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"TRACE ELEMENTS LEVEL IN TYPE 2 DIABETIC NEPHROPATHY"

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DECLARATION

I, Efua Amuaba Appiah, declare that this dissertation "Trace elements level in type 2 Diabetic Nephropathy" is my work, which was supervised by Prof. George Asare of Chemical Pathology Unit, Department of Medical Laboratory Sciences, SBAHS, University of Ghana, Legon and Dr. Robert A. Ngala of Department of Molecular Medicine, SMS, KNUST. All authors of references made to this work are duly acknowledged.

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This piece of work is dedicated to my mum, **Mrs. Comfort Egyirba Appiah**. Thanks mummy for being there for me, may the good Lord richly bless you.

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My profound appreciation goes to my Heavenly Father for seeing me through this work successfully.

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LIST OF ABBREVIATIONS

ACEI	Angiotensin-converting enzyme inhibitors
ADA	American Diabetic Association
AGE	Advanced glycation end products
AGEs	Advanced glycosylation end products
ARB	AT1 receptor blockers
COX-2	cyclooxygense-2
Cr	Chromium
CTGF	Connective tissue growth factor;
Cu	Copper
DM	Diabetes Mellitus
DN	Diabetes Nephropathy
DPP-4	Dipeptidyl peptidase-4
ESKD	End Stage Kidney Disease
ESKD	End stage kidney disease
GFR	Glomerular filteration rate
GLP-1	Glucagon like peptide-1
GPx	Glutathione perioxidase
HPLC	High Performance Liquid Chromatography
IDF	International Diabetes Federation
IL-6	Interleukin-6
MAPK	Mitogen-activated protein kinase

MAU	Microalbuminuria
MCP-1	Monocyte-chemoattractant protein-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB;
NO	Nitric oxide
Nrf2	NFE2-related factor 2
NSAID	Nonsteroidal anti-inflammatory drugs
OHA	Oral Hypoglycemic agents
PKC	protein kinase C
PPAR-γ	peroxisome proliferator-activated receptor-γ
PPS	Pentosan polysulfate.
Se	Selenium
SHP-1	Src homology-2 domain-containing phosphatase-1
TGF-β	Transforming growth factor-beta
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
Zn	Zinc

ABSTRACT

Background: Trace elements are essential nutrients with numerous biological functions resulting from their action as major components of enzymes throughout metabolism. The trace elements (Cu, Zn, Se & Cr) for this study play a key role in the pathogenesis of diabetes and its complications. Decreased levels of these trace elements have been found to be associated with DN type 2 because they are excreted by the kidney through the urine, thus hyperzincuria is a cause of low plasma zinc in diabetics.

Aim: The basic aim of the study is to determine whether Zn, Cu, Se and Cr are involved in the development of DN in Ghanaian type 2 diabetic patients.

Method: Blood samples of 5-10 mL were aseptically collected from the median antecubital or cephalic veins of the study subjects, after an overnight (12 hr) fast. The blood was then dispensed into labelled plain BD vacutainer®, tubes, and fluoride oxalate coated tubes (to prevent glycolysis) for fasting blood glucose. Samples for blood glucose assay were immediately analysed. After clotting, blood sample in the plain tubes were centrifuged at 3000 g for 3 min and the serum stored at 20°C until ready for analysis of kidney function test and trace element respectively. The trace elements (Zn, Cu, Se & Cr) analysis was performed at Ghana Atomic

Energy Commission using the International neutron activation analysis (lNAA) which is a sensitive, non-destructive method for determining the elemental composition of the sample. An early morning urine sample was collected in a sterile wide mouth container and screw capped for urine protein which was immediately analyzed.

Results: A total of 80 diabetics and 48 non-diabetics were involved in this case-control study. The mean age of the diabetics was 51.70 ± 6.23 years with 51.2% being the majority between the ages of 50-59 years whereas the mean age of the non-diabetics was 49.50 ± 6.17 years with majority between the ages of 40-49 years. Blood pressure and BMI showed no significant differences between the two groups. However, 17.5% and 2.5% of the diabetics had grade 1 and 2 hypertension respectively. Also, 30.0% were found to be obese with 8.8% morbid obese respectively. On the other hand, 16.7% and 14.6% of the non-diabetics had grade 1 and 2 hypertension respectively. A percentage of 37.5 were found to be obese with 10.4 being morbid obese respectively. There was a significant difference ($p<0.0001$) in the blood glucose levels among the diabetics and non-diabetics. The levels of Zn ($p=0.001$) and Cu ($p=0.002$) showed a significant difference in the diabetics compared to the nondiabetics whereas that of Se and Cr were not significantly different. Renal function among the patients with diabetes shows significantly increased urea and potassium with decreased creatinine levels on comparison with the non-diabetic patients. Significant differences

(p=0.0001) was also observed when proteinuria was compared between the two groups with 48.8% and 4.2% of the diabetics and non-diabetics respectively having increased urine protein.

Conclusion: This study revealed that Zn, Cu, Se & Cr have an interactive connection with type 2 diabetes mellitus. The high levels of trace elements in the diabetics estimated in this study may also be related to the kidney damage complication associated with the disease. Since the levels of trace elements were increased in diabetics with nephropathy compared to those without nephropathy.

Keywords: Zinc, Copper, Selenium, Chromium, Proteinuria, DM type 2 diabetes and Nephropathy.

CHAPTER 1

1.0 INTRODUCTION

Trace elements are essential nutrients with numerous biological functions as a result of their action as major components of enzymes throughout metabolism (Lobo, Torres et al. 2010). They are required for good health because of their various metabolic characteristic and functions. They play a role in production of reactive oxygen species (ROS), thereby contributing to oxidative stress which leads to the pathogenesis of diabetes mellitus (Shrabani, Bharatkumar et al. 2013). Clinical data suggests homeostasis of trace elements is disrupted by diabetes. Subsequently early onset of imbalances of trace elements disturb normal glucose metabolism and insulin action (Tasneem, Hassan et al. 2008). Therefore impaired kidney function in diabetes (DN) can disrupt homeostasis of trace elements. Therefore assessing their levels in patients with DN may help to understand their relationship with normal glucose metabolism and insulin action.

Diabetes mellitus (DM) affects over 170 million people world-wide and 90% or more are DM type 2 patients (Wild, Roglic et al. 2004). DM type 2 is represented by resistance of liver, skeletal muscle and fat tissue to insulin (Schinner, Scherbaum et al. 2005). Another relevant process determining its cause is increased apoptosis and the loss of β-cell mass which can reach up to 60%. This is demonstrated in obese diabetic patients where the apoptotic rate is three times higher than in the obese non diabetic patients (Butler, Janson et al. 2003). This difference is even more pronounced when non obese diabetics and non-diabetics are

compared: the apoptosis rates are ten times as great in the diabetics (Butler, Janson et al. 2003). Type 2 diabetes is also associated with increased oxidative stress (Pan, Zhang et al. 2010). One of the most important clinical features of diabetes is its harmful effects on the kidneys small blood vessels (microangiopathy) which may progress to diabetic nephropathy (Bakris 2011).

Diabetic nephropathy (DN) is a chronic end-stage kidney disease or damage that can occur in people with diabetes and accounts for 30 – 40% of cases associated with DM type 2 cases ((Saha and Tuttle 2010). Diabetic nephropathy occurs in approximately one- third of individuals with DM type 2, and it is associated with high morbidity and mortality (ADA 2006). It is characterized by persistence albuminuria (excretion >300 mg/day, proteinuria or macroalbuminuria), a relentless decline in glomerula filteration rate (GFR), raised arterial blood pressure and rapid progression of other complications like eye problems, nerve disorders, diabetic foot ulcers and blood pressure, once proteinuria develops irreversible deterioration in renal function and renal failure occurs (Bahnam, Ghayour et al. 2010). During progression to DN and in microalbuminuria (MAU) stages, morphological and structural changes occur in the glomerular and basement membrane of the kidneys. These include the thickening of glomerular basement membrane (GBM), hyperfilteration, mesangial expansion and podocyte loosing. All of these changes will cause leakage of albumin along with other enzymes and zinc to the urine leading to marked increase of these markers (Zargar,

Bashir et al. 2002).

Several studies conducted where the levels of trace elements have been altered in diabetic nephropathy, of which Zn, Cr, Se and Cu are no exception. In one study performed, it was observed that levels of copper had increased and also had a positive association with glucose and microalbumin (Talaei, Jaban et al. 2011). The high levels of copper in the serum of the diabetic nephropathy patients was due to poor glycemic control and osmotic diuresis (Pham, Pham et al. 2007). High copper levels have been found to be in disease condition of the structure of the arterial walls, stress, infection, and diabetes mellitus (Kazi, Afridi et al. 2008). Copper acting as a pro-oxidant may be involved in the production of metal catalyzed formation of free radicals and high levels of these free radicals are involved in the development of DM type 2 (Shrabani, Bharatkumar et al. 2013). Human studies also demonstrated that individuals with DM type 2 may have abnormal levels of serum copper (Olaniyan, Awonuga et al. 2012).

Low copper levels was observed in individuals with DM type 2 in another study, and this was consistent with findings of Hasan (Hasan 2013). Low Cu level results in glucose intolerance, decreased insulin response, increased glucose level, which lead to a lowered activity of CuZn Super Oxide Dismutase (SOD) thereby increasing the amount of free radicals resulting in increased oxidative damage. High Cu levels caused stimulated glycation and release of copper ions leading to oxidative stress in one study performed on DM type 2 patients (Bozkurt, Tekin et al. 2013).

Deficiency of zinc occurs in patients with DM and thus the status of zinc with progression of nephropathy has been explored. In a study performed

on DM type 2 patients, the average zinc level in the serum of the diabetics were significantly lower compared to their non- diabetic counterparts. Diabetic patients with MAU and low values of e-GFR had a lower mean serum zinc level than other diabetic nephropathy groups. There was a significant inverse relationship between serum zinc and MAU with a positive relationship between serum zinc and e-GFR. These findings supports the fact that diabetes affects zinc status and therefore significant decrease was seen in serum level at the onset of diabetic nephropathy (Al-Timimi, Mahmoud et al. 2011). Increased urinary losses of Zn leads to proteinuria and zincuria in nephrotic syndrome patients (Freeman, Richards et al. 1975). The proximal tubules reabsorbs filtered proteins from the glomerulus but in instances of excessive breakdown of these proteins it becomes defective (Shah and Yan 2008). Once this occurs, reabsorption of trace elements and filtered amino acids cannot take place in the proximal tubules. The absorption of zinc becomes impossible resulting in reduced levels of zinc.

Selenium is an important component of antioxidant enzymes, especially in Gluthatione Peroxidase (GPx) makeup (Zachara, Gromadzińska et al. 2006). The GPx structure is mostly made up of selenocysteine, deficiency of Se would lead to a reduced GPx activity and increase oxidative stress (Beytut, Erisir et al. 2004). DM type 2 patients usually have low level of Se and GPx compared to those without diabetes (Kornhauser 2008). The relation between plasma Se, GPx levels with albuminuria and severity of DN type 2 patients is still unclear (Aslan, Sabuncu et al. 2007) even though MAU is the best predicator for developing diabetic nephropathy

(Abdeyazdan, Hashemipour et al. 2007). One study came up with findings that, there was a negative association between plasma Se, GPx levels and albuminuria in type 2 diabetic patients (Hernandez-Marco, Codoner-Franch et al. 2009). This trend was observed again, in another study showing the same result in type 2 diabetic patients. GPx level in plasma was also found to be lower in diabetic patients with MAU than those without it or those without the disease condition (Kornhauser 2008). Some studies have demonstrated that antioxidant status in diabetic nephropathy shows low levels of Se and GPx in diabetic patients with MAU than other study groups (Hernandez-Marco, Codoner-Franch et al. 2009). Studies done in rats tissues showed positive correlation between GPx activity at the cellular level with Se concentration in the plasma (Aliciguzel, Ozen et al. 2003). In a human study however, there was no association between plasma Se concentration and MAU among American adults (Ford, Giles et al. 2005).

A study demonstrated that in DM patients with high insulin resistance, the level of chromium excreted in the urine is very high as compared to those who have a lesser degree of insulin resistance (Bahijri and Alissa 2011). This suggests that chromium deficiency may be associated with advancing renal dysfunction and chromium loss. There was an overall significant lower chromium level in patients with diabetes and a negative correlation between serum levels and hemoglobin A1c independent of renal function evaluation (Ahmed and Helal 2012). A reduced fasting plasma glucose was observed in the patients on diet containing yeast supplemented with chromium (Sharma, Agrawal et al. 2011). The effects

of chromium picolinate as well as chromium histidinate on renal dysfunction in the laboratory utilizing a rat kidney model has also been demonstrated. It was concluded that the administration of chromium may be of benefit in reducing renal disease in the patients with diabetes mediated through the modulation of nuclear factor-kappa B pathways (Selcuk, Aygen et al. 2012).

1.2 Problem Statement

Diabetes mellitus is an epidemic in most countries and the increasing trend has become a public health concern in Ghana. Nephropathy as a complication adds up to the problem of diabetes mellitus. In recent years, sub-Saharan Africa has experienced progress in economic growth leading to an improvement in the living conditions of its inhabitants. There been an increased in some of the risk factors as far as noncommunicable diseases (NCDs) are concerned and these are poor eating habits, absence of physical activity or exercise, excessive intake of alcohol and tobacco because of the direct influence of the western culture on the African continent. If this adopted lifestyle should continue till 2020, then the major cause of death will be NCDs such as diabetes, coronary vascular disease and cancers on the Africa continent. The burden on the economic becomes heavier in the disease condition, especially in managing the disease as far as the health system and medication is concerned. Caretakers will have to be employed to send patients to seek medical care, the cost involved and sometimes disability leading to premature death all adds up to the burden. The International Diabetic Foundation (IDF) gave

an estimated healthcare funding of US$ 111 for an individual with diabetes in Africa in 2010, which already amounts to 7% of national healthcare expenditure. Here in Africa, the management and cost of diabetes cannot be met fully on individual basis, funds from the national coffers are minimal and support from the government is scanty. Furthermore it affects the active age group leading to a reduced economy input yielding a less output then an evident burden on both the national economy and in the individual homes. Cu, Zn, Se & Cr, are the trace elements being studied in this work, these are involved in the development of diabetes and its complications. Decreased levels of the trace elements have been found to be associated with DN type 2 because they are excreted by the kidney through the urine eg. hyperzincuria being a cause of low plasma zinc in diabetics. The possible cause being renal tubular defect in absorption of the trace element, glucose induced diuresis and possibly abnormal binding of the trace elements to tissues. In other instances levels of copper was also increased due to high glucose enhancing glycation which caused the buildup of reactive oxidants leading to tissue damage. Diabetes mellitus has a connection with abnormal metabolism of trace elements. The issue now is to deduce if high glucose levels and the diabetes influence trace element metabolism or changes in mineral homeostasis influence carbohydrate metabolism. The health benefits and levels of trace elements in various disease condition including diabetes and its complications remain controversial and many questions still remain unanswered, further research is needed for clarification.

1.3 Justification

Diabetic nephropathy is common in developing countries, but its prevalence is unknown in most of these countries. At present many developing countries are in an epidemiological transition from communicable to non-communicable chronic diseases, conditions like hypertension, diabetes mellitus, cardiovascular disease as well as chronic kidney disease (CKD) are emerging as public health problems. Without detection and absence of treatment for microalbuminuria in individuals with diabetes they will eventually develop proteinuria, diabetic nephropathy, and ultimately end stage renal disease (ESRD). High cost of renal replacement therapy, and limited budgets in countries such as Ghana makes (ESRD) a death sentence. Urinary excretion of proteins accompany that of trace elements of which Zn, Cu, Se & Cr are not exempted. Up to date only few research information on nephropathy is available in Ghana and even that is only in the urban setting, representing a handful of the populace. A study done in Ghana showed that females are more prone to developing diabetes than males with a percentage of 7.7 to 5.5 by (Amoah, Owusu et al. (2002). An early detection and treatment of microalbuminuria and macroalbuminuria is therefore very important as a preventive measure in detecting diabetic nephropathy.

1.4 Hypothesis

Trace elements do not play a role in the development of diabetic nephropathy a microvascular complication of DM type 2.

1.5 Aim

The aim of the study was to determine whether Zn, Cu, Se and Cr are involved in the development of DN in Ghanaian type 2 diabetic patients.

1.6 Objectives

1. To establish the level of DN among Ghanaian type 2 diabetic patients
2. To determine which of the trace element level were associated with DN or otherwise presents a risk factor.(Suzuki, Hashiura et al. 2010)

CHAPTER 2

Diabetes is a long-standing disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces (WHO 2013). The prevalence of diabetes in the age groups between 20 to 70 years worldwide was estimated to be 8.3% in 2013 and projected to reach 10.1% in 2035. The total number of adults with diabetes is projected to rise from 382 million in 2013 to 592 million in 2035. In 2013, an estimated 5.1 million people died from consequences of hyperglycemia. More than 80% of diabetes deaths occur in low- and middle-income countries (IDF 2013). According to the International Diabetes Federation (IDF), the cost for the treatment of diabetes globally in 2010 was about $376 billion (11.6% of total health spending). The IDF predicts that this cost will increase by 2030 to $490 billion, with considerable strain being put on health systems. Type 2 diabetes can be

prevented or delayed through healthy diet, regular physical activity, maintaining a normal body weight, and avoiding smoking (IDF 2013). Diabetes mellitus comes with several complications with the advancement and duration of the disease, as part of the study DN as a microvascular complication will be considered in connection with DM type 2.

Diabetic nephropathy (DN) is typically defined by macroalbuminuria that is, a urinary albumin excretion of more than 300 mg in a 24-hour collection or macroalbuminuria and abnormal renal function as represented by an abnormality in serum creatinine, calculated as creatinine clearance, or glomerular filtration rate (GFR). Clinically, DN is characterized by a progressive increase in proteinuria and decline in GFR, hypertension and a high risk of cardiovascular morbidity and mortality (K/DOQI 2002). The kidneys play a central role in fluid, electrolyte and acid base homeostasis in humans. In chronic kidney disease (CKD), irreversible damage results in an inability of the kidneys to perform its vital homeostatic, excretory and synthetic functions. CKD is the presence of kidney damage with abnormal albumin excretion. There is a decreased kidney function that lasts longer than three months as quantified by measured or estimated glomerular filtration rate (eGFR). Progressive renal disease usually leads to the common end point - end stage kidney disease (ESKD) - of a shrunken, fibrotic kidney. The cost for renal replacement services for ESKD is enormous. In the UK and Italy, 0.02 - 0.06% ESKD population accounts for an estimated 0.7 -1.8% of the health service budget (K/DOQI 2002). In the United States, the expenditure on ESKD was estimated as US $28 billion in 2010 (Xue, Ma et al. 2001). CKD affects

between 5-15% of the adult population in the developed world (Chen, Wang et al. 2009). In Africa, CKD is estimated to affect about 10.4% of some populations (Afolabi, Abioye-Kuteyi et al. 2009) making it a significant public health issue. It has been found to account for 8-10% and 5% of medical admissions in Nigeria (Akinsola, Odesanmi et al. 1989) and Ghana (Plange-Rhule, Phillips et al. 1999) respectively. The risk factors for CKD abound in the sub - Saharan African population. A prevalence of 46.9% among hypertensives with KD was observed in a Ghanaian outpatient setting (Osafo, Mate-Kole et al.
2011), similar to the findings from an earlier review of autopsy data (Mate-Cole *et al.*, 1993). In Burkina Faso, 44% of hospitalised hypertensives had chronic renal failure (Lengani, Laville et al. 1994). Chronic glomerulonephritis remains an important cause of CKD in tropical Africa (Eghan, Amoako-Atta et al. 2009).

2.1 Pathophysiology of DN

A significant risk factor for the development of diabetic nephropathy is a long-standing hyperglycemia which may directly result in the expansion and injury by an increase in the mesangial cell glucose concentration.
Initially the mesangium expands by cell proliferation and hypertrophy. This expansion is caused by transforming growth factor β (TGF-β) and later fibrosis sets in through the stimulation of collagen fibronectin. Glucose can reversibly and irreversibly bind to proteins in the kidneys and in circulation to form advanced glycosylation end products (AGEs). With long standing hyperglycemia, AGEs can form complex cross-links which can lead to renal damage by stimulation of growth and fibrotic factors

through receptors for AGEs. Moreover, intermediate agents of proliferation and expansion like platelet-derived growth factor, TGF-β, and vascular endothelial growth factor (VEGF), which are elevated in diabetic nephropathy can contribute to further renal and microvascular complications (Fukami, Yamagishi et al. 2008).

Proteinuria is a marker of diabetic nephropathy and a potential contributor to renal injury. With increase in glomerular permeability, plasma proteins escape into the urine. These proteins in the proximal tubular cells cause an inflammatory response, which contributes to interstitial scarring and eventually leading to fibrosis. Hyperglycemia, angiotensin II, TGF-β and proteinuria itself, play roles in stimulating this fibrosis (Wu, Chen et al. 2010). In advanced stages of diabetic nephropathy, tubulo-interstitial fibrosis is a better indicator of renal failure than glomerular sclerosis. A change in the epithelialmesenchymal tubules causes the proximal tubular cell to be converted to fibroblast-like cells. These cells can then migrate into the interstitium and produce collagen and fibronectin (Wu, Chen et al. 2010).

In DN, the activation of the local renin-angiotensin system occurs in the proximal tubular epithelial cells, mesangial cells and podocytes. Angiotensin II (ATII) itself contributes to the progression of diabetic nephropathy because even though increased levels are seen with the disease, the intra renal level of ATII is mostly high, even in the presence of lower systemic concentrations. The constriction of the efferent arteriole in the glomerulus causes a higher glomerular capillary pressure. The hemodynamic effects of ATII stimulates renal growth and fibrosis through

ATII type 1 receptors, which secondarily up regulate TGF-β and other growth factors (Ruggenenti, Cravedi et al. 2010).

Control of hypertension is important and powerful intervention in decreasing the progression of diabetic nephropathy. In diabetics who have disordered auto regulation at the level of the kidney, systemic hypertension can contribute to endothelial injury. Human studies of type 2 diabetes have shown that blood pressure lowering, regardless of the agent used, retards the onset and progression of diabetic nephropathy (Bakris, Sarafidis et al. 2010). In animal studies, the degree and severity of the diabetic nephropathy were strongly linked to systemic blood pressure (Ruggenenti, Cravedi et al. 2010).

The fact that most type 2 diabetics do not develop diabetic nephropathy (DN) suggests that other factors may be involved. Genetic factors clearly play a role in the development of this disease condition in family members who have DN and its linkage to specific areas on the human genome is evolving (Krolewski, Ng et al. 2001). The theory of a reduction in nephron number at birth indicates that individuals born with a reduced number of glomeruli may be predisposed to subsequent renal injury and progressive nephropathy. If this linkage is true in humans, that would have important implications concerning the role of maternal factors in the eventual development of kidney disease (Hall 2006).

2.2 Signs and symptoms

The early signs and symptoms associated with DN is unusual but becomes more pronounced when kidney disease has progressed. Some of which

include the presence of albumin or protein in urine. An increased blood pressure, blood urea nitrogen (BUN) and serum creatinine. There is frequent urination at night, weakness, paleness, itching, and anemia. In the presence of nausea with its accompanying vomiting and morning sickness, there is no need for the intake of anti-diabetic medications and insulin.

Table 2.1: Methods of measuring urinary protein and ranges for abnormal excretion

Methods	Normal value	Abnormal Values	
		Microalbuminuria	Macroalbuminuria
24-hr urine collection	<150 mg/day Estimate 1 g creatinine excretion/1.73 m2 (ratio of 0.15=150 mg protein/24hr/1.73 m^2)	150 – 300 mg/day	>300 mg/day
Spot protein-tocreatinine ratio(mg/mg)	20 mg/kg creatinine excretion for men 15 mg/kg for women	Ratio:0.18-0.36 for 60 kg male, >0.27 for 60 kg male, 0.135-0.27 for 60 kg female	Ratio: >0.36 for 60 kg male, for 60 kg female
Albumin-tocreatinine ratio(ug/mg)	<30 ug/mg	30-300 ug/mg	>300 ug/mg

This is mostly for screening purposes and does not correlate well with 24-hr urinary protein at higher (nephrotic) levels of proteinuria. It does not account for patient weight or muscle mass. The ratio of 17-249 µg/mg for women and 25-354 µg/mg for men to define microalbuminuria. *Source:- Butt, 2010*

2.3 Laboratory and other diagnosis

Laboratory testing is most appropriate for screening as far as diabetic nephropathy is concerned. There should be an early diagnosis of diabetes and glucose intolerance to identify patients who are at risk for developing microalbuminuria. Further investigation for other risks factors associated with type 2 diabetes such as hypertension, lipid abnormalities or central obesity can be done later. As noted, approximately one third of type 2 diabetics are believed to be undiagnosed. A routine check of urinary protein level is done to guide therapy and prognosis only when type 2 diabetes is confirmed. This is because it is uncommon to find albuminuria that is either micro/macro in type 2 diabetic patients at the initial stages of the disease. Since patients might have undiagnosed diabetes (Holt, Gunnarsson et al. 2014) for years and would have contributed to hypertension or other processes that may cause proteinuria independent of diabetes, eg. small vessel artherosclerosis. Patients with diabetes can develop nephroticrange proteinuria (higher than 3.5 g/24 hr), but normally after a longstanding diabetes. A bland urine sediment supports the diagnosis of diabetes, although it is not uncommon to have some microscopic hematuria with advanced diabetic nephropathy. A renal biopsy should be considered if only there is an increased urine protein or a rise in the decline of GFR and the presentation of an acute nephrotic syndrome (Suarez, Thomas et al. 2013).

Another method used for assessment would be the imaging studies where renal ultrasound is mostly obtained to observe for kidney size. In the early stages of diabetic nephropathy, kidney size may be enlarged from

hyperfiltration and eventually a diminish in kidney size from glomerulosclerosis with progression of disease (Fukami, Yamagishi et al. 2008). A renal ultrasound can also be used to assess for hyperechogenecity suggestive of CKD and can assist in ruling out obstruction. (McClellan and Powe 2009)

The presence of red cell or white cell casts in the urine should also suggest a biopsy. Renal biopsy findings consistent with diabetic nephropathy in the early stages are mesangial expansion and glomerular basement membrane thickening. Eventual progression of diabetic nephropathy can lead to nodular glomerulosclerosis also referred to as Kimmelstiel-Wilson disease (ADA 2012).

Table 2.[1] Description and stages of kidney disease

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[1] .4 Treatment

The control of blood pressure and maintenance of a tight glycemic control are the basis for prevention and management of DN. A reduction in GFR was observed in most patients immediately after blood pressure lowering (Parving, Andersen et al. 1983). A slowing in the rate of reduction of GFR from an average of 0.91 to 0.39 mL/min/month was observed after a 2 year follow up with a significant preservation of GFR above that expected from pretreatment measurements (Parving, Andersen et al. 1983). There was renal preservation because of the initial drop in GFR due to the effect of the antihypertensive treatment.

Stage	Description	eGFR (ml/min/1.73 m2)
1	Kidney damage with normal or increased GFR	>90
2	Kidney damage with mildly decreased GFR	60 – 89
3	Moderately decreased GFR	30 – 59
4	Severely decreased GFR	15 – 29
5	Kidney failure	<15

Source:- Butt, 2010.

creatinine levels are stable with an increase of less than 30% of baseline (Palmer 2002). It is important to emphasize that diuretics are extremely beneficial adjuncts for blood pressure control and are often missing in patients who are not close to meeting blood pressure goals.

Diuretics are first-line agents for many hypertensive and is routinely used as a second-line agent after angiotensin blockade in diabetics. Thiazide diuretics work well, even at low dosages for patients with normal renal function. For example, just 12.5 to 25 mg/day of hydrochlorothiazide is often effective (Palmer 2002). When the GFR is below 60mL/min, an institute loop diuretics is often used for a better natriuretic effect. Short-acting loop diuretics such as furosemide work better when given at least twice daily to avoid rebound sodium retention (Palmer 2002).

A study recommended maintaining antihypertensive therapy in renal disease even if therapy causes some hemodynamic drop in GFR, if

The use of agents specific for blocking the renin-angiotensin system is of most beneficial in preventing or slowing the progression of diabetic nephropathy. ATII can increase glomerular capillary pressure by specifically constricting the renal efferent arteriole (Lewis, Hunsicker et al. 1993). It can also activate renal cell growth and fibrosis independent of hemodynamic effects. Even with blood pressure at a goal and without microalbuminuria, initiation of low dosages of angiotensin-blocking agents is considered, with titration up as tolerated.

Other antihypertensives may not offer this antiproliferative effect and may cause adverse hemodynamic effects. For example, the calcium channel blockers (dihydropyridines) cause dilation of the afferent artery leading to increase glomerular capillary pressure. These dihydropyridines perform better when reserved as third- or fourth-line agents in patients with diabetes, only after angiotensin blockade and diuretics have already been administrated (Lewis, Hunsicker et al. 1993). Diet is another factor for consideration, since many animal studies on glomerulopathies have shown a reduction in renal function by restricting dietary protein. However that in human studies remains inconclusive but it is advised to avoid protein supplements (Pan, Guo et al. 2008). The American Diabetes Association recommends moderate protein restriction (0.8-1.0 g/kg/day) for patients in the initial stages of DN, and a reduction to 0.8 g/kg/day for patients in a more advanced stage of this complication. A low sodium intake is also preferred since increase intake of salt increases blood pressure (ADA 2008).

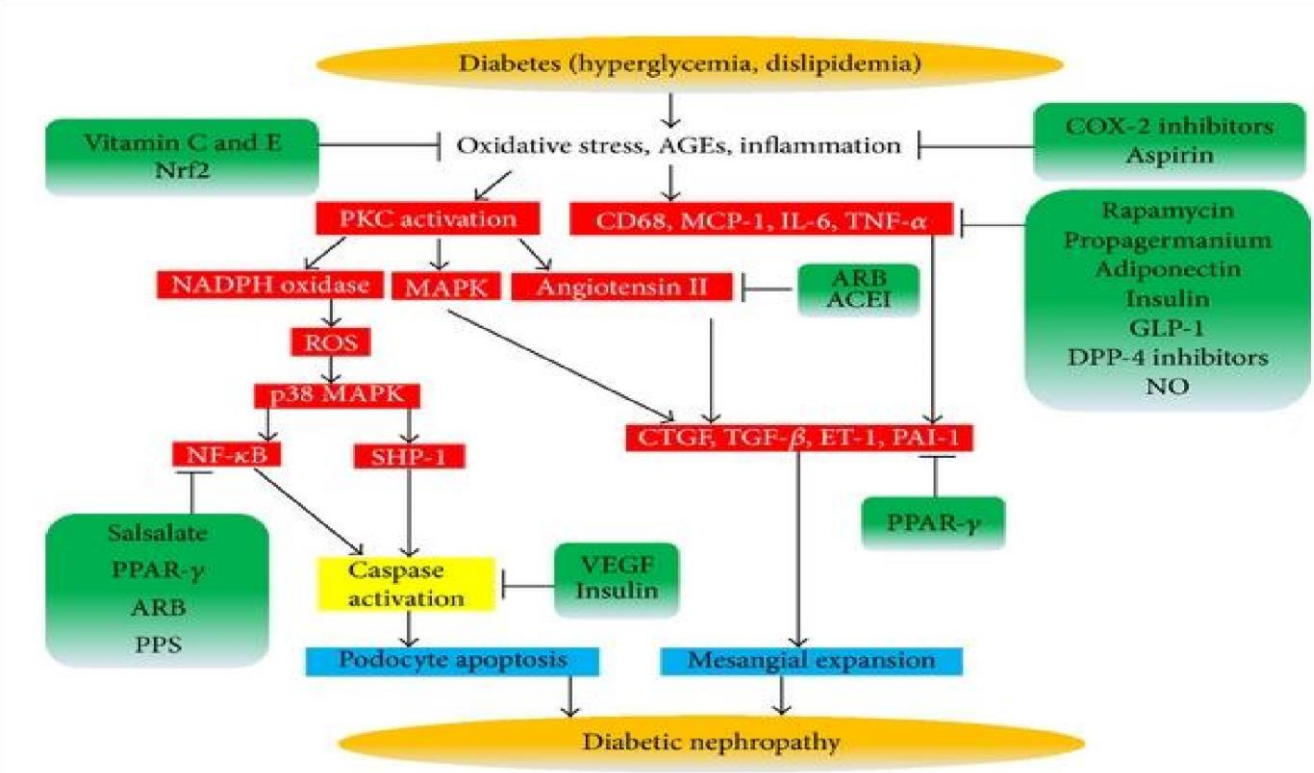
Another group of agents to avoid as far as DN is concerned are nephrotoxins and nonsteroidal anti-inflammatory drugs (NSAIDs) which

can cause a significant drop in GFR in patients with diabetic nephropathy, especially when used with angiotensin-blocking agents. Daily low-dosage aspirin is safe in diabetics, and the cardiac benefits greatly outweigh any risk. However, aspirin at higher dosages and other NSAIDs should be avoided if possible. Cyclooxygenase 2 (COX-2) inhibitors are similar to other NSAIDs in their potential for renal toxicity (Mogensen, Neldam et al. 2000).

Radiocontrast media are also particularly nephrotoxic for diabetics. Even with a normal serum creatinine level, patients with diabetes and proteinuria should be volume-loaded 12 hours before and after exposure to contrast, if possible. Diuretics should be temporarily discontinued, and hyperglycemia should be controlled. Other agents such as dopamine-like agonists and acetylcysteine may help prevent contrast nephropathy in diabetics but require further study (Owen, Hiremath et al. 2011).

2.5 Role of oxidative stress in DN

Hyperglycemia increases superoxide anion and other reactive oxygen species (ROS) production in patients with diabetes (Pricci, Leto et al. 2003). Increased oxidative stress is an important factor in the pathogenesis of diabetic nephropathy (Martin-Gallan, Carrascosa et al. 2007). Some studies clearly indicate that both diabetic state and insulin resistance play a central role in producing oxidative stress. Free glucose activates aldose reductase activity in the polyol pathway and decreases NADPH/$NADP^+$ ratios (Tesfarmariam and Cohen 1992). Higher levels of intracellular glucose activates protein kinase C (PKC) pathway through *de*



novo synthesis of diacylglycerol (DAG) (Ishii, Jirousek et al. 1996 B). Activation of PKC in the glomeruli has been associated with processes increasing mesangial expansion, thickening basement membrane, endothelial dysfunction, smooth muscle cell contraction, and activation of cytokines and transforming growth factor-β (TGF-β) (Koya and King 1998). PKC induces oxidative stress by activating mitochondrial NADPH oxidase (Bedard and Krause 2007).

Figure 2.1: Schematic diagram on the progression of diabetic nephropathy and its inhibition.

AGEs: advanced glycation end products; **PKC**: protein kinase C; **COX-2**: cyclooxygense2; **Nrf2**: NFE2-related factor 2; **NADPH**: nicotinamide adenine dinucleotide phosphate; **MAPK**: mitogen-activated protein kinase; **MCP-1**: monocyte-chemoattractant protein-1; **IL-6**: interleukin-6; **TNF-α**: tumor necrosis factor-α; **GLP-1**: glucagon like peptide-1; **DPP-4**: dipeptidyl peptidase-4; **NO**: nitric oxide; **ARB**: AT1 receptor blockers; **ACEI**: angiotensin-converting enzyme inhibitors; **NF-κB**: nuclear factor-κB; **CTGF**: connective tissue growth factor; **TGF-β**: transforming growth factor-β; **VEGF**: vascular endothelial growth factor; **SHP-1**: Src homology-2 domain-containing phosphatase-1; **PPAR-γ**:

peroxisome proliferator-activated receptor-γ; **PPS**: pentosan polysulfate. *Source:- Mima, 2013*

Vascular NADPH oxidase consists of multiple subunits including phox47, phox67, and Nox isoforms (Bedard and Krause 2007). ROS generated from Nox isoforms might induce endothelial dysfunction, inflammation, and apoptosis. Excess FFA, mainly derived from insulinresistant state can also increase oxidant production by β oxidative phosphorylation via mitochondrial metabolism (Duckworth 2001). Studies using rodents indicate that increases in oxidative stress could be responsible for developing DN, inhibition of the polyol pathway with aldose reductase inhibitors could reduce the effects of hyperglycemia on DN (Dunlop 2000). Further, administration of vitamin C or E has been shown to be effective in ameliorating rodent model of DN (Bursell and King 1999). Another study has also shown that high doses of vitamin E normalized parameters of oxidative stress and inhibited vascular abnormalities caused by DAG-PKC activation in the kidney (Koya, Lee et al. 1997).

2.6 Inflammation in DN

The main causes of DN are metabolic and hemodynamic factors, some studies have shown that DN is an inflammatory process and immune cells could be involved in the development of disease condition (NavarroGonz´alez, Mora-Fern´andez et al. 2011). Hyperglycemia may induce macrophage production of interleukin 12 (IL-12), which can stimulate CD4 cell production of IFN-γ. Free fatty acids, hyperglycemia, and obesity may activate nuclear factor κB (NF-κB) through PKC and ROS

to rapidly stimulate the expression of cytokines (Chen, Lee et al. 2004). Following activation, NF-κB translocate to the nucleus, there is the rapid stimulation of transcription genes such as endothelin-1 (ET-1), VCAM-1, intercellular adhesion molecule-1 (ICAM-1), IL-6, and TNF-α that promote the development of DN.

It is well known that elevated levels of advanced glycation end products (AGE) can be found in kidney. AGE interacts with its receptor (RAGE), and AGE/RAGE interactions have been reported in the development of DN (Myint, Yamamoto et al. 2006). Exposure of activated lymphocytes to AGE enhances the expression of IFN-γ which may accelerate immune responses that contribute to developing DN (Galkina and Ley 2006). Furthermore, in a clinical study of type 2 diabetic patients, positive correlations were observed between plasma IFN-γ, proteinuria, and estimated glomerular filtration rate (eGFR) (Wu, Chen et al. 2010). Also, plasma IL-2R levels found in DM type 2 overt nephropathy, and apparent positive correlation was also observed between plasma IL-2R and proteinuria (Wu, Chen et al. 2010).

2.7 Role of trace elements in diabetes

Serum albumin is most abundant protein in plasma, which serves as a carrier for a variety of nutrients and metabolites (Lu, Stewart et al. 2008). The metal binding capacity of albumin has been known for a longtime, it binds to essential and toxic metal ions including Cu, Zn, Cr and Se just to mention a few. In some instances low levels of albumin in serum have led to equally reduced levels of these micronutrients. This is the reason

why the knowledge of the level and influence of these trace elements becomes necessary in this study, as there is the loss of protein associated with nephropathy.

2.8 Copper

Copper is the third most abundant trace element in the body. Its role as a cofactor component of cytochrome oxidases, superoxide dismutase, tyrosinase, uricase, dopamine ß-hydroxylase, lysyl oxidase and ceruloplasmin make it a key micronutrient for oxidative pathways. The bioavailability of copper from the diet is about 65- 70% depending on a variety of factors including chemical form, interaction with other metals, and dietary components.

2.8.1 Biological functions of Cu

Its biological half-life in diet is 13-33 days with biliary excretion being the major route of elimination (Weiss, Lozoya et al. 2008). The normal range of serum copper is 0.60-1.8 mg/L (Walker, Walker et al. 2010). Its primary functions in relation to enzymatic property includes Phase-I detoxifying enzymes (i.e., the cytochrome C oxidase family of enzymes) (Huskisson, Maggini et al. 2007) and in addition it is also necessary for the development of connective tissue and nerve coverings (myelin sheath) (Shenkin 2008). It participates in the iron metabolism (Huskisson, Maggini et al. 2007). It may be accumulated in the adult body (liver and brain) up to a limit of 80 mg (Guerrero-Romero and Rodriguez-Moran 2005), supporting deficient dietary intake, without inducing clinical symptoms of toxicity for a short period of time. Deficiency of Cu is not

frequent in humans, although it can cause several hematological symptoms such as normocytic, hypochromic anemia, leucopenia and neuropenia, and skeletal disturbances (Huskisson, Maggini et al. 2007). Toxic levels have been related to liver damage in chronic intoxication and gastrointestinal effects with cramps, nausea, diarrhoea and vomiting in acute episodes (Guerrero-Romero and
Rodriguez-Moran 2005).

2.8.2 Copper and DN

It has been also suggested that abnormal renal Cu homeostasis may play a key role in the development of diabetic nephropathy, and selective Cu (II) chelation can protect against pathogenic mechanisms that lead to diabetic nephropathy and might be clinically useful in the treatment of early-stage diabetic kidney disease (Gong, Lu et al. 2008). On the other hand, an overload of urinary copper damages renal tubules and may play a role in the progression of nephropathy in patients with advanced nephropathy (Ito, Fujita et al. 2001).

Since duration of diabetes and HbA1c results do not affect the level of copper excreted in urine, then nephropathy is the cause of excretion of high levels of copper. Urinary copper excretion may also be due to dissociations from both copper-albumin and ceruloplasmin-copper complexes filtered through the damaged glomerulus (Gong, Lu et al. 2008). It does not exclude that overloading of urinary copper to damaged renal tubules may play some role in the progression of nephropathy in patients with advanced nephropathy (Gong, Lu et al. 2008).

High urinary copper may be a marker of elevated renal tissue copper, as evidenced by animal studies, in which, copper chelation improved markers of renal damage (Gong, Lu et al. 2008). The exact roles of copper in diabetic patients may be really more complicated. For example, metallothioneins (MT) a group of intracellular metal-binding and cysteine-rich proteins act as regulator of metal homeostasis such as copper in tissues. MTs were found to be potent antioxidants and adaptive (or stress) proteins to protect cells and tissues from oxidative stress (Li, Cai et al. 2007). Oxidative stress is a well-known mechanism of chronic diabetic complications like nephropathy. Genetically or pharmacologically enhanced MT expression in various organs including the kidney provides significant protection from diabetes-induced organ dysfunction such as nephropathy (Li, Cai et al. 2007).

Copper is a renal toxin that induce renal failure by a direct effect on the kidneys or indirectly through hemoglobinuria from intravascular hemolysis and myoglobinuria. These are manifested through microscopic hematuria, proteinuria, glucosuria, kidney enlargement, uremia, edema, and renal tubular acidosis. In most instances copper is largely bound to protein with little glomerular filtration. Most filtered copper is either reabsorbed into the blood or sequestered into storage vesicles by ATP7A and ATP7B mediated transport, respectively (Linz, Barnes et al. 2008). In Wilson's disease, copper is deposited in the epithelium of the proximal and distal convoluted tubules and in the glomerular mesangium. The resulting basement membrane thickening interferes with the resorptive function of the tubule (Zhuang, Mo et al. 2008).

2.9 Zinc

Zinc is required for the structure and activity of more than 100 enzymes (Shenkin 2008), for the synthesis of nucleic acids and proteins, for cellular differentiation, for glucose use and insulin secretion (Lukaski 2004). It takes part in the Zn fingers associated with DNA, hemoglobin, myoglobin and cytochromes (Guerrero-Romero and Rodriguez-Moran 2005).

The normal range of serum zinc is 72-144 ug/dl (Walker, Walker et al. 2010). Its bioavailability is reduced by the presence of large amounts of other elements such as Fe or Cu, its absence negatively affects the immune system efficacy, and the sensibility of taste and smell senses, and impairs DNA synthesis (Shenkin 2008). It has also been described that Zn deficiency produces hair loss and hypochromic anaemia whilst its toxicity shows both acute and chronic effects (Shenkin 2008). Intakes of 150–450 mg per day over an extended period of time has been associated with poor Cu levels, altered Fe, immune functions and reduced levels of HDL (Guerrero-Romero and Rodriguez-Moran 2005).

2.9.1 Zinc in Diabetes

Zinc is useful in the synthesis, storage and secretion of insulin. Zinc may improve glycaemia therefore a restored zinc status in patients with type 2 diabetes may counteract the deleterious effects of oxidative stress which helps to prevent complications associated with diabetes.

Zinc is involved in the synthesis, storage, secretion, and conformational integrity of insulin monomers and assembles as a dimeric form for storage and secretion as

crystalline insulin. Lower levels of Zn may affect the ability of pancreatic islet cells responsible for the production and secretion of insulin, such as in type-2 diabetes (Kazi, Afridi et al. 2008). The predominant effect on zinc homeostasis of diabetes is hypozincemia which may be the result of hyperzincuria or decreased gastrointestinal absorption of zinc or both (Al-Timimi, Mahmoud et al. 2011).

Zn plays a key role in this process because it is highly concentrated in the pancreas especially within the islets (ADA 2011). The conversion of proinsulin to insulin in combination with an acidic medium allows for crystallization of insulin within the mature granule. Insulin can associate into dimers that can further associate to form hexamers in the presence of zinc. These zinc hexamers can then be packed together to form a stable structure which dissociate upon secretion and enables insulin to function in the bloodstream. Thus, zinc is essential for the correct processing, storage, and secretion of insulin (Emdin, Dodson et al. 1980) and is said, therefore to cosecrete along with insulin. Even though the oversecretion of insulin can deplete the β-cells of zinc, the actions of Zn in the pancreas are not limited to the β-cell because it regulates the α-cell response to hypoglycemia (Zhou, Zhang et al. 2007). Zn was able to reduce both fasting glucose and insulin as well as increase pancreatic zinc in db/db mice (Simon and Taylor 2001). It also impaired oxidative changes in the retina of diabetic rats (Moustafa 2004) but supplementation before treatment with the pancreatic toxic agent alloxan or dithiozone prevented hyperglycemia and destruction of islets

(Jansen, Karges et al. 2009). The mechanisms of the effects of zinc on diabetes are only partially known. Given the importance of zinc on insulin storage and secretion, one of

the most relevant findings has been the identification of the role of the zinc transporter ZnT8 (Chimienti,

Rungby et al. 2004).

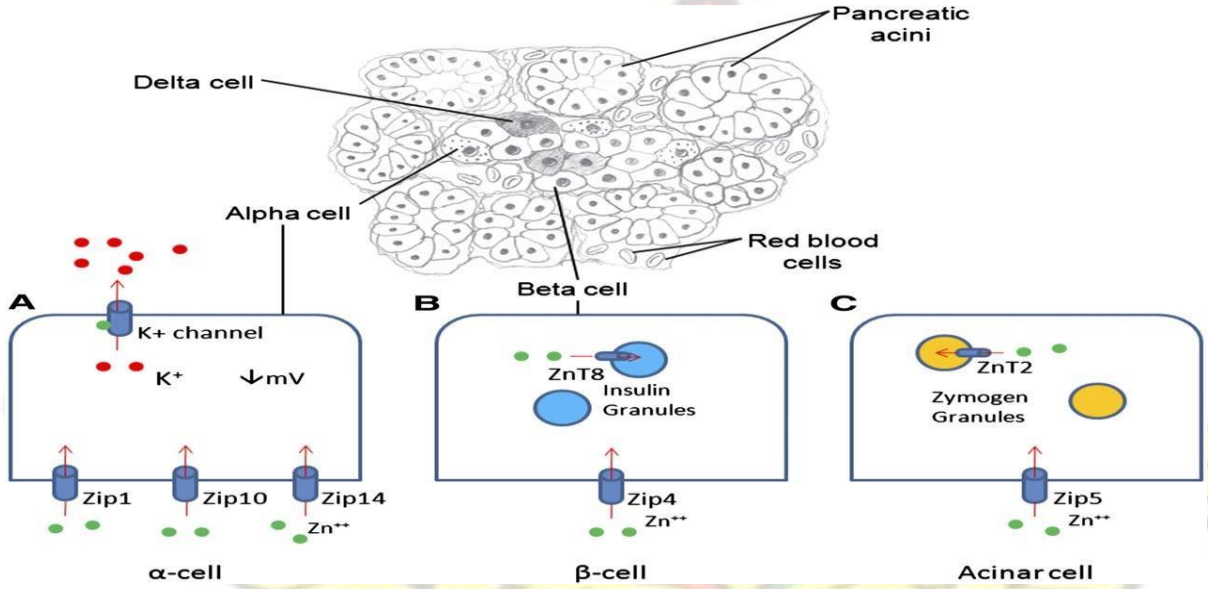
2.9.2 Zinc and its transport protein (ZnT8)

ZnT8 was initially described as pancreatic β-cell specific. Subsequent studies showed that it can also be expressed in subcutaneous fat tissue, pancreatic α-cells and peripheral blood mononuclear cells (Overbeck, Uciechowski et al. 2008). Overexpression of ZnT8 in cultured cells is associated with increased intracellular zinc which did not cause toxicity to the cells but rather protected them from apoptosis and also decreased glucagon secretion by 50% (Wijesekara, Chimienti et al. 2009). On the other hand, in the absence of ZnT8 a dramatic defects in insulin processing and secretion at the β-cell occurred (Wijesekara, Chimienti et al. 2009).

A single nucleotide polymorphism in the ZnT8-encoding gene has been shown to increase the risk of type 2 diabetes (Frayling 2007). Inflammatory cytokines play a major role in β-cell destruction in type 2 diabetes. Interleukin 1β (IL-1β) is involved in alteration of insulin secretion and islet destruction, apparently these effects are mediated by the activation of NF-kB (Jansen, Karges et al. 2009).

Zinc has relevant effects on cytokine synthesis and activity. For instance, supplementation of zinc inhibits the release of some inflammatory cytokines and its reduction in HL-60 cells increased IL-1β, as shown by some studies that zinc may have protective effects in diabetes by

suppressing IL-1β release and inhibiting NF-kB activation (Jansen, Karges et al. 2009). It was observed from another study that zinc transport, particularly that mediated by ZnT8 in β-cells, was highly cytokine sensitive (Egefjord, Jensen et al. 2009). Although these authors explored some effects in two apoptosis genes (Bax and Bcl2), there were a number of unanswered questions in this regard.



(A) Localization of Zip1, Zip10, and Zip14 to pancreatic α-cells suggests that these transporters are responsible for importing Zn into the cell. Zn binds to and opens ATP dependent K (+) channels, allowing the efflux of Zn from the α-cell and inactivation of voltage dependent calcium channels, resulting in decreased glucagon secretion. **(B)** Zn is transported into pancreatic β-cell cells via Zip4. ZnT8 is responsible for the transport of Zn into insulin granules. Auto antibodies to ZnT8 and polymorphisms of ZnT8 are associated with the onset of DM.
(C) Zip5 is responsible for the transport of Zn into pancreatic acinar cells. Zn is transported into zymogen granules by ZnT2 where it binds to and activates digestive enzymes that are subsequently secreted.

Figure 2.2: The transport of Zn into the various pancreatic cells (alpha, beta and acinar cells) with the aid of its transporters ZnT8 and ZnT2 respectively with ZnT8 being the main transporter of Zn into insulin granules in the beta cell. The processes occurring in the cells are explained.
Source:- Kelleher, 2011

2.9.3 Zinc effect on oxidative stress

Zinc can induce synthesis of metallothionein and glutathione, which have protective roles against the effects of reactive oxygen species and is part of the enzyme superoxide dismutase (SOD), which acts on the superoxide radical to convert it into hydrogen peroxide. It competes with Fenton's catalytic agents, such as copper and iron, and stabilizes disulfide bridges in proteins (Powell 2000). Overexpression of metallothionein and SOD is β-cell protective (Butler, Janson et al. 2003). On the other hand, some polymorphisms of different isoforms of metallothionein lead to lower plasma zinc and greater glycated hemoglobin (HbA1c) levels (Giacconi, Cipriano et al. 2005). In addition to the roles described above, zinc is able to influence protein–protein interactions of redox-sensitive proteins that are part of signaling process. Some authors have regarded zinc as a signaling ion itself. It has been suggested that zinc is involved in the regulation of NF-kB, phosphorylation of protein kinase C (PKC), and activation of the phosphoinositide 3'-kinase (PI3K)/Akt signaling pathway, among others
(Jansen, Karges et al. 2009).

2.9.4 Zinc in DN

Although several factors are involved in the onset of diabetic nephropathy, low zinc status appears to contribute to the diabetes associated renal injury (Parham, Amini et al. 2008). Measurement of serum zinc levels may be considered medically necessary in diabetic patients and might be used

as a marker of nephropathy associated with other nephropathy detection markers.

From studies performed, diabetic patients having microalbuminuria with lower values of e-GFR had a mean serum zinc value lower than other diabetic nephropathy groups (Al-Timimi, Mahmoud et al. 2011). A significant inverse relationship between serum zinc and microalbuminuria was observed, with a significant positive relationship between serum zinc and e-GFR. These findings gave supports in the observation that diabetes affects zinc status and significant decrease was observed in the serum level of zinc at the onset of diabetic complications such as hypertension and nephropathy (Al-Timimi, Mahmoud et al. 2011). Therefore, hypozincaemia observed in diabetics can be attributed to hyperzincuria. The results of the study were consistent with finding reported by others (Al-Timimi and Bakir 2009).

2.10 Chromium

The kidney is the main route of excretion of essential trace elements and chromium is no exception. Dietary chromium as trivalent chromium is absorbed by passive diffusion from the gastrointestinal tract and quickly passes to the bloodstream where it binds to transferrin and is then transported to the tissue (Vincent and Stearns 2011). It is well known that Cr is essential for normal blood glucose and lipid metabolism and also an insulin-coadjuvant (Shenkin 2008). Other biochemical actions for Cr are the involvement in gene expression, energy production, lipoprotein or lipid synthesis as well as metabolism regulation(Shenkin 2008).

Deficiencies in Cr are accompanied by glucose intolerance, weight loss and peripheral neuropathy (Shenkin 2008). Moreover, low Cr levels may increase the risk of cardiovascular diseases (Thomas and Gropper 1996). Decreased levels of Cr are observed in an organism because it is not easily absorbed and this explains the absence of data on its toxicity. However, high doses of Cr have been related to chromosomal damage, alterations in the kidney and liver as well as metallic-mineral disorders (Guerrero-Romero and Rodriguez-Moran 2005).

2.10.1 Chromium in diabetes

Chromium and lower molecular weight chromium binding substance (LMWCr) act together to increase the action of insulin (Cefalu and Hu 2004). LMWCr is a naturally occurring oligopeptide that has been purified from many sources: rabbit liver, porcine kidney, bovine liver, colostrum, dog, rat and mouse liver (Vincent 2000). It is widely distributed in mammals with a very large binding constant ($K \approx 10^{21}\ M^{-4}$) and forms a strong bond with four chromic ions. LMWCr exists in its inactive or apo form within the cytosol and nucleus of insulin-sensitive cells (Vincent 2000).

When insulin concentration in the blood rises, insulin binds to the external subunit of its receptor proteins and causes a conformational change. This change results in the autophosphorylation of the tyrosine residue located on the internal ß-subunit of the receptor, thereby activating the receptor's kinase activity (Vincent 2000). An increase in

insulin levels is a signal for the movement of transferrin receptors from the vesicles of insulin-sensitive cells to the plasma membrane.

Transferrin, the protein responsible for Cr transport, binds to these receptors, and becomes embedded through the process of endocytosis. Chromium can then be released from transferrin, when there is a decrease in pH of the vesicles containing the transferrin molecules through the action of ATP-driven proton pumps. The free chromium within the cell is then removed by LMWCr (Cefalu and Hu 2004). The binding of LMWCr to chromium converts it into its holo or active form and once activated, LMWCr binds to the insulin receptors which helps to maintain and increase the activity of insulin receptor tyrosine kinase.

In one study that was performed on bovine liver, it was observed that LMWCr could increase the activity of protein kinase receptors by up to 7-fold in the presence of insulin. Furthermore, evidence suggests that the action of LMWCr is most effective when it is bound to four chromic ions (Vincent 2000).

When the insulin signaling pathway is shut off, the insulin receptors on the plasma membrane relax and become deactivated. This causes the release of holo-LMWCr from the cell and excreted from the body through the urine (Vincent 2000). LMWCr cannot be converted back into its inactive form due to the high binding affinity of this oligopeptide for its chromium ions.

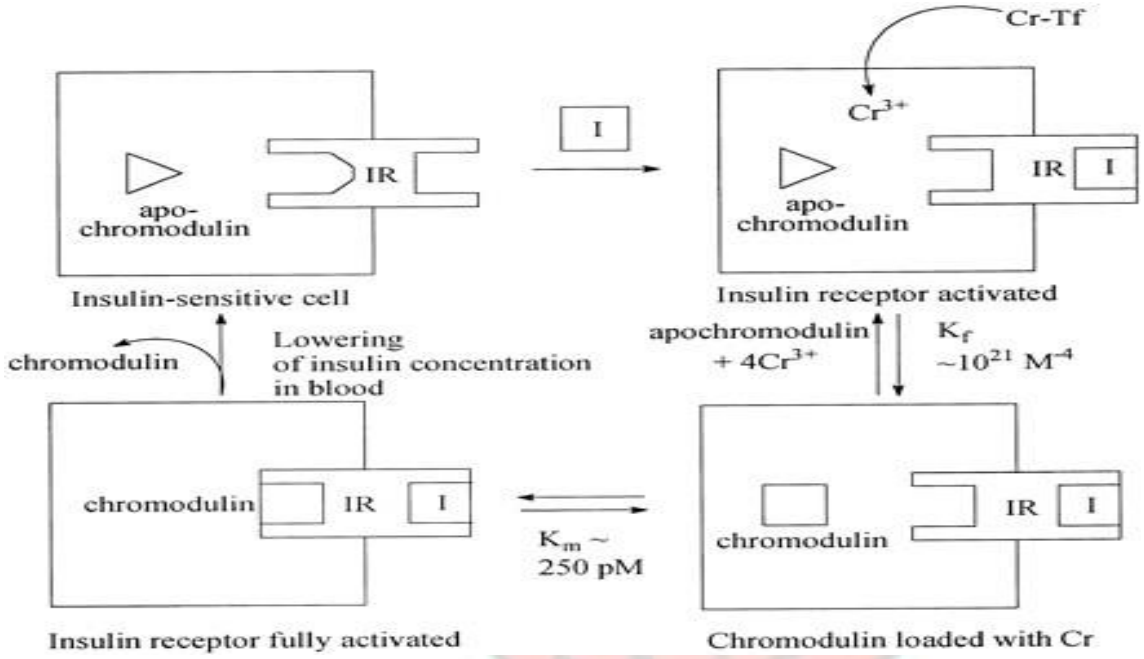


Figure 2.3: The mechanism for the activation of insulin receptor kinase activity by chromodulin in response to insulin.

Source:- Vincent, 2001.

The inactive form of the insulin receptor (IR) is converted to the active form by binding insulin (I). This triggers a movement of chromium (presumably in the form of Cr-transferrin (Cr-Tf) from the blood into insulin-dependent cells, which in turn results in the binding of chromium to apochromodulin (triangle). Finally, the holochromodulin (square) binds to the insulin receptor, further activating the receptor kinase activity. Apochromodulin is unable to bind to the insulin receptor and activate kinase activity. When the insulin concentration drops, holochromodulin is released from the cell to relieve its effects.

2.10.2 Chromium in DN

The level of chromium was found to be 6 to 20 times higher in concentration than that in red blood cells or liver from studies done on

the trachea of animals using radioactive ^{51}Cr (Weber 1983). *In vitro* studies using rat renal cortical slices support these observations ((Berndt 1976). Both trivalent and hexavalent chromium add up to 80 fold above the incubation medium concentration in the slice preparation by a process that appears to represent active (energy requiring) biologic transport (Berndt 1976).

The amount of chromate present in the renal cortex is detected by autoradiography which represent it's absorption in the convoluted portion of proximal tubules at the site of cellular necrosis (Weber 1983). Although it is evident that the trivalent form of chromium is less nephrotoxic than the hexavalent form, tubular necrosis and acute renal failure have been reported from both species of the metal (Laborda, DiazMayans et al. 1986). The reasons for these different findings are not clear but may relate to the absence of a reliable measure of cumulative past absorption and uncertainty about the oxidation state of chromium because of its instability in biological materials. Urinary LMW proteins may originate from either the tubule cell (e.g., BB-50 or N-acetylglucosaminidase) or from outside the kidney (e.g., β_2-microglobulin or retinol-binding protein). Extra-renal LMW proteins are filtered at the glomerulus and taken up by endocytosis at the luminal surface of proximal tubule cells. The intracellular LMW proteins are then catabolized within lysosomes to form amino acids. These filtered LMW proteins, eventually do not appear in the urine.

LMW proteinuria exhibits a tubular abnormality often called "tubular proteinuria." This tubular abnormality is usually reversible and is not considered a sign of renal

disease unless it predicts the appearance of renal failure shown by a decrease in glomerular filtration rate and when urinary chromium exceeds 15 ug/g creatinine (Gurson and Saner 1978). Although it is possible that chromium filtered at the glomerulus is largely reabsorbed by the tubule. The renal clearance calculations are uninterpretable because of the complex binding nature of the various chromium species to serum proteins and the dependence of chromium excretion on urine flow rates. Tubular proteinuria, the excretion of a few milligrams of protein under 20kDa molecular weight are absent in urine and must be distinguished from classical glomerular proteinuria. Tubular proteinuria being reversible, cannot by itself be considered evident of chronic renal disease since it occurs after a wide variety of physiologic stresses, (Gurson and Saner 1978).

2.11 Selenium

Selenium (Se) is an essential trace element with important biological roles in human health. Se participates as a cofactor for several enzymes (selenoproteins) which act in the regulation of thyroid hormone metabolism, enzymatic antioxidant defenses and the immune system (Beckett and Arthur 2005). It plays an important role in the expression of several selenoproteins (Beckett and Arthur 2005), changes in serum or tissue Se levels could be associated with disturbances in specific functions of these Se dependent enzymes.

Se deficiency has been associated with an increased risk for cardiovascular disease (Flores-Mateo, Navas-Acien et al. 2006), coronary heart disease mortality and all-cause mortality (Eaton, Abdul Baki et al. 2010), the development of malignant neoplasms (Bleys, Navas-Acien et al. 2008) and viral infectious diseases (Hoffmann and Berry

2008) in the general population. A clear relationship between the kidney and Se metabolism has been documented (Kohrle, Jakob et al. 2005). Different selenoproteins are synthesized in the kidney. For example, type 1 5'deiodinase (D1) is extensively expressed in the kidney, primarily in the basolateral plasma membrane and human kidney proximal tubules are the main source of plasma glutathione peroxidase (GPx) (Avissar, Ornt et al. 1994).

Changes in Se levels have been associated with alterations in several selenoproteins. In this regard, animal studies have shown that severe Se deficiency reduces both D1 and GPx proteins and their activity in a tissue-specific manner, and Se repletion increases them (Gross, Oertel et al. 1995). Acute kidney injury (AKI), chronic kidney disease (CKD) and end-stage renal disease (ESRD) are often associated with low serum and tissue levels of Se. The relationship between hyposelenemia and the complications associated with renal disease has not been extensively evaluated. However, one study suggest an association between hyposelenemia and death risk, especially due to infectious disease in CKD (Fujishima, Ohsawa et al. 2011).

2.11.1 Biological functions of Se

Selenium is incorporated as selenocysteine (Se-Cys) at the active site of a wide range of Se-dependent enzymes, also known as selenoproteins. At least 30 selenoproteins coded by 25 selenoprotein genes in humans have been identified (Kryukov, Castellano et al. 2003), although the physiological functions of many of them are still unknown (Rayman 2000). These enzymes are important in different biological functions including the formation of thyroid hormones,

antioxidant defense systems, redox signaling and immune function (Hawkes and Alkan 2010). In healthy individuals, Se circulates in human serum at concentrations ranging from 0.5 to 2.5 μmol/L (Alfthan and Neve 1996). The antioxidant defense effects of Se derive from the actions of different selenoproteins (Hawkes and Alkan 2010). Selenoprotein P (SelP), the most common plasma selenoprotein which makes up 60% of plasma Se, acts as an antioxidant protecting endothelial cells from damage induced by peroxynitrite, a compound formed *in vivo* from superoxide and nitric oxide that can mediate oxidation, nitration or nitrosation reactions, leading to impaired function, toxicity and alterations in signaling pathways (Sies and Arteel 2000).

Other selenoproteins such as GPx act as antioxidant enzymes reducing oxidative stress by means of reduction of several reactive oxygen species (ROS), such as superoxide anions and peroxides (hydrogen peroxide and lipid hydroperoxides), protecting vascular and immune cells from oxidative damage (Park and Oh 2011). Another selenoprotein with antioxidant action is thioredoxin reductase (TrxR) which catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of thioredoxin, participating in the regeneration of several antioxidants, possibly including vitamin C (Mustacich and Powis 2000).

Se also contributes to the regulation of immune function (Williams and Kwon 2004). It has been reported that selenoproteins are critically required for limiting ROS production in T cells and thus preventing ROSmediated suppression of T-cell activation (Carlson, Yoo et al. 2010).

2.11.2 Se in Diabetes

Selenoproteins and low molecular weight selenium compounds may interfere at different stages with insulin-induced signal transduction, eventually leading to dysregulation of carbohydrate metabolism as represented in **figure 2.4**.

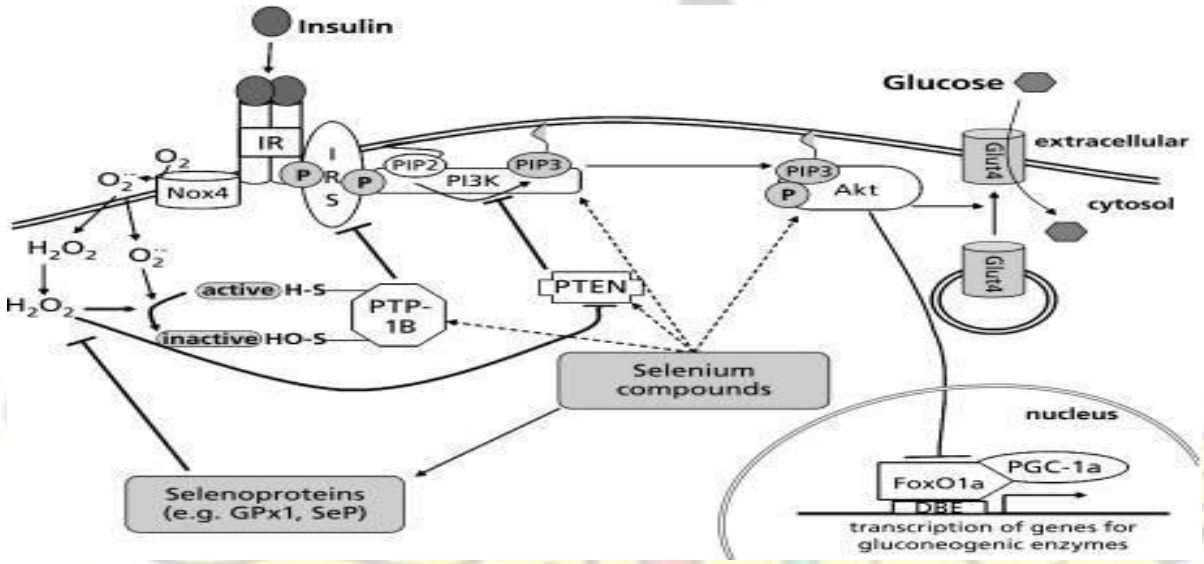


Figure 2.4: Depicts a potential influence of selenium on components of the insulin signaling cascade.

Source:- Streinbrenner, 2011.

The clue to answer the pivotal question of whether and how selenium exerts adverse effects on insulin-regulated metabolic pathways in humans may lie in the apparent "redox paradox" of insulin signalling, a concept that refers to facilitated insulin action by insulin-stimulated reactive oxygen species (Goldstein, Mahadev et al. 2005). Upon binding to its receptor at the plasma membrane of adipocytes, insulin elicits a transient burst of ROS (superoxide and H_2O_2) (May and de Haen 1979). Insulin activates the NAD (P) H oxidase (Nox) 4 to generate superoxide, which is subsequently converted to H_2O_2 (Mahadev, Motoshima et al. 2004). These

insulin-stimulated small amounts of H_2O_2 serve as second messengers, which attenuate the activity of phosphatases with redoxsensitive cysteine residues and thereby enhance the phosphorylation of components downstream in the insulin signalling cascade (Goldstein, Mahadev et al. 2005).

2.11.3 Se in DN

Plasma Se levels have been found to be reduced in critically ill states and are inversely correlated with the severity and outcome of the disease (Gärtner 2009). This finding has also been reported in patients with acute kidney injury (AKI) (Wiesen P., Van Overmeire et al. 2011). Among the possible mechanisms involved in the impaired Se status in CKD patients are increased Se requirements, low dietary intake, impaired intestinal absorption, reduced Se binding proteins and elevated urinary and dialysis losses (Zachara, Salak et al. 2004a). Se is excreted from the organism mainly in the urine and feces, but urine contains 50-70% of the ingested element (Sanz 1993). The amount of urine Se excretion increased progressively with its intake, but the form excreted in the urine remained unknown for a long time. It was formerly thought to be trimethylselenonium ion (TMSe) (Suzuki 2005) and it represented 2050% of Se excreted from rat urine (Palmer 2002). Later on TMSe was identified in human urine (Francesconi and Pannier 2004). High performance liquid chromatography (HPLC) separations coupled with atomic and molecular mass spectrometric detection have provided new insights into this area.

Se-containing carbohydrates (selenosugars) are now known to be the major urinary metabolites in humans and in rats. The major metabolite is beta-methylseleno-N-acetyl-D-galactosamine (Suzuki 2005). This selenosugar plateaus with a dose higher than 2.0 µg/mL in water or diet, and TMSe tends to increase in high Se intakes, indicating that TMSe can be a biomarker of excessive and toxic doses of Se. TMSe is now considered to be a less significant metabolite (Francesconi K. 2006). In mice the element is metabolized mainly in the liver and kidney (Suzuki, Hashiura et al. 2010) when Se supplementation was administered. In the kidney, the excess amount is excreted mainly as a selenosugar in urine. Some authors have identified another Se metabolite in human urine – Semethylselenoneine (Klein, Ouerdane et al. 2011). It cannot be ruled out that the use of other methods of analysis will discover more selenium compounds in urine.

Studies on this enzyme have shown that although GSH-Px 3 is synthesized in a range of tissues, the renal proximal tubular epithelial cells are the main source from which it is secreted into plasma (Whitin, Tham et al. 1998). Many reports indicate that patients with CKD have very low plasma GSH-Px activity including those undergoing HD (Zachara, Gromadzińska et al. 2006). Reports by several authors have shown that plasma GSH-Px activity in uremic patients is reduced by 3452% as compared with healthy controls (Zachara, Gromadzińska et al. 2006).

2.12 Role trace element in diabetes

The level of trace elements (Zn, Cu, Se & Cr) in the body is minute but they play significant roles in most of the biological systems in the body. They are mostly found in the diet we take. The scope of this work is not to address the dietary aspect but to address the hypothesis.

CHAPTER 3

3.0 Methodology

3.1 Study Design

The study design was a case – control study

3.2 Study Site

The study site was at the National Diabetic and Research Centre at Korle - bu teaching hospital in Accra.

3.3 Sample size

The statistical formula for calculating the sample size is as follows:

$$n = \frac{Z^2 \cdot P(1-P)}{E}$$

Where n = sample size
Z = α = 95% = 1.96 (confidence level)
P = estimated prevalence (0.64)
E = precision (0.05)

$$n = \frac{(1.96)^2 \cdot 0.064(1 - 0.064)}{(0.05)^2}$$

n = 93.

110 samples were originally taken for the study but 80 was recorded because the rest did not observe the 10 – 12 hour fast and some could not provide any urine sample at the time of sampling.

3.4 Criteria

3.4.1 Inclusion Criteria

Individuals diagnosed with type 2 DM reporting to National Diabetic and

Research Centre at the Korle-bu teaching hospital within the ages of 40 – 60 years and do not have any medical conditions symptomatic to the metabolism of trace elements.

3.4.2 Exclusion Criteria

Individuals without diabetes and outside the specified age range for this study as well as control group on medication and or supplements that could affect trace element levels were excluded.

3.5 Control Subjects

The control subjects for the study were non – diabetic healthy individuals, who had no clinical condition and were not on any dietary supplements or medication that will affect the trace element levels. This information was captured in a questionnaire with an approved consent form filled by individuals. They were age matched with cases of the study.

3.6 Analyses

3.6.1 Sample collection

5.0 mL of venous blood samples was aseptically collected from the median antecubital or cephalic veins of the study subjects, after an overnight (12 h) fast was aseptically collected. The blood was then dispensed into labelled plain BD vacutainer®, tubes, and fluoride oxalate coated tubes (to prevent glycolysis) for fasting blood glucose (Becton Dickenson, Plymouth, UK). Samples for blood glucose assay were immediately analysed. After clotting, blood sample in the plain tubes were centrifuged at 3000 g for 3 min and the serum stored at -20°C until ready for analysis

of kidney function test and trace element analysis respectively. Hemolysed samples were eliminated from the test prior to analysis.

Early morning urine sample was collected in a sterile wide mouth container and screw capped for urine protein which was immediately analyzed using the dipstick method.

3.6.2 Determination of Serum Glucose using the JAS Diagnostics procedures

3.6.2.1 Principle of Glucose

$$D-Glucose + H_2O + O_2 \xrightarrow{Glucose\ oxidase} H_2O_2 + D-Gluconate$$

$$H_2O_2 + 4-Aminoantipyridine + Phenol \xrightarrow{Peroxidase} Quinone\ dye + H_2O$$

The intensity of the red color formed is proportional to glucose concentration in the sample. The enzymatic method for glucose determination uses the Glucose oxidase to catalyse the oxidation of glucose to hydrogen peroxide and glucoronic acid. The hydrogen that is formed is measured by the oxidation of a chromogen.

The present procedure is based on the above principle but utilizes a noncorrosive phenol substitute for added safety and convenience. The sample to reagent ratio was 1:100. A serum of 3 µl was added to 300 µl of glucose reagent and incubated for 10 minutes. It was run at a wavelength of 500 nm at 37^{0}C. The reagent is calibrated and the integrity of the reaction monitored by a two level control with a known concentration. The JAS chemistry control comes as a low and high

controls (1 & 2).

3.6.3 Determination of serum creatinine using the classic Jaffe method

3.6.3.1 Principle of Creatinine

$$Creatinine + Sodium\ Picrate \xrightarrow{Alkaline\ medium} Creatinine\ picrate\ complex\ (yellow - orange)$$

Creatinine reacts with picric acid in alkaline medium to form a color complex. The intensity of the color is proportional to the creatinine in the sample.

The Jaffe method (1886), for the determination of creatinine involves a protein free filtrate and a reaction with picric acid in an alkaline solution. Although other methods are used but the classic Jaffe method is mostly preferred. The kinetic procedures of the classic method have become popular because it is fast, simple and devoid of interferences. The sample to reagent ratio was 1:10. A serum of 0.1 ml was added to 1.0 ml of creatinine reagent and incubated for 10 minutes. It was run at a wavelength of 510 nm at 37^0C. The reagent is calibrated and the integrity of the reaction monitored by a two level control with a known concentration. The JAS chemistry control comes as a low and high controls (1 & 2).

3.6.4 Determination of serum blood urea nitrogen

3.6.4.1 Principle of blood urea nitrogen

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

Glutamate dehydrogenase

$$NH_3 + \propto -Ketoglutarate + NADH \longrightarrow L - Glutamate + NAD$$

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The liberated ammonia reacts with αketoglutarate in the presence of NADH to yield glutamate. An equimolar quantity of NADH undergoes oxidation during the reaction resulting in absorbance that is directly proportional to the urea nitrogen concentration in the sample.

Urea can be determined by the direct method condenses with diacetyl to form a chromogen and indirect method where ammonia is measured as a product of Urease action on urea.

Talke and Schubert introduced a totally enzymatic procedure in 1965 utilizing Urease and Glutamate dehydrogenase. The present procedure is based on a modification of the method. The sample to reagent ratio was 1:100. A serum of 0.003 µl was added to 0.300 ml of blood urea nitrogen reagent and incubated for 10 minutes. It was run at a wavelength of 340 nm at 37^0C. The reagent was calibrated and the integrity of the reaction monitored by a two level control with a known concentration. The JAS chemistry control comes as a low and high controls (1 & 2).

3.6.5 Electrolytes estimation (sodium and potassium)

The ion-selective electrode (ISE) or the specific ion electrode (SIE), method was used for the estimation of the electrolyte. It is a sensor which convert the activity of a specific ion in solution into an electrical potential, which can be measured by a pH meter or voltmeter. The voltage is theoretically

dependent on the logarithm of the ionic activity, according to the Nernst equation.

3.6.5.1 Principle of electrolyte estimation

$$KCl \overset{aqueous}{\underset{medium}{\Longleftrightarrow}} K^+ + Cl^-$$

The Nernst equation gives a formula that relates the numerical values of the concentration gradient to the electrical gradient that balances it. If a concentration gradient is established by dissolving potassium chloride (KCl) in half of a divided vessel, full of H_2O. A membrane is placed between the two halves which is permeable to K^+ ions. After a period of relaxation, an equilibrium is reached causing a chemical concentration gradient to cause the ions to move from a region of high concentration to that of a lower concentration and is exactly balanced by an electrical gradient that opposes the movement of charge.

3.7 Neutron Activation Analysis

There are series of methods for the estimation of trace elements, the Flame Atomic Spectrophotometer and others but the International neutron activator analysis (INAA) was used for this work.

3.7.1 The principle of the analysis:-

The International neutron activation analysis (lNAA) is a sensitive, nondestructive method for determining the elemental composition of a sample. NAA is predicated on the fact that stable, naturally occurring isotopes of many chemical elements have the ability to absorb or

"capture" slow-moving thermal neutrons into their atomic nuclei.

3.7.2 Sample preparation:-

The samples were allowed to thaw and mixed thoroughly in their containers to ensure uniformity before weighing. A serum of 500 mg was weighed into new small polyethylene vials. The polyethylene capsule of diameter 1.2 cm and height 2.35 cm were then transferred into a bigger polyethylene capsule of diameter 1.6 cm and height 5.5 cm (Rabbit capsule) i.e. double encapsulation. It was smoothly heat sealed with a soldering rod to prevent spillage. Single Standard Reference materials of concentration 10 (ppm) and 20 (ppm) of the various elements of interest from the National Institute of Standards and Technology and blanks were equally prepared in the same manner as the test samples.

3.7.3 Methodology

Samples and controls were irradiated in the Ghana Research Reactor (GHARR-1) at the Ghana Atomic Energy Commission, operating at 15 KW at a thermal flux of 5×10^{11} n.cm $^{-2}$. s^{1}.

Samples were transferred into irradiation sites via pneumatic transfer system at a pressure of 0.60 Mpa. The irradiation was categorized according to the half-life of the element of interest. For, Se, Cu, Zn and

Cr, samples were irradiated for 5 minutes and counted for 10 minutes. After the irradiation, radioactivity measurement of induced radionuclide was performed by a PC-based γ-ray spectrometry set-up. It consists of an

n-type high performance germanium (HPGe) detector coupled to a computer based multi-channel analyzer (MCA) via electronic modules. The relative efficiency of detector was 40 % and its energy resolution of 1.8 keV at a γ-ray energy of 1332 keV belonging to ^{60} Co. Through appropriate choice of cooling-time, detector's dead time was controlled to be less than 10%. Identification of γ-ray of product radionuclide was identified through the energies and quantitative analysis of the concentration was achieved using the γ-ray spectrum analysis software, ORTEC MEASTRO-32.

Application of the relative comparator method requires Standard Reference Materials (SRM's) or Certified Reference Materials (CRM's) for element(s) of interest. NIST Single Standard Reference materials of concentration 10 ppm and 20 ppm of the various elements of interest from the National Institute of Standards and Technology were used alternatively for calibration and validations where appropriate. The purpose of this alternate calibrations and validations was to ensure that, only high photo peaks (well defined peaks and of good statistics) were used for calibrations in order to obtain good results (calibration factors).

3.8 Estimation of Glomerular Filteration Rate (GFR)

There are many formulae for the estimation of GFR but here the Cockcroft Gault formula was used:- CKD-EPI equation expressed as a single equation:

$GFR = 141 \times \min(S_{cr}/\kappa, 1)^{\alpha} \times \max(S_{cr}/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] × 1.159 [if black] where:

S_{cr} is serum creatinine in mg/dL, κ is 0.7 for

Stage	Description	(GFR)
At increased risk	Risk factors for kidney disease (e.g., diabetes, high blood pressure, family history, older age, ethnic group)	More than 90
1	Kidney damage with normal kidney function	90 or above
2	Kidney damage with mild loss of kidney function	89 to 60
3a	Mild to moderate loss of kidney function	59 to 44
3b	Moderate to severe loss of kidney function	44 to 30
4	Severe loss of kidney function	29 to 15
5	Kidney failure	Less than 15

females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of S_{cr} /κ or 1, and max indicates the maximum of S_{cr} /κ or 1.

These are described below in the table below:-

The GFR number or results tell how the kidney is functioning and as the number reduces with time its gives a fair idea if the kidney is getting worse, that is approaching kidney failure. *Source:- Butt, 2010.*

3.9 Statistical analysis:-

Results were expressed as mean ± S.D. except where otherwise stated. Statistical analysis was performed using SPSS version 20.0 (SPSS Inc.) and Graph Pad prism 5 for Windows. Normal distribution and homogeneity of the variances were tested using Kolmogorov-Smirnov and Levène tests, respectively. Student t-test was used to compare the significance of the difference in the mean values of any two groups and

chi-square analysis was used to compare frequency between the two groups. Linear regression analysis was used to study the association between the parameters. Correlations between parameters were analyzed using the Pearson R test for variables with normal distribution and the Spearman test for variables with non-normal distribution. $P<0.05$ was considered statistically significant.

3.10 Ethical Clearance

This study was approved by the Committee of Human Research, Publications and Ethics of the School of Medical Sciences, Kwame Nkrumah University of Science and Technology.

CHAPTER 4

4.0 Results

4.1 Baseline characteristics of the participant

Table 4.1 presents the baseline characteristics of the diabetic patients (subjects) and non-diabetic patients. A total of 80 diabetics and 48 nondiabetics were involved in this case-control study. The mean age of the diabetics was 51.70 ± 6.23 years with 51.2% being the majority between

the ages of 50-59 years whereas the mean age of the non-diabetics was 49.50 ± 6.17 years with majority between the ages of 40-49 years. Blood pressure and BMI showed no significant differences between the two groups. However, 17.5% and 2.5% of the diabetics had grade 1 and 2 hypertension respectively. A percentage of 30.0 obesity and 8.8% morbid obesity as well. On the other hand, 16.7% and 14.6% of the nondiabetics had grade 1 and 2 hypertension respectively. With 37.5% obesity and 10.4% morbid obesity respectively (Table 4.1).

Table 4.1: Demographics, clinical characteristics and measures of Anthropometry Diabetics of (Patients) and Non-diabetics (Control)

Age (Mean ± SD)	51.70 ± 6.23	0.061

Gender n (%)			0.098
Male	22 (27.5)	20 (41.7)	
Female	58 (72.5)	28 (58.3)	
Age group n (%)			0.170
40-44	15 (18.8)	15 (31.2)	
45-49	13 (16.2)	11 (22.9)	
50-54	17 (21.2)	9 (18.8)	
≥ 55	35 (43.8)	13 (27.1)	
Occupation n (%)			0.194
None	7 (8.8)	1 (2.1)	
Informal	26 (32.5)	21 (43.8)	
Formal	47 (58.8)	26 (54.2)	
Educational status n (%)			**<0.0001**
None	5 (6.2)	0 (0.0)	
Basic	34 (42.5)	5 (10.4)	
Secondary	22 (27.5)	29 (60.4)	
Tertiary	19 (23.8)	14 (29.2)	
Blood pressure (mmHg			
SBP	133.48 ± 22.46	141.52 ± 27.26	0.073
DBP	78.26 ± 11.80	81.96 ± 14.35	0.116
BP Categories			0.076
Normal	21 (26.2)	12 (25.0)	
Pre-hypertension	43 (53.8)	21 (43.8)	
Grade 1	14 (17.5)	8 (16.7)	
Grade 2	2 (2.5)	7 (14.6)	
Family History of Diabetes			**<0.0001**

Yes	25 (31.2)	11 (22.9)	
No	55 (68.8)	37 (77.1)	
BMI n (Kg/m2)	30.40 ± 8.95	30.24 ± 6.18	0.914
BMI n (%)			0.765
Underweight	1 (1.2)	0 (0.0)	
Normal	16 (20.0)	10 (20.8)	
Overweight	32 (40.0)	15 (31.2)	
Obese	24 (30.0)	18 (37.5)	
Morbid Obesity	7 (8.8)	5 (10.4)	

Variable	**Patients**	**Controls**	**P-value (n= 80)**	**(n = 48)**
				49.50 ± 6.617

Values are Mean ±SD, Differences is significant at P<0.05, WC = Waist Circumference, WHR = Waist to Hop ratio, BMI = Body Mass Index

4.2 Levels of Trace elements, renal function and dipstick proteinuria of the diabetic patients and the non-diabetics

The clinical characteristics presented in table 4.2 shows a statistically significant difference in the levels of blood glucose among the diabetics and non-diabetics (p<0.0001). Concentration levels of the trace metal were increased in the diabetics compared to the non-diabetics with Zn (p=0.001) and Cu (p=0.002) showing significant differences whereas Se and Cr were not significantly different. Renal function among the patients with diabetes shows significantly increased urea and potassium with decreased creatinine levels on comparison with the non-diabetic patients (Table 4.2). Significant differences (p<0.0001) was also observed when proteinuria was compared between the two groups with 48.8% and 4.2% of the diabetics and non-diabetics respectively having increased urine protein (Table 4.2).

Table 4.1: Levels of Trace elements, renal function and dipstick proteinuria of the diabetic patients and the non-diabetics

Variable	Patients (n= 80)	Controls (n = 48)	Ref. Ranges	P-value
FBG (mmol/l)	9.65 ± 4.11	5.34 ± 0.89	3.1 – 6.3	**<0.0001**
Trace elements (mg/L)				
Zinc (Zn)*	1.25 ± 0.14	1.17 ± 0.54	0.59-1.22	**0.001**

Copper (Cu)*	1.00 ± 0.12	0.60 ± 0.14	0.60-1.80	**0.002**
	0.016	± 0.017	± 0.005-	
Selenium (Se)*	0.003	0.006	0.10	0.258
Chromium (Cr)*	1.04 ± 0.15	0.59 ± 0.13	0.04-0.48	0.100
Renal function				
Urea (mmol/L)	4.5 ± 1.75	3.8 ± 0.99	2.1 – 7.1	**0.013**
Creatinine (umol/L)	78 ± 26.91	93 ± 34.94	53 – 124	**0.008**
		129	± 135 – 145	
Sodium (Na+) (mmol/L)	129 ± 5.52	15.72		0.901
Potassium (K+)			3.6 – 5.0	
(mmol/L)	4.4 ± 0.51	4.1 ± 0.42		**<0.0001**
Proteinuria				**<0.0001**
Negative	41 (51.2)	46 (95.8)		
Trace	15 (18.8)	2 (4.2)		
1+	10 (12.5)	0(0.0)		
2+	5 (6.2)	0 (0.0)		
3+	9 (11.2)	0 (0.0)		

Values are Mean ±SD, Differences is significant at P<0.05.
****Median test comparison (Mann-whitney median test)***

4.3 Prevalence of Proteinuria, estimated glomerular filtration rate (eGFR) and stages of Chronic Kidney Disease (CKD)

Estimated glomerular filtration rate was reduced in the non-diabetic patients than the diabetics. However, a greater proportion of the diabetics have proteinuria (48.8%). Glomerular filtration rate (GFR) < 60 *mL/min/1.73 m²* was observed in 5.0% of the diabetics and 14.6% of the non-diabetics. Moderately and severely reduced renal function was observed in 3.8% and 1.2% of the patients with diabetes and 12.5% and 2.1% in the non-diabetics respectively (Table 4.3). Chronic kidney disease (CKD) was prevalent in 50.0% of the diabetics and 18.8% of the patients with no diabetes. Furthermore, 30.0% of the diabetics and 4.2% of the

non-diabetics had stage 1 kidney failure whereas 1.2% of the diabetics and 2.1% of the non-diabetics had stage 4 renal failure (Table 4.3).

Table 4.2: Prevalence of Proteinuria, estimated glomerular filtration rate (eGFR) and stages of Chronic Kidney Disease (CKD) among the diabetic patients and the non-diabetics

eGFR, mL/min/1.73 m²	96.68 ± 24.42	40.46	0.548
Proteinuria			**< 0.0001**
Positive	39 (48.8)	2 (4.2)	
Negative	41 (51.2)	46 (95.8)	
eGFR n (%)			0.292

≥ 90	47 (58.8)	26 (54.2)	
60-89	29 (36.2)	15 (31.2)	
30-59	3 (3.8)	6 (12.5)	
15-29	1 (1.2)	1 (2.1)	
CKD n (%)			**0.0002**
Stage 1: eGFR ≥90 + Alb	24 (30.0)	2 (4.2)	
Stage 2: eGFR 60-89 + Alb	12 (15.0)	0 (0.0)	
Stage 3: eGFR 30-59	3 (3.8)	6 (12.5)	
Stage 4: eGFR15-29	1 (1.2)	1 (2.1)	
Total CKD, n (%) (Stages 1-5)	40 (50.0)	9 (18.8)	

Variable	**Patients**	**Controls**	**P-value (n= 80)**	**(n = 48)**
				93.22 ±

Kidney Disease Outcome Quality Initiative (K/DOQI). Clinical practice guidelines for chronic kidney disease: evaluation, classification and stratification. *Am J Kidney Dis* 2002; 39(Suppl): S221–S266

4.4 Clinical characteristics of the diabetic patients in relation to gender

Prevalence of Grade 1 hypertension was higher in the female diabetic patients (78.6%) and Grade 2 was more prevalent in the male diabetics (100%) (Table 4.4). Obesity was observed in 75% of the females and 25% of males, however, morbid obesity was only seen in the females 7(100%). Majority of the patients were on Oral hypoglycemic agents (OHA) and 12.5% males and 75.0% females have been on their medications for more than 15 years (Table 4.4).

Table 4.3: Age distribution, Blood pressure, measure of Anthropometry, and duration on medication among the diabetic patients stratified by gender

Variable	Male (n= 22)	Female (n= 58)	Male/Female Ratio	Pvalue
Age (Mean ± SD)	50.59 ± 6.81	51.74 ± 6.26		0.476
Age group n (%)				0.651
40-44	4 (18.2)	11(19)	26.7/73.3	
45-49	5 (22.7)	8 (13.8)	38.5/61.5	
50-54	3 (13.6)	14 (24.1)	17.6/82.4	
≥ 55	10 (45.5)	25 (43.1)	28.6/71.4	
Blood pressure (mmHg)				
SBP	134.91 ± 21.98	132.93 ± 22.81		0.727
DBP	79.23 ± 11.28	77.90 ± 12.06		0.655
BP Categories				0.128
Normal	5 (22.7)	16 (27.6)	23.8/76.2	
Pre-hypertension	12 (54.6)	31 (53.5)	27.9/72.1	
Grade 1	3 (13.6)	11 (18.9)	21.4/78.6	
Grade 2	2 (9.1)	0 (0.0)	100	
BMI n (Kg/m²)	27.98 ± 6.81	51.74 ± 9.96		0.138
BMI n (%)				0.222
Underweight	1 (4.50)	0 (0.0)	100	
Normal	5 (22.7)	11 (19.0)	31.2/68.8	
Overweight	10 (45.5)	22 (37.9)	31.2/68.8	
Obese	6 (27.3)	18 (31.0)	25.0/75.0	
Morbid Obesity	0 (0.0)	7 (12.1)	100	
DM Medication				0.478
OHA	18 (81.8)	51 (87.9)	26.1/73.9	

Insulin	4 (18.2)	7 (12.1)	36.4/63.6	
Duration on medication (years)	7.69 ± 4.89	9.08 ± 5.83		0.325
Duration on medication n (%)				0.38
<5	8 (36.4)	13 (22.4)	38.1/61.9	
5-9	6 (27.3)	17 (29.4)	26.1/73.9	
10-14	6 (27.3)	14 (24.1)	30/70	
≥ 15	2 (9.0)	14 (24.1)	12.5/87.5	

4.5 Levels of Trace elements, renal function and dipstick proteinuria of the diabetic patients stratified by gender

The clinical characteristics presented in table 4.2 shows no statistically significant difference in the levels of blood glucose among the diabetic males and females. Concentration levels of the trace metal were increased in the diabetic females compared to the male with none showing significant differences whereas Se was lower. Renal function among the males with diabetes shows increased urea, creatinine, sodium and potassium levels on comparison with the females (Table 4.5). No significant difference (P=0.328) was also observed when proteinuria was compared between the two groups.

Table 4.4: Levels of Trace elements, renal function and dipstick proteinuria of the diabetic patients stratified by gender

Variable	Male (n= 22)	Female (n= 58)	P-value

FBG (mmol/l)	8.66 ± 2.66	10.02 ± 4.50	0.187
Trace elements (mg/L)			
Zinc (Zn)*	1.00 ± 0.19	1.35 ± 0.08	0.453
Copper (Cu)*	0.81 ± 0.21	1.07 ± 0.12	0.106
Selenium (Se)*	0.016 ± 0.005	0.017 ± 0.004	0.706
Chromium (Cr)*	1.09 ± 0.26	1.03 ± 0.18	0.802
Renal function			
Urea (mmol/L)	4.6 ± 1.81	4.5 ± 1.75	0.834
Creatinine (mmol/L)	78.9 ± 19.13	78.2 ± 29.47	0.914
Sodium (Na+) (mmol/L)	130 ± 5.94	129 ± 5.36	0.329
Potassium (K+) (mmol/L)	4.5 ± 0.64	4.3 ± 0.45	0.113
Proteinuria			0.328
Negative	8 (19.5)	33 (80.5)	
Trace	7 (46.7)	8 (53.3)	
1+	3 (30.0)	7 (70.0)	
2+	2 (40.0)	3 (60.0)	
3+	2 (22.2)	7 (77.8)	

4.6 Prevalence of Proteinuria, estimated glomerular filtration rate (eGFR) and stages of Chronic Kidney Disease (CKD) among the diabetic patients in relation to gender

Glomerular filtration rate (GFR) < 60 $mL/min/1.73\ m^2$ was observed in 4 (100%) diabetic females. Chronic kidney disease (CKD) was prevalent in 14 (35.0%) males and 26 (65.0%) females with diabetes (Table 4.6). Furthermore, 34.0% males and 66.0% females had stage 1 kidney failure whereas 100% females had stage 4 renal failures (Table 4.3).

Table 4.5: Prevalence of Proteinuria, estimated glomerular filtration rate (eGFR) and stages of Chronic Kidney Disease (CKD) among the diabetic patients in relation to gender

			P-

Variable	Male (n= 58)	Female (n= 22)	value
eGFR, mL/min/1.73 m²	107.8 ±21.70	92.45 ± 24.22	0.011
Proteinuria			0.101
Positive	14 (35.9)	25 (64.1)	
Negative	8 (19.5)	33 (80.5)	
eGFR n (%)			0.362
≥ 90	16 (34.0)	31 (66.0)	
60-89	6 (20.7)	23 (79.3)	
30-59	0 (0.0)	3 (100)	
15-29	0 (0.0)	1 (100)	
CKD n (%)			0.270
Stage 1: eGFR ≥90 + Alb	11 (45.8)	13 (54.2)	
Stage 2: eGFR 60-89 + Alb	3 (25.0)	9 (75.0)	
Stage 3: eGFR 30-59	0 (0.0)	3 (100)	
Stage 4: eGFR15-29	0 (0.0)	1 (100)	
Total CKD, n (%) (Stages 1-4)	14 (35.0)	26 (65.0)	

4.7 Percentage occurrences of complications of diabetes

Across the various age groups, diabetic nephropathy was prevalent in the age group 50-59 years (45.0%) followed by 40-49 years (40.0%) as presented in the figure 4.1 below.

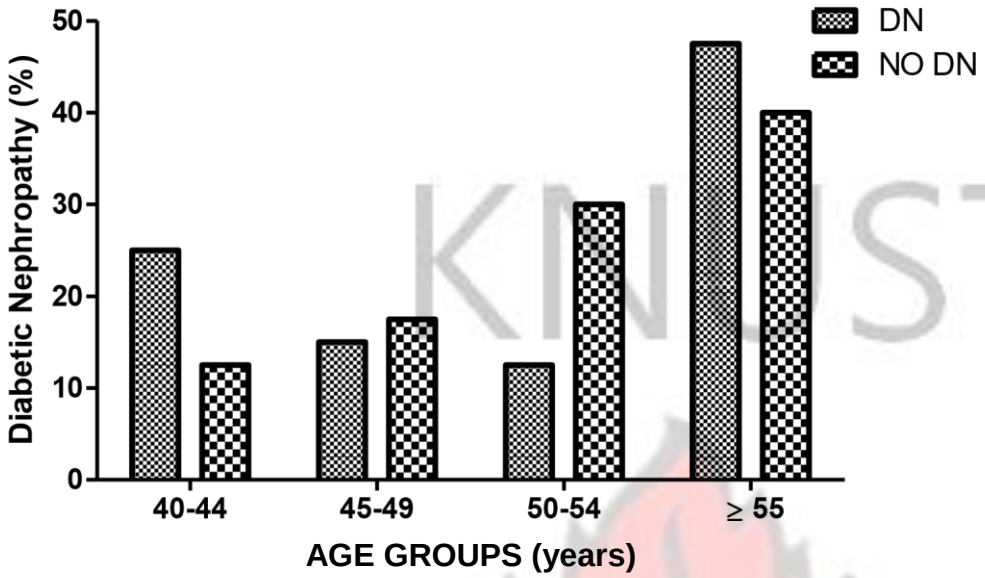


Figure 4.1: Percentage occurrences of complications of diabetes across the various age distributions

4.8 Clinical characteristics of patients with diabetes and those that have developed nephropathy

In the patients with diabetic nephropathy, 17.5% and 2.5% had grade 1 and 2 hypertension respectively as presented in table 4.7. Obesity and morbid obesity was observed in 27.5% and 7.5% of the patients with nephropathy respectively. Among the patients with diabetic nephropathy, 32.5% have been on their medication for less than 5 years and 17.5% for more than 15 years (Table 4.7).

Table 4.6: Clinical characteristics of patients with diabetes and those that have developed nephropathy

Variable	DN STATUS		P-value
	YES (n= 40)	NO (n= 40)	
Blood pressure (mmHg)			
SBP	133.88	± 133.08	±

	20.36	24.65	0.875
DBP	79.00 ± 10.15	77.53 ± 13.33	0.579
BP Categories			0.995
Normal	10 (25.0)	11 (27.5)	
Pre-hypertension	22 (55.0)	21 (52.5)	
Grade 1	7 (17.5)	7 (17.5)	
Grade 2	1 (2.5)	1 (2.5)	
BMI n (Kg/m²)	30.29 ± 11.06	30.51 ± 6.33	0.912
BMI n (%)			0.450
Underweight	0 (0.0)	1 (2.5)	
Normal	11 (27.5)	5 (12.5)	
Overweight	15 (37.5)	17 (42.5)	
Obese	11 (27.5)	13 (32.5)	
Morbid Obesity	3 (7.5)	4 (10.0)	
DM Medication			0.745
OHA	35 (87.5)	34 (85.0)	
Insulin	5 (12.5)	6 (15.0)	
Duration on medication (years)	7.63 ± 5.34	9.76 ± 5.69	0.090
Duration on medication n (%)			0.516
<5	13 (32.5)	8 (20.0)	
5-9	12 (30.0)	11 (27.5)	
10-14	8 (20.0)	12 (30.0)	
≥ 15	7 (17.5)	9 (22.5)	

4.9 Levels of Trace elements and renal function of the diabetic patients and those that have develop nephropathy

Trace element concentrations levels were increased in the diabetics with nephropathy compared to those without nephropathy with none showing

significant differences (P>0.05) whereas Cr was lower. Renal function among the diabetics with nephropathy shows increased levels of urea, creatinine, sodium and potassium on comparison to those without the complication (P> 0.05) (Table 4.5).

Table 4.7: Levels of Trace elements and renal function of the diabetic patients and those that have develop nephropathy

Variable	DN STATUS		P-value
	YES (n= 40)	NO	
FBG (mmol/l)	10.81 ± 4.72 (n= 40)	8.49 ± 3.02	**0.011**
Trace elements (mg/l)			
Zinc (Zn)	1.27 ± 0.22	1.23 ± 0.18	0.502
Copper (Cu)	1.09 ± 0.18	0.91 ± 0.12	0.655
Selenium (Se)	0.018 ± 0.005	0.016 ± 0.005	1.000
Chromium (Cr)	1.03 ± 0.16	1.06 ± 0.26	0.823
Renal function			
Urea (mmol/L)	4.53 ± 1.86	4.49 ± 1.66	0.928
Creatinine (mmol/L)	82.88 ± 35.63	73.97 ± 12.52	0.140
Sodium (Na+) (mmol/L)	129.44 ± 6.69	129.09 ± 4.11	0.779
Potassium (K+) (mmol/L)	4.46 ± 0.58	4.32 ± 0.43	0.250

4.10 Relationship between blood glucose, anthropometry and measures of renal function among diabetic patients

Fasting blood glucose was negatively correlated to age, sodium, potassium levels, SBP and eGFR. On the other hand, the duration a diabetic patient has been on medication was directly correlated with BMI

(r=0.084, P= 0.460), urea (r= 0.193, P=0.086), creatinine (r=0.031, P=0.782) and blood pressure (SBP/DBP) and inversely with sodium, potassium and eGFR (P>0.05).

Table 4.9 Relationship between blood glucose, anthropometry and measures of renal function among diabetic patients (Pearson's correlation)

Parameters		FBG	AGE	DDM	BMI	BUN	Creatinine	Na^+	K^+	SBP	DBP	eGFR
FBG	R	1	-0.005	0.105	0.099	0.148	0.084	-0.099	-0.022	-0.11	0.057	-0.102
	P-value		0.965	0.354	0.383	0.191	0.46	0.382	0.849	0.333	0.615	0.369
AGE	R		1	-0.063	0.161	0.002	0.262*	-0.074	0.005	-0.057	-0.033	-0.292**
	P-value			0.581	0.153	0.983	0.019	0.516	0.965	0.613	0.774	0.008
DDM	R			1	0.084	0.193	0.031	-0.021	-0.043	0.225*	0.195	-0.046
	P-value				0.46	0.086	0.782	0.857	0.702	0.044	0.083	0.688
BMI	R				1	-0.046	-0.165	-0.489**	-0.199	-0.116	0.126	0.079
	P-value					0.684	0.143	0.000	0.077	0.306	0.265	0.488
BUN	R					1	0.456**	0.172	-0.044	-0.047	0.079	-.434**
	P-value						0	0.127	0.698	0.678	0.487	0.000
Creatinine	R						1	0.13	-0.083	0.254*	0.087	-0.859**
	P-value							0.25	0.464	0.023	0.44	0.000
Sodium (Na^+)	R							1	-0.189	0.100	-0.157	-0.097
	P-value								0.094	0.378	0.163	0.39
Potassium(K^+)	R								1	-0.218	-0.024	0.144

	P-value	0.052	0.83	0.203
SBP	R	1	0.559**	-0.135
	P-value		0.000	0.233
DBP	R		1	-0.093
	P-value			0.409
eGFR	R			1
	P-value			

R=Correlation coefficient, ** Correlation is significant at the 0.01 level (2-tailed),* Correlation is significant at the 0.05 level (2 tailed).FBG=Fasting Blood Glucose, DDM= Duration on Diabetic Medication, SBP=Systolic Blood Pressure, DBP=Diastolic Blood Pressure

4.11 Correlation between fasting blood sugar and trace elements The correlation between blood glucose, Zn, Cu and Cr shows inverse relationship with no significance (P> 0.05). However, blood glucose levels positively correlated with Se though not significant (P=0.389). Zn was significantly related to Cu (r= 0.348, P= 0.002) and Cr (r= 0.247, P=0.027) (Table 4.10).

Table 4.10: Correlation between fasting blood sugar and trace elements (Spearman's rho)

Parameters		FBG	Zn	Cu	Se	Cr
FBG	R	1	-0.11	-0.053	0.098	-0.042
	Pvalue	.	0.329	0.642	0.389	0.711
Zn	R		1	0.348**	0.064	0.247*
	Pvalue		.	0.002	0.574	0.027
Cu	R			1	-0.004	0.321**
	Pvalue			.	0.97	0.004
Se	R				1	-0.126
	Pvalue				.	0.265
Cr	R					1
	Pvalue					.

R=Correlation coefficient, ** Correlation is significant at the 0.01 level (2-tailed),* Correlation is significant at the 0.05 level (2 tailed)

4.12 Logistic regression of determinants for developing diabetic

nephropathy

The risk of developing nephropathy was low (OR= 0.46, P= 0.137) being a female with diabetes as presented in Table 4.11. Pre-hypertension, Grade 1 and 2 hypertensions were determined to be high risk factors associated with Diabetic nephropathy (OR>1). Obesity, however was a low risk factor whereas overweight was high risk to developing nephropathy in diabetes (Table 4.11). The risks of developing nephropathy was also associated to the type of medication and how long the patient was on treatment although the risk was determined to be low and not significant (OR> 1, P>0.05).

Table 4.11: Logistic regression of determinants for developing diabetic nephropathy

Variable	OR (95% CI)	P-value
Gender		
Male*	Reference	
Female	0.46 (0.17-1.28)	0.137
BP Categories		
Normal*	Reference	
Pre-hypertension	1.15 (0.41-3.28)	0.790
Grade 1	1.10 (0.28-4.26)	0.890
Grade 2	1.10 (0.06-20.01)	0.949
BMI		
Underweight	-	-
Normal*	Reference	
Overweight	2.49 (0.70-8.83)	0.157
Obese	0.96 (0.33-2.77)	0.938
Morbid obesity	0.85 (0.16-4.43)	0.847
DM Medication		
Insulin*	Reference	

OHA	1.23 (0.34-4.43)	0.746
Duration on medicatio	Reference	
<5*		
5-9	0.67 (0.20-2.23)	0.516
10-14	0.41 (0.12-1.44)	0.164
≥ 15	0.48 (0.13-1.80)	0.275

OR=Odds Ratio, CI= Confidence Interval

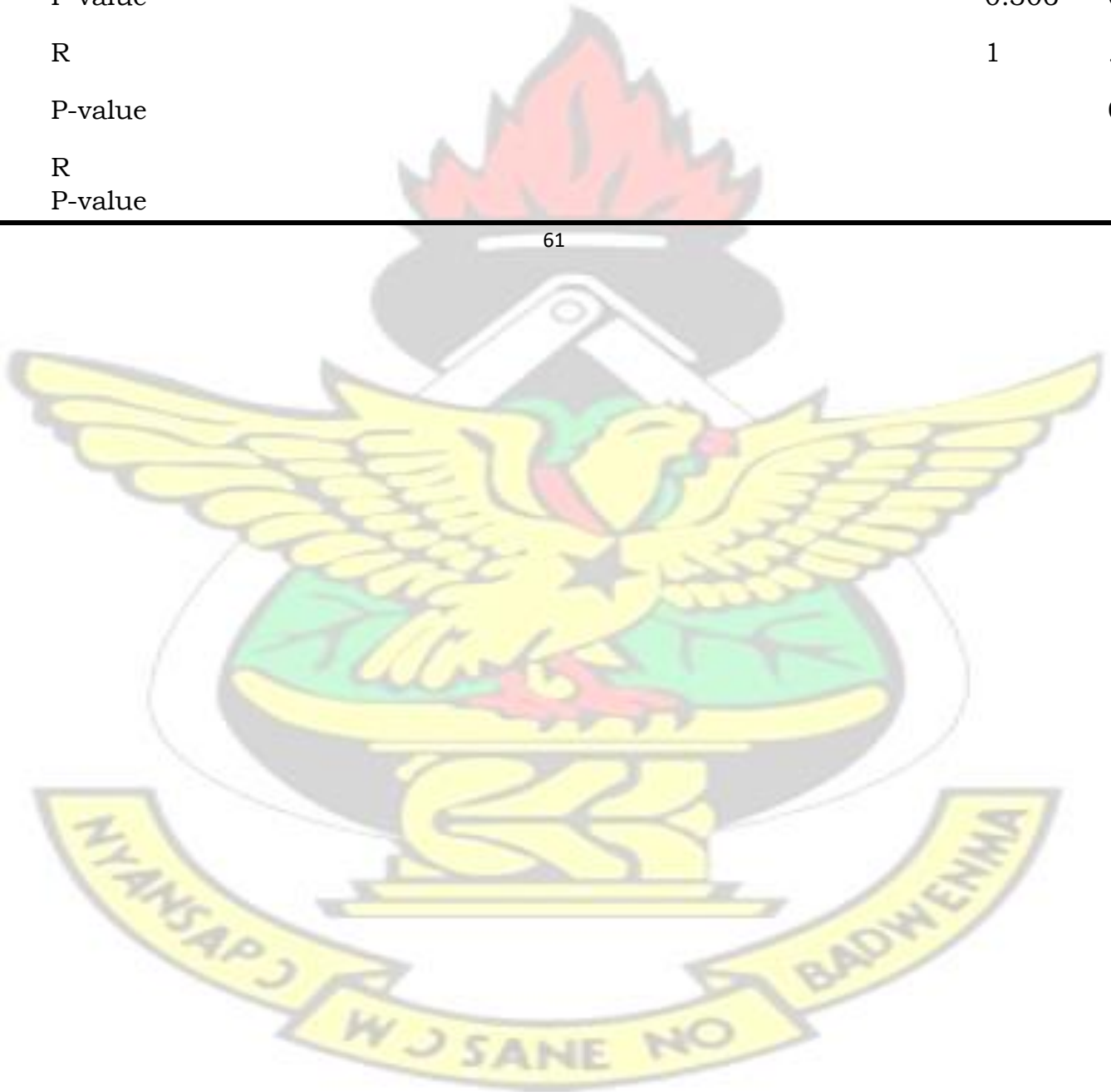
4.13 Relationship between trace elements, blood glucose, anthropometry and blood pressure among diabetic patients (Pearson's correlation)

Presented in 4.12 is the relationship between trace elements, blood glucose, anthropometry and blood pressure among diabetic patients. Levels of Zn, Cu and Cr except for Se negatively correlated with BMI. The trace elements in correlation with SBP and DBP showed negative non-significant r, except for the correlation between Zn and DBP which was significant (r= -0.242, P=0.030).

Table 4.12 Relationship between trace elements, blood glucose, anthropometry and blood pressure among diabetic patients (Pearson's scorrelation)

Parameters		Zn	Cu	Se	Cr	FBG	AGE	DDM	BMI	SBP	DBP
Zn	R	1	.271*	0.078	0.182	-0.058	-0.131	-0.016	-0.039	-0.107	-.242*
	P-value		0.015	0.489	0.107	0.609	0.248	0.888	0.73	0.343	0.03
Cu	R		1	-0.089	.387**	0.025	-0.067	-0.136	-0.058	-0.046	-0.111
	P-value			0.433	0	0.829	0.557	0.228	0.612	0.687	0.329
Se	R			1	-0.074	0.065	.256*	0.197	0.024	-0.057	-0.089
	P-value				0.512	0.566	0.022	0.08	0.834	0.616	0.431
Cr	R				1	-0.056	-0.041	0.018	-0.074	-0.012	-0.043
	P-value					0.624	0.716	0.872	0.511	0.913	0.703
FBG	R					1	-0.019	0.105	0.099	-0.11	0.057
	P-value						0.87	0.354	0.383	0.333	0.615
AGE	R						1	0.025	0.192	-0.09	-0.041
	P-value							0.827	0.087	0.428	0.717
DDM	R							1	0.084	.225*	0.195
	P-value								0.46	0.044	0.083

BMI	R	1	-0.116	0.126
	P-value		0.306	0.265
SBP	R		1	.559**
	P-value			0
DBP	R			1
	P-value			



4.14 Relationship between trace elements, measures of renal function among diabetic patients

Table 4.13 shows the relationship between trace elements, measures of renal function among diabetic patients. Zinc showed non-significant inverse relationship with BUN and Creatinine, however, a direct relationship was observed for sodium, potassium and eGFR. Levels of Cu and Se showed negative correlation with potassium and eGFR but positive for Sodium although the relationship was not significant (P>0.05). Chromium on the other hand was directly correlated with sodium and eGFR and inversely with BUN, creatinine and potassium.

Table 4.83: Relationship between trace elements, measures of renal function among diabetic patients

Parameters		Zn	Cu	Se	Cr	BUN	Creatinine	Na^+	K^+	eGFR
Zn	R	1	.271*	0.078	0.182	-0.002	-0.095	0.112	0.015	0.173
	P-value		0.015	0.489	0.107	0.986	0.403	0.321	0.894	0.126
Cu	R		1	-0.089	.387**	-0.072	0.008	0.161	-0.004	-0.012
	P-value			0.433	0.000	0.527	0.946	0.155	0.97	0.914
Se	R			1	-0.074	-0.006	-0.013	0.005	-0.093	-0.035
	P-value				0.512	0.955	0.908	0.964	0.41	0.756
Cr	R				1	-0.116	-0.114	0.047	-0.106	0.116
	P-value					0.306	0.315	0.679	0.35	0.304
BUN	R					1	.456**	0.172	-0.044	-.434**
	P-value						0	0.127	0.698	0
Creatinine	R						1	0.13	-0.083	-.859**
	P-value							0.25	0.464	0
Sodium (Na^+)	R							1	-0.189	-0.097
	P-value								0.094	0.39

Potassium(K^+)	R		1	0.144
	P-value			0.203
eGFR	R			1
	P-value			

Table 4.94: Concentration ranges (mg/L) of trace elements in serum

Trace element	Literature data*
Zn	0.59-1.22
Cu	0.60-1.80
Cr	0.04-0.48 ng/ml
Se	0.005-0.10

***ranges from references (Caroli, Alimonti et al. 1994, Forrer, Gautschi et al. 2001, Forrer, Gautschi et al. 2001)**

Table 4 105: Results of 20 mmol/L single standard elements used for the validation

Elements	No of Measurements	Prepared concentration	Measured value	Units
Cr	3	20	19.85	mmol/L
Se	3	20	20.23	mmol/L
Zn	3	20	20.10	mmol/L
Cu	3	20	19.57	mmol/L

Chapter 5

5.0 Discussion

5.1 Characteristics of the study participants

Globally, the largest proportion of people with DM type 2 are aged between 40-59 years (Whiting, Guariguata et al. 2011) which was reflected in this study where the mean age of the diabetics was 51.70 ± 6.23 (Table 4.1). The high proportion of females with diabetes (72.5%) in this study may be due to the fact that more women seek medical attention than men (27.5%) and the fact that diabetes is more prevalent in females than males (Crook, Earle et al. 1994, Amoah, Owusu et al. 2000). The largest percentage (43.8%) of diabetic patients in this study was found in the age group, 55-59 years. However, a sizeable percentage (16.2%), was aged 45-49 years. In subSaharan Africa, prevalence of diabetes increases with age, with most reports indicating a peak at either 65 years or older (Ahren and Corrigan 1984, Ducorps, Baleynaud et al. 1996, Fichtlscherer, Rosenberger et al. 2000) or 55–64 years (Mollentze, Moore et al. 1995). There was positive correlation between diabetes and age, indicating that age is a risk factor as far as the disease is concerned in the African population (Elbagir, Eltom et al. 1996, Christensen, Friis et al. 2009).

The high prevalence of females (61.3%) than males (38.7%) with diabetes in the present study is consistent with the observation by Wild, Roglic et al. (2004). The present study also recorded more elderly women than men

(Table 4.4). The combined effect of a greater number of elderly women than men in most populations, and the increasing prevalence of diabetes mellitus with age is the most likely explanation for this observation. This pattern, however, confirms that the prevalence of diabetes mellitus increases with age for both males and females. Furthermore in developing countries, the majority of people with diabetes mellitus are in the 45 – 64 age range (King, Aubert et al. 1998, Hillier and Pedula 2001, Wild, Roglic et al. 2004). In Nigeria, Ekpenyong, Akpan et al. (2012) also found diabetes to be higher among females than males.

5.2 Anthropometric and Blood pressure variables in DM Type 2

In most studies from sub-Saharan Africa, adiposity (encompassing bodymass index, waist and hip circumference, and waist-to-hip ratio and adiposity indices) has generally been associated with diabetes. Data indicate that prevalence of the disorder rises with increasing body-mass index, waist-to-hip ratio, and waist circumference (Cooper, Rotimi et al. 1997, Welborn, Dhaliwal et al. 2003, Motala, Omar et al. 2008). Mean BMI was not significantly different between diabetic patients and non-diabetics, however overweight and obesity was prevalent in the diabetic patients for the present study. These results are similar to other findings in several studies where overweight and obesity were recorded in patients with diabetes (Kaushik 2006, Nguyen, Magno et al. 2008, Oghagbon, Odili et al. 2009). It was revealed that 8.8% of the diabetic patients were morbidly obese, with a BMI

≥ 40 kg/m^2 whilst 30.0% were obese with a BMI ≥ 30 kg/m^2. Thus, 78.8 % were overweight or obese or had a BMI ≥25.0 kg/m^2, which is higher than a value of 75.0% overweight or obese type 2 diabetic patients reported in Mexico (Aguilar-Salinas, Velazquez Monroy et al. 2003). Researchers from South Africa, reported very high rates of obesity (58–65%) in individuals with diabetes compared with people from Tanzania (9·1%) and Sudan (7·7%) (Levitt, Katzenellenbogen et al. 1993, Motala, Omar et al. 2008).

The mean BMI of males (27.98 ± 6.81 kg/m^2) and females (31.74 ± 9.96 kg/m^2) both indicate overweight and obesity respectively. Similarly, the percentage of female diabetics who were obese (80.6%) and overweight (68.8%) were higher than the corresponding values of 19.4% and 31.2% for male diabetics (P = 0.222). This is consistent with earlier report by Akbar (2002) that indicated that obesity was more common in females than males in type 2 diabetic patients. Females have been known to be more prone to abdominal obesity compared with their male counterparts due to their gender makeup and their venerability to developing diseases. On the average, women have more body fat than men and are more likely to be obese in adulthood if they were nutritionally deprived in childhood (Stephen 2007, Ekpenyong, Akpan et al. 2012). This could be attributed to the impact of oestrogen as it reduces their ability to burn energy after eating which results in increase storage of fat in the body (Stephen 2007, Ekpenyong, Akpan et al. 2012). Obesity characterized by excess body fat is probably the most notable risk factor for the development of type 2 diabetes (Edelstein, Knowler et al. 1997,

Wild, Roglic et al. 2004). This, however, could account for the higher prevalence of obesity in the diabetic females in this study. Thus, a higher percentage of Ghanaian female are diabetic (72.5%) than males (27.5%) as a result of obesity being evident. These results corresponds with the findings in several studies where high overweight and obesity prevalence were recorded in female patients with diabetes (Kaushik 2006, Oghagbon, Odili et al. 2009, Mitolo, Dare et al. 2015).

Type 2 diabetes is associated with devastating complications usually classified as microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (coronary vascular disease, stroke, and peripheral vascular disease). Cardiovascular disease (CVD) complications are far more common than microvascular complications and are the leading cause of death and disability in type 2 diabetes (Shantaram 1999). Multiple risk factors are associated with CVD in type 2 diabetic patients, including hypertension, hyperlipidaemia and obesity (Haffner, Mykkänen et al. 2000).

These risk factors are also the main features of the metabolic syndrome. Persons with elevated blood pressure are 2.5 times more likely to develop diabetes mellitus within 5 years (Sowers and Bakris 2000) than persons without either condition. This is because the coexistence of hypertension and diabetes mellitus is particularly pernicious because of strong linkage of the two conditions with cardiovascular disease (Fagan and Sowers 1999, Haffner, Greenberg et al. 2002). This is contrary to the findings in the present study due to the compliance to medication (Table 4.1). Gender difference in

blood pressure was not significant; however, SBP and DBP were increased in the males than the females. On the contrary, several coexisting factors have been attributed to females developing hypertension since they are more prone to obesity, which is a common denominator to developing diabetes and hypertension (Sowers and Bakris 2000).

5.3 Renal function in DM Type 2

Significant increase in urea and decrease in creatinine in diabetics than the non-diabetics was observed in this study. Probably as a result of loss of muscle mass since diabetics are prone to muscle wasting. Increment of blood urea level with the increment of blood sugar level clearly indicates that the increase blood glucose level causes damage to the kidney (Zimmet, Alberti et al. 2001, Shrestha, Gyawali et al. 2008). This collaborates with the findings of Shrestha, Gyawali et al. (2008) that hyperglycemia is one of the major causes of progressive renal damage. Furthermore, Adler, Stevens et al. (2003) in their study submitted that raised plasma creatinine and urea levels in diabetic patients may indicate a pre-renal problem such as volume depletion. Judykay (2007) in his study suggested that high creatinine levels observed in diabetic patients may be due to impaired function of the nephrons.

In addition, the male diabetics showed slightly higher creatinine levels than the females but were not significant. This result is supported by various researchers who showed that gender variation occurs only in serum creatinine level but not in blood glucose level and urea level. High serum

creatinine level was seen in males than females, which could be because of storage of creatinine as a waste product in muscle mass and the presence of high muscle mass in males (Anjaneyulu and Chopra 2004, Ashavaid, Todur et al. 2005, Singh, Khan et al. 2014). Research conducted by Anjaneyulu and Chopra (2004) had found that increase urea and serum creatinine in diabetic indicates progressive renal damage.

Patients with type 2 diabetes have an increased risk for cardiovascular and chronic kidney disease. Superimposed hypertension further increases the risk and is associated with increased dietary sodium intake (Provenzano, Stark et al. 2014). However, decrease in sodium levels in the diabetic patients were observed in this study. This could be attributed to dietary counseling and changes adopted by the diabetic patients.

According to the guidelines of the National Kidney Foundation for the diagnosis and stratification of chronic kidney diseases, renal function is moderately decreased if GFR is <60 mL/min/1.73 m^2 and severely decreased if GFR is <30 mL/min/1.73 m^2 (Levey, Coresh et al. 2003). Renal state of the diabetic patients showed that 5% of the diabetics had GFR <60 mL/min/1.73 m^2. Recent studies have reported poor renal function among patients with diabetes (Dukas, Schacht et al. 2005, Kengne, Amoah et al. 2005). Proteinuria was also prevalent in the diabetics (48.8%) indicating severe renal dysfunction with decreased eGFR. The presence of albuminuria is a risk factor for more advanced nephropathy (Adler, Stevens et al. 2003). In comparison with this study, other studies show that a greater proportion of

patients with diabetes developed microalbuminuria over a shorter time period (Gall, Hougaard et al. 1997, Forsblom, Groop et al. 1998).

Approximately 30% of patients with diabetic nephropathy eventually progress to end-stage renal failure and the rest usually die from cardiovascular disease before reaching end stage. All develop microalbuminuria and, subsequently, proteinuria. Therefore, albuminuria is an important risk factor in these patients, and all diabetic patients should have a microalbuminuria assessment yearly (Atkins 2005). A prevalence of 50.0% diabetic nephropathy was recorded in this study with 3.8% stage 3 and 1.2% stage 4. It is estimated that the rate of DN prevalence is 4-8% of patients monitored in diabetic centers. In addition, a significant portion of diabetics, especially the type 2 diabetic patients, are affected by the non-diabetic type nephropathy of primarily atherosclerotic etiology (Krolewski, Niewczas et al. 2014).

5.4 Variations in the levels of trace elements in DM Type 2

Interest in the biochemical and clinical consequence of trace element metabolism has been steadily increasing. Trace elements have important physiological effects when present at concentrations other than those associated with classical toxicity or with extreme deficiency. There is accumulating evidence that the metabolism of several trace elements is altered in diabetes mellitus (Fujimoto 1987, Walter, Uriu-Hare et al. 1991). The trace elements estimated in this study includes; Zinc (Zn), Copper (Cu),

Selenium (Se) and Chromium (Cr).

5.4.1 Zinc

Zinc has been found to have insulin-like effects, in that it causes or enhances glucose up-take. It has been proposed that zinc enhances glucose up-take by inhibiting glycogen synthetase (Ilouz, Kaidanovich et al. 2002). This inhibition blocks the conversion of glucose to glycogen. Furthermore, it has the ability to regulate insulin receptor intracellular events that determine glucose tolerance and the ability to support a normal pancreatic reaction to glucose load (Chris 2005). Some past studies have reported low zinc level in diabetics (Nakamura, Higashi et al. 1991). Some of the reasons for this observation are, excessive zinc loss in urine in diabetes (Pidduck, Wren et al. 1970) and low zinc absorption rate (Selinus 2002). Nevertheless, the present study showed significantly increase in zinc levels in the diabetics than the non-diabetics. Consistent with this report is that of (Zargar, Bashir et al. 2002) and Rusu, Marutoiu et al. (2005) where same or higher level of zinc in type 2 diabetic subjects was observed than the controls. In addition, reports by Diwan, Pradhan et al. (2006) indicated comparable serum levels of zinc in the type 2 diabetic patients and normal subjects have been reported elsewhere. This however suggests that the relationship between diabetes, insulin and zinc is complex, with no clear cause and effect relationships.

5.4.2 Copper

Cooper, Chan et al. (2005) showed that copper metabolism in diabetics is abnormal. Many studies have reported higher copper level in diabetics, especially in those with complications (Walter *et al.,* 1991). These conforms to the findings of significant increase in Cu among the diabetic patients in this study. In contrary to this however, Akinleye (2007) reported no significant difference between blood copper levels in diabetics and the controls, and also the findings of Smith, Heise et al. (1988) and Ito, Fujita et al. (2001). Sjögren, Florén et al. (1986) found that despite elevated plasma copper concentrations, type 2 diabetic subjects had lower concentrations of copper in muscle biopsies compared with control subjects, indicating possible copper depletion in muscle. They therefore stated that whether abnormal copper status in the face of elevated plasma copper occurs in other tissues such as the eye and blood vessels remains to be investigated (Sjögren, Florén et al. 1986).

5.4.3 Selenium

Contrary to previous observation of an increased risk of diabetes with high selenium concentrations in diabetic patients, a study conducted by Bleys *et al.* in 1994 and reported in 2007 (Bleys, Navas-Acien et al. 2007), the mean plasma Se content in the diabetics present in that study was comparably lower than in controls. The lower Se level observed in this study also agreed with the work of Akinloye, Ogunleye et al. (2013) which showed lower Se

concentration in diabetic patients than in healthy control subjects. Se acts as an antioxidant and peroxynitrite scavenger when incorporated into selenoprotein. The low concentration of Se and other relevant antioxidants in serum could potentially expose the subject to oxidative stress which is known to be associated with the pathogenesis of diabetes mellitus. Also, low Se level has been shown to reduce insulin secretion and increase insulin resistance, thereby possibly playing a role in the pathogenesis of type 2 diabetes.(Failla and Gardell 1985, Akinloye, Ogunleye et al. 2013).

5.4.4 Chromium

Chromium is required for normal carbohydrate metabolism and as a critical cofactor for insulin action (Tuman and Doisy 1977, Kimura 1996). Chromium is involved in increasing the number of insulin receptors present in a target organ and also increases the binding of insulin to its receptors (Anderson, Åberg et al. 2002). The observation in the present study conforms with the findings of higher chromium levels observed in the diabetics by Akinleye (2007). However, Cooper, Phillips et al. (2005) found that diabetes did not alter chromium balance (difference between elemental intake and output), and urinary or fecal excretion rates. The findings in this study are also contrary to work done by Anderson, Cheng et al. (1997) which showed Cr deficiency in type 2 diabetic subjects indicating that reduction in levels of Cr in type 2 diabetes might be due to high level of glucose since hyperglycaemia

have been suggested to increase the excretion of Cr (Anderson, Cheng et al. 1997).

5.5 Variation in trace elements among patients with Diabetic nephropathy

Zinc has been found to enhance the effectiveness of insulin *in–vitro* and hence, a zinc deficiency may aggravate the insulin resistance in type 2 diabetes. This may cause complications (Zargar, Shah et al. 1998, Hashemipour, Kelishadi et al. 2009). In the present study, patients with diabetic nephropathy had higher serum concentrations of Zn than the diabetics without nephropathy. A clear relationship between the kidney and Selenium metabolism has been documented (Kohrle, Jakob et al. 2005). Plasma Se levels have been found to be reduced in critically ill states and are inversely correlated with the severity and outcome of the disease (Gärtner 2009). This finding has also been reported in patients with acute kidney injury (AKI) (Wiesen, Van Overmeire et al. 2011). Among the possible mechanisms involved in the impaired Se status in CKD patients are increased Se requirements, low dietary intake, impaired intestinal absorption, reduced Se binding proteins and elevated urinary and dialysis losses (Zachara, Gromadzińska et al. 2006). Contrary to previous studies, Se level was found to be slightly increased in the diabetics with nephropathy than those without the nephropathy. This could result from other factors such as dyslipidemia and hypertension among the diabetics with

nephropathy since a positive associations of selenium concentrations with lipid levels (Bleys, Navas-Acien et al. 2008) and with hypertension (Laclaustra, Navas-Acien et al. 2009) have been documented. This increasing trend in serum selenium levels highlights the importance of understanding the association between serum selenium levels and diabetes.

These trace element deficiencies appear to be an additional risk factor in the development and progress of disease and they contribute to the pathogenesis of diabetes mellitus and its complications. Their repletion may be an effective therapeutic intervention in prevention of the progression of the diabetes and its complications, along with a glycemic control and control of other risk factors (Lindeman, Adler et al. 1967, Kimura 1996).

5.6 Relationship between trace elements, plasma glucose and Anthropometry

In accordance with some findings in literature, zinc, chromium and copper showed no significant relationships with plasma glucose (Ruiz, Alegria et al. 1998, Ugwuja, Nwibo et al. 2014). Ghosh, Bhattacharya et al. (2002) found that plasma chromium was lower by 33% and urine chromium by 100% higher in diabetics and that plasma chromium were inversely correlated with plasma glucose during the onset of type 2 diabetes. However, serum Se levels were significantly correlated with serum glucose and consistent with the findings of Steinbrenner, Speckmann et al. (2011)

Furthermore, zinc, copper and selenium were positively correlated. However zinc and copper were inversely correlated with age and blood pressure variables. Zn status, particularly circulating level, is known to be altered in conditions such as obesity and type 2 diabetes (Costarelli, Muti et al. 2010). Obese people experience chronic inflammation resembling that found in infections (Klevay and Obes 2010). Se functions as a part of proteins known as selenoproteins. Through these selenoproteins, Se may play roles as a defensive mechanism for oxidative stress (Boosalis 2008). However, it is known that the therapeutic window of Se is narrow, and adverse health effects may occur due to supranutritional Se intake even below the levels required for intoxication (Steinbrenner, Speckmann et al. 2011). Diets rich in Se may stimulate the release of glucagon, promoting hyperglycaemia, or may induce overexpression of glutathione peroxidase-1 and other antioxidant selenoproteins resulting in insulin resistance and obesity (Steinbrenner, Speckmann et al. 2011). These could however be attributed to the direct relationship of Se with plasma glucose and BMI in this study. Chromium levels were not directly related with BMI in the diabetics which agrees with the finding of Yerlikaya, Toker et al. (2013). It has been demonstrated that chromium supplementation reduces body weight, regulates hunger, and also decreases body fat (Mertz 1969, Hasten, Rome et al. 1992). Such difference may be due to race, lifestyle, geographical influence and even analytical methods. Findings from this study showed an association between serum Cr levels and diabetes. Dietary deficiency of Cr is believed to be positively

associated with the risk of diabetes and its complications (Sreejayan, Dong et al. 2008).

5.7 Risk factors associated with Diabetic nephropathy

In the light of the present study, hypertension and overweight were predictive of developing nephropathy in diabetes. This is evident in several studies were high blood pressure and obesity have been linked to chronic kidney disease in diabetics (Gall, Hougaard et al. 1997, Adler, Stevens et al. 2003). The common causes of diabetic complications are poor control of diabetes either due to non-adherence, poor attitude towards the disease and its complications, unhealthy diet, and insufficient physical activity, and due to poor management by the health care professionals (Fitzgerald, Anderson et al. 1995, Ajayi and Ajayi 2009, Sharma, Grover et al. 2011). In addition, the coexistence of hypertension and diabetes mellitus is particularly pernicious because of strong linkage of the two conditions with cardiovascular disease (Fagan and Sowers 1999, Haffner, Greenberg et al. 2002). Obesity characterized by excess body fat is probably the most notable risk factor for the development of type 2 diabetes and probably complications such as nephropathy (Edelstein, Knowler et al. 1997, Wild, Roglic et al. 2004). On the other hand, gender and duration of medicine intake by patients showed lesser odds of nephropathy. However patients placed on Oral Hypoglycemic Agents treatments were at higher risk of developing diabetic nephropathy (OR=1.23, P=0.746).

CHAPTER 6

6.0 CONCLUSION

A prevalence of 50% chronic kidney disease (CKD) was observed among the diabetics and 18.8% among the patients with no diabetes. Furthermore, 30.0% of the diabetics and 4.2% of the non-diabetics had stage 1 kidney failure whereas 1.2% of the diabetics and 2.1% of the non-diabetics had stage 4 renal failures. In the light of this present study, hypertension and overweight were determined to increase the odds ratio of developing nephropathy in diabetes. These findings are evident in the rise in diabetic nephropathy.

The study revealed that trace elements have an interactive connection with type 2 diabetes mellitus. The high blood levels of the trace elements in the diabetics as estimated in this study may also be related to the kidney damage complication associated with the disease. The concentration levels of Zn, Cu and Se were found to be increased in diabetics with nephropathy compared to those without nephropathy.

6.1 Limitations

1. In order to better understand the role of trace elements in diabetes, further clinical studies are required to enroll larger number of patients and the use of more sophisticated techniques like the Flame Atomic Spectrophotometry and the International Neutron Activator methods for clinical analysis.

2. To give a more detailed information on trace elements and its relation to DN, microalbuminuria would have been a better marker than urine protein which was used in this study.
3. A dietary information from participants in this study would have given a deeper concept on their trace element status as far this study is concerned.

6.2 Recommendation

1. From the findings in this study, if Zn, Cu, Se and Cr levels can be added to the routine test done for DM type 2 individuals it would enable the clinician to recommend a supplementation to help reduce or prevent some of the complications associated with the disease.
2. Based on these findings, it is therefore recommended that healthcare providers would consider testing people living with diabetes for complications associated with Zn, Cu, Se and Cr as part of the treatment regime.
3. Also, in kidney damage complications, a dietary regime could be set in place to reduce the high level of trace elements as observed in this study.

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