolism of these parameters and the integrity of the nephron is the same regardless of the sex (Uslu *et al.,* 2005; Sahira (2004). However, serum creatinine reference range differed between males and females. This can be explained by the fact that males are more masculine than the females and creatinine predominantly originates from the muscles where it exists as phosphocreatine. In patients with renal failure the serum creatinine is not sex dependent due to the fact that the kidney is no longer functioning normally. This argument is supported by the findings of Ursula and Dieter (1998) who demonstrated significantly elevated levels of serum creatinine in healthy male subjects compared to healthy female subjects from Switzerland. In addition, Waithaka *et al.* (2009) reported significant elevation of serum levels of creatinine in healthy male subjects compared to healthy female subjects from Kenya.

The usefulness of diagnostic tests, that is their ability to detect a patient with disease or exclude a person without a disease, is usually described by terms such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The sensitivity of a test is defined as the proportion of people with disease

who will have a positive test and specificity of a test is the proportion of people without the disease who will have a negative result. A highly sensitive test is most helpful to the clinician when the test result is negative while a highly specific test is most helpful to the clinician when the test result is positive. The positive predictive value of a test is defined as the proportion of people with a positive test result who actually have the disease whereas the negative predictive value is the proportion of people with a negative test result who actually do not have the disease.

In this study the sensitivity and specificity of the various urinary parameters were first established. The positive and negative predictive values were also determined using urinary N-acetyl-β-D-glucosaminidase as the gold standard. Uslu *et al.* (2005) and Sahira (2004) showed that the enzyme is a good renal tubular marker that can be used to detect early kidney damage in diabetes mellitus. Urinary biomarkers used were lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase and micro protein using N-acetyl-β-D-glucosaminidase as the gold standard. In the current study, a cut-off value of 1.0 U/mmol creatinine alkaline phosphatase resulted in a sensitivity of 74%, a specificity of 92% and an overall accuracy of 82%. A cut-off value of 3.6 U/mmol creatinine gamma glutamyl transferase yielded a sensitivity of 89%, a specificity of 91% and an overall accuracy of 90%. A cut-off value of 1.3 U/mmol creatinine lactate dehydrogenase yielded a sensitivity of 83%, a specificity of 90% and an overall accuracy of 88%, while a cut-off value of 8.8mg/mmol creatinine micro protein yie lded a sensitivity of 46%, a specificity of 98% and an overall accuracy of 74%. Notably, despite having the same origin, alkaline phosphatase and gamma glutamyl transferase demonstrated different sensitivities, possibly because

alkaline phosphatase enzyme is less stable in urine than gamma glutamyl transferase, which is relatively stable in urine (Taha *et al.*, 2007).

Urinary gamma glutamyl transferase had the highest sensitivity followed by lactate dehydrogenase, alkaline phosphatase and the least sensitive was micro protein. The parameter with the highest specificity was micro protein followed by alkaline phosphatase, gamma glutamyl transferase and finally lactate dehydrogenase. The findings are also supported by Shokeir (2008) who reported that the same urinary biomarkers had a sensitivity of between 74.3 and 100%, specificity of 80 - 90% and overall accuracy of 81.5 - 94% in the diagnosis of congenital obstructive uropathy in children.

This can be explained by the fact urinary gamma glutamyl transferase is a smaller molecule compared to the other enzymes and therefore able to penetrate through the cell membranes faster than the others and is also relatively stable in the urine. Alkaline phosphatase enzyme is a relatively larger molecule and quite unstable in the urine and therefore degenerates faster than the others. Urinary micro protein normally measures the glomerular integrity and in most diabetic nephropathy cases, tubular insult precedes the glomerular damage. Therefore proteins start appearing in urine after the tubular enzymes have already been elevated (Jung *et al.*, 1988).

In this study the urinary enzyme levels were significantly higher in normoproteinurics than in normal healthy individuals. These findings are in accordance with the observations in other studies that have shown urinary enzymes to be sensitive indicators of kidney injury as their levels increase before other renal function tests

(especially urinary micro protein, serum creatinine and urea) become pathologically elevated (Piwowar *et al.,* 1999; Sahira, 2004; Jung *et al.,* 1988). In addition, urinary enzyme levels were significantly higher in the microproteinuric compared to normoproteinuric patients, while urinary micro protein, serum creatinine and urea levels remained within the normal ranges. Similar observation on the relationship between the urinary enzyme levels and the degree of nephropathy in diabetic patients has previously been reported (Piwowar *et al.,* 1999). This is due to the pathological damage caused to the tubules by persistent and chronic hyperglycemia.

The present study showed significant differences in the measured urinary enzymes between the normal healthy group and the normoproteinuric diabetic group. There was also a significant difference in the same parameters between the normoproteinuric and microproteinuric diabetic groups as well as between the renal and the microproteinuric diabetic groups. This can be explained by the fact that the degree of pathological damage to the renal tubules varies depending on the stage and duration of the disease suggesting that progressive damage to the tubules raises the levels of excreted enzymes because of the damaged cell membranes. There was no significant difference in the measured urinary micro protein, serum urea and serum creatinine between the referent group and the normoproteinuric diabetic group. The studies confirmed findings of other investigators that proximal tubular biomarkers were significantly increased among the subjects exposed to risks of renal injury and were better markers of renal dysfunction (Sahira, 2004; Ibrahim *et al.,* 2003).

Likewise, there was no significant difference in the levels of serum urea and creatinine between the normoproteinuric and microproteinuric diabetic groups. The

only significant difference in serum urea and creatinine was between the microproteinuric diabetic group and renal patients. Increased N-acetyl-β-D-glucosaminidase excretion has been reported by several authors in diabetic patients (Jung *et al.,* 1988; Piwowar *et al.,* 2006). In this study, diabetic patients with microproteinuria excreted significantly high levels of urinary N-acetyl-β-D-glucosaminidase compared to diabetic patients with normoproteinuria. Several studies have shown that N-acetyl-β-D-glucosaminidase reflects lysosomal dysfunction of both glomerular and proximal tubular epithelium (Morta *et al.,* 1991; Perez-Branco *et al.,* 1989). Lactate dehydrogenase is functionally involved in the catalyzation of reversible steps common in both glycolysis and glyconeogenesis. Urinary micro protein is a measure of pathological glomerular integrity (Rosenthal *et al.,* 2004; Golov *et al.,* 1995; Chouinard *et al.,* 1992).

The location and function of the brush-border membrane make it a good target for the primary involvement in the pathogenesis of diabetic renal complications. Alkaline phosphatase and gamma glutamyl transferase are brush-border enzymes, which reflect damage of proximal tubules (Limaye *et al.,* 2003; Nuyts *et al.,* 1994). Structural alterations of the diabetic brush-border membrane such as an increase in protein oxidation and lipid peroxidation with a reduction in fluidity result in functional changes in membrane-associated activities of alkaline phosphatase and gamma glutamyl transferase (Nouwen *et al.,* 1994).

Increased excretion of urinary enzymes found in the proximal renal tubular cells, has been demonstrated to be more specific for renal tubular pathology (Flynn et al., 1992). The molecular weights of these enzymes are large enough to preclude passage

through the normal glomerular basement membrane. Thus increased excretions of tubular enzymes reflect active tubular damage and have also been reported in patients with glomerulonephritis or under nephrotoxic drug treatment (Hultberg *et al.,* 1983; Shennan *et al.,* 1983; Swedenberg *et al.,* 1981). The increase in urinary enzymes excretion found in diverse renal pathologies is consistent with the underlying disease process of injury to the proximal tubular cells. Increased glomerular filtration of protein by itself can seemingly cause tubular pathology, since patients with nephrotic syndrome due to minimal change glomerulonephritis were shown to have large lipid and protein-laden vacuoles in their proximal tubular cells.

In this study there were no positive correlations between serum urea and the other measured urinary renal markers except in patients with renal disease. The same finding was observed with serum creatinine. This can be explained by the fact that serum urea and serum creatinine are poor early renal markers and are usually elevated when too much pathological damage has already occurred in the kidney when almost 50% of the renal mass is destroyed (Uslu *et al.,* 2005). The other reason is that serum urea and creatinine usually measure the level of glomerular dysfunction, which in most of the time is preceded by tubular dysfunction. They therefore usually measure the glomerular filtration capacity of the kidney. All the urinary enzymes were correlated with one another in all the four study groups but only correlated with urinary micro protein in patients with microproteinuria and individuals with renal disease. This can be explained by the fact that all the studied urinary enzymes are proximal tubular enzymes whose excretion is influenced by the same pathological conditions (Yagoo *et al.,* 1993). On the other hand urinary micro protein is a measure

of glomerular filtration capacity of the kidney and the glomerular damage normally occurs after tubular damage.

The urinary tubular enzymes showed positive correlations with one another even in the normal health individuals (control group) because of their high sensitivity and originate from the same part of the kidney. Increased urinary enzymes excretion may suggest tubular dysfunction and their detection could be useful for assessing the pre-clinical stage of diabetes nephropathy (Rosenthal *et al.,* 2004; Dedov *et al.,* 1989). Oxidative stress has been considered a common pathogenic factor in diabetes mellitus and its complications, including nephropathy (Baynes, 1991; Ha *et al.,* 2001). Hyperglycemia leads to enhanced reactive oxygen species production, and as a result tubular cell damage and hence development of abnormal urinary enzyme excretion (Yagoo *et al.,* 1993; Ishii *et al.,* 1995). The findings are also supported by studies by Sahira (2004) and Ibrahim *et al.* (2003).

The study also showed that the urinary parameters N-acetyl-β-D-glucosaminidase, lactate dehydrogenase, gamma glutamyl transferase, alkaline phosphatase and micro protein as well as serum creatinine are not age dependent. The glomerular filtration rate decreases with increasing age but the effect is not manifested in the concentration of serum creatinine and other urinary markers unless there is a kidney failure. These finding are also supported by a study done by Uslu *et al.* (2005). Serum urea concentration was however shown to increase with age. Urea, being a small molecule can be affected by urine flow; this can happen especially in men who may have slight urinary bladder outlet obstruction in case of prostate hypertrophy, which was also described by Piwowar *et al.* (1999). Glomerular filtration rate (GFR), which is a renal

function index, is a useful tool in clinical nephrology. Precise renal function measurement can be done using inulin clearance or radioactive markers, but these methods are cumbersome and not useful in routine daily practice. The use of endogenous markers has the great advantage of speed and simplicity (Mussap *et al.*, 2002). In this study, urinary enzyme levels were compared with other conventional renal function markers such as serum creatinine, serum urea and urinary micro protein for predicting renal damage.

Urea is readily filtered, but approximately 40-50% of the filtered urea is normally reabsorbed by the proximal tubules. Many factors such as hydration status, the status of the liver and protein intake, may influence the urea level while the glomerular filtration rate remains constant. Therefore, urea is not a reliable renal function marker and cannot be used for that purpose. Serum creatinine level is a function of muscle mass in normal persons and shows little response to dietary changes. It is freely filtered at the glomerulus and is not reabsorbed by the tubules but the kidney function in such a way that almost 50% of the kidney nephrons could be lost before there is any elevation in serum creatinine. This is supported by a study done by Perrone *et al.* (1992), which makes it also unreliable early kidney marker.

4.2 Conclusion

In conclusion, the present study demonstrated:

1. High sensitivity of gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase and a low sensitivity of micro protein using N-acetyl-β-D-glucosaminidase as the gold standard (89, 70, 83 and 46%, respectively).
2. High specificity of gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase and micro protein using N-acetyl-β-D-glucosaminidase as the gold standard (91, 92, 90 and 98%, respectively).
3. The reference ranges for the studied urinary renal biomarkers were: N-acetyl-β-D-glucosaminidase, 9.2-21.7 U/mmolcr; alkaline phosphatase, 0.1-1.0 U/mmolcr; lactate dehydrogenase, 0.5-1.3 U/mmolcr; gamma glutamyl transferase, 1.7-3.6 U/mmolcr and micro protein, 1.2-8.8 mg/mmolcr.
4. Urinary enzyme levels N-acetyl-β-D-glucosaminidase, lactate dehydrogenase, alkaline phosphatase and gamma glutamyl transferase were elevated in normoproteinuric diabetic patients compared to healthy normal individuals. Urinary micro protein, serum urea, and creatinine concentrations were not significantly elevated. These urinary enzymes are therefore early indicators of diabetic renal injury.
5. The results suggest that the renal tubular insult may precede increased glomerular permeability in diabetic renal disease as indicated by relatively normal levels of serum urea and creatinine even in patients with microproteinuria.
6. Serum creatinine and serum urea may not be appropriate in the detection of early kidney damage due to their insensitivity.

The null-hypothesis, that there are no significant differences in the levels of measured Urinary NAG, LDH, ALP and γ-GT enzymes between normal healthy individuals and normoproteinuric diabetic patients, was consequently rejected, and the alternative hypothesis accepted.

4.3 Recommendations

1. Longitudinal studies are required to confirm the clinical usefulness of urinary biomarkers in the diagnosis and follow-up of patients with diabetic renal dysfunction in relation to other traditional renal markers.

2. There is need for the Ministry of Health to adopt a policy on the screening of diabetic patients for early signs of renal dysfunction by use of sensitive biomarkers such as urinary enzymes.

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APPENDICES

Appendix 1: Explanation and consent form

Evaluation of urinary tubular enzymes as screening markers of renal dysfunction in patients suffering from diabetes mellitus type 2

Information sheet on ethical issues of the study

a) **Information to the subjects**

Iam doing a study on "The use of tubular enzymes as screening markers of early renal dysfunction in diabetic patients" in this Hospital. To be eligible to participate in this study, you should preferably be 18 years of age. You will be requested to give consent to this study at your will. If you give consent, you will be asked some questions and given a form to sign. You will be requested to give 5ml of blood and 50ml of urine. Your consent will give me access to your information in the Hospital file. However, this information will be used for this study only. It will be confidential. Your name will not appear anywhere except in the consent form and the questionnaire. The final results will not show your name. Further, your information will not be revealed to any one else without your consent. You are under no pressure or any obligation to consent this study. You are encouraged to ask any question you may be having before signing the consent and in a language you understand. If you need to consult before making this decision, you are free to do so.

b) Purpose of the Study

The study will investigate whether some substances found in the kidney e.g. enzymes can be useful in the detection of early kidney damage due to diabetes mellitus. Routinely, we have been screening for protein (Albumin) in urine to detect kidney damage but we would prefer more sensitive, reliable and specific parameters in order

to detect the damage in the very early stages. This will go a long way in reducing cases of diabetic patients developing chronic renal failure.

c) Patient's Responsibility

After willingly enrolling for the study, you will be requested to give 5ml blood and 50ml urine to me, which will be taken to the laboratory for the analysis. You will be requested to give authority to the use of your information in the Hospital file such as the type of medication you are on and any other medical condition that is relevant to this study.

d) Risks

During sample collection, you will feel some pain. This will be minimal. A scar is likely to form although not always. Personal information in this study will be kept confidential. Study forms will be kept in a locked cabinet to which no one else except me will have access. The study forms will not be identified with your name but only with a study number.

e) Benefits

If you are found to have diabetic nephropathy, you will be treated and given medical advice. This study will allow us to make a diagnosis and appropriate management.

f) Other pertinent information

i). You will not be required to pay anything for the estimation of the various parameters (NAG, LDH, ALP, γ-GT, UREA, CREATININE, and MICROPROTEIN).

ii). The study will be voluntary. You may refuse or withdraw from the study at any time without penalty, intimidation, threat or loss of benefits you are entitled.

iii). The monitor, the auditor, ERC and the regulatory authority will be granted direct access to the study.

iv). Your identity will not be revealed. Codes and numbers will be used. When the results will be published, your identity will remain confidential.

Before I involve you in the study, I kindly request you to append your signature below in the consent form.

I..... have read and understood the purpose and benefits of the study and I hereby agree to participate in the study.

Participant's signature.....Date.....

Appendix 2: Questionnaire

Evaluation of urinary tubular enzymes as screening markers of renal dysfunction in patients suffering from diabetes mellitus type 2

STUDY CASE NUMBER .....

AGE (YRS) ☐☐ SEX 1. MALE ☐ 2. FEMALE ☐

1. ARE YOU DIABETIC? 1. **YES** ☐ 2. **NO** ☐ IF YES, WHAT DURATION
2. DO YOU SUFFER FROM RENAL FAILURE? 1. **YES** ☐ 2. **NO** ☐
3. DO YOU HAVE URINARY TRACT INFECTION? 1. **YES** ☐ **2. NO** ☐
4. ARE YOU TAKING ANY ANTIBIOTICS? 1. **YES** ☐ **2. NO** ☐ IF YES, WHICH ONES.....
5. HAVE YOU EVER HAD RENAL TRANSPLANT? 1. **YES** ☐ **2. NO** ☐
6. WHAT (IF ANY) OTHER AILMENT DO YOU SUFFER FROM?.....
7. ARE YOU USING ANY MEDICATION FOR DIABETES? 1. **YES** ☐ **2.** ☐ **NO** IF YES, WHICH ONE? .....
.....
8. DO YOU CONSUME ALCOHOL? 1. **YES** ☐ **2. NO** ☐
9. DO YOU SMOKE? 1. **YES** ☐ **2. NO** ☐

Appendix 3: Screening Form for Controls

Evaluation of urinary tubular enzymes as screening markers of renal dysfunction in patients suffering from diabetes mellitus type 2

STUDY CASE NUMBER.....

SEX.....DATE OF BIRTH.....

DO YOU HAVE ANY MEDICAL PROBLEM?.....YES/NO

IF YES, WHICH ONE (S).....

URINALYSIS

PH

S.G

PROTEIN..... POSITIVE/NEGATIVE

BLOOD..... POSITIVE/NEGATIVE

KETONES..... POSITIVE/NEGATIVE

NITRITES..... POSITIVE/NEGATIVE

BILIRUBIN..... POSITIVE/NEGATIVE

UROBILINOGEN..... POSITIVE/NEGATIVE

LEUCOCYTES..... POSITIVE/NEGATIVE

GLUCOSE..... POSITIVE/NEGATIVE

Appendix 4: KNH- ERC approval letter.

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 20723, Nairobi.
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP", Nairobi.
Email: KNHplan@Ken.Healthnet.org

Ref: KNH-ERC/ 01/ 4526

9th July 2007

Mr. Wilfred Kimani Gatua
Dept. of Clinical Chemistry
KNH

Dear Mr. Gatua

REVISED RESEARCH PROPOSAL: "URINARY ENZYMES AS SCREENING MARKERS OF RENAL DYSFUNCTION IN PATIENTS SUFFERING FROM DIABETES MELLITUS TYPE 2" (P128/5/2007)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and approved your revised research proposal for the period 9th July 2007 – 8th July 2008.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimen must also be obtained from KNH-ERC for each batch.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF. A.N. GUANTAI
SECRETARY, KNH-ERC

c.c. Prof. K.M. Bhatt, Chairperson, KNH-ERC
The Deputy Director CS, KNH
Supervisors: Prof. C.S. Kigondu, Dept. of Clinical Chemistry, UON
Dr. Paul Ngugi, KNH
Dr. Joseph N. Makumi, Kenyatta University