EVALUATION OF NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN (NGAL) IN TYPE 2 DIABETIC PATIENTS WITH DIABETIC NEPHROPATHY

BY

AISHA ABUBAKAR JIBRIL
SPS/16/MML/00046

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DECLARATION

I, Aisha Abubakar Jibril hereby declare that this dissertation work was carried out by me, in partial fulfilment of the requirement for the award of Master of Science degree in Medical Laboratory Science.

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Aisha Abubakar Jibril Date

CERTIFICATION

This is to certify that this dissertation was carried out by Aisha Abubakar Jibril (SPS/16/MML/00046) of the Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Collage of Health Sciences, Bayero University Kano, under my supervision.

29/7/19

Mohammad Ahmad Bello — Date

(Supervisor)

29/07/2019

Saleh Idris Tudun Wada — Date

(Supervisor)

29/07/19

Dr. Isah Abubakar Aliyu — Date

Head of Department/Chief Examiner,

APPROVAL

DEDICATION

This work is dedicated to my husband Sulaiman Muhammad Sadisu and my mother Kaltume A D Abubakar who gave me all the encouragement to see me attained greater heights in the field of my studies and also to my late father Alhaji Abubakar Jibril who inspired and shaped me in the field of education.

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ABBREVIATIONS AND SYMBOLS USED

BMI = Body Mass Index

DM= Diabetes Mellitus

DN=Diabetes Nephropathy

ECM= Extracellular Matrix

EGFR= estimated Glomerular Filtration rate

ESRD= End Stage Renal Disease

ESRF=End stage renal Failure

FPG=Fasting Plasma Glucose

HB= Haemoglobin

HbA1C= glycated haemoglobin

NGAL= Neutrophil Gelatinase Associated Lipocalin

SA= Serum Albumin

SC= Serum creatinine

Kg= kilogram

Kg/m^2=kilogram per meter square

mmol= millimole

ng/ml=nanogram per mill

µmol=micromole

Yr= Year

ABSTRACT

Diabetic nephropathy (DN) is a devastating chronic microvascular complication that represents the major cause of end-stage renal failure leading to the development and progression of diabetic syndrome. The aim of this study was to evaluate serum neutrophil gelatinase associated lipocalin (NGAL) in type 2 DM with diabetic nephropathy. Eighty (80) type 2 diabetic patients with DN and apparently healthy controls were respectively recruited. Blood samples were collected and tested for serum NGAL, creatinine, albumin, fasting plasma glucose and HbA1c. Creatinine and albumin were analyzed using Abbot autoanalyser, HbA1c was analyzed using fine care system and serum NGAL using the ELISA method. Estimated GFR (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula. Statistical analysis was performed using statistical package for social science (SPSS) software version 20.0. Student t-test, one way analysis of variance (ANOVA) and Pearson's correlation were used for comparisons and correlation of data respectively with level of significance set <0.05. The mean values of the serum NGAL, FPG, HbA1c, BMI and eGFR in both DN group and control group were found to be 3.72±2.62 vs 1.08±0.78µg/ml, 7.06 ±3.46 vs 4.08± 0.39mmo/l, 6.73±1.08 vs 4.71 ±0.39%, 27.33±5.29 vs 25.08±3.65ml/min/1.73m^2 and 76.57 ±11.20 vs 118.23 ±12.11ml/min/1.73m^2 respectively. The study found a high and significant difference in the mean values of the DN group compared to the control group. A positive and significant relationship was observed between serum NGAL and eGFR and duration of diagnosis of diabetes mellitus. Hence, serum NGAL could therefore be used as a biomarker to diagnose DN even earlier to incipient nephropathy.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The prevalence of non-communicable diseases (NCD's) is on the increase globally with the low-middle-income countries (LMIC's) being disproportionate afflicted with the burden of this increase. Diabetes mellitus (DM) is a major cause of concern because of it increasing prevalence and related complications including microvascular as well as macrovascular (Siddiqi *et al*., 2017). The prevalence of diabetes mellitus is reported to be about 3.5% (1.33 million). In terms of mortality, about 38 million deaths have been attributed to diabetes mellitus in 2012 (WHO, 2016). In Nigeria, one in every five adults between the ages of 30 -70 years die prematurely due to non-communicable disease with diabetes mellitus accounting for 2% of the deaths (WHO, 2016). One of the major complications of diabetes mellitus is diabetic nephropathy (DN). It is a devastating chronic microvascular complication that represents the major cause of end stage renal disease (ESRD). The mechanisms leading to the development and progression of DN are mainly poor metabolic and hemodynamic control (Gnudi, 2015). Development of diabetic nephropathy increases morbidity and mortality; and health care burden well before the development of end-stage renal disease (Lalibarte *et al*., 2009).

Glomerular filtration rate (GFR) is considered the best measure of kidney function and measures the rate at which the kidneys two million nephrons filter the plasma to remove waste products from the circulation. Injury to the kidneys such as that occurring in acute and chronic kidney disease gradually declines the remaining functional ability of the kidney which can be estimated

by measuring or by estimating glomerular filtration rate (e-GFR). In a normal-sized person the normal value of GFR is about 100 - 150 mL/min. Other methods used for the determination of GFR include serum creatinine, and creatinine clearance (Pradeep, 2010).

However, formula-derived e-GFR has become widely used in clinical practice. The National Service Framework for Renal Services in the UK recommends the adoption of formula-derived e-GFR in the annual evaluation of all patients with diabetes mellitus (Ahmad *et al.*, 2006). The adoption of such protocol is anticipated to aid in the early identification and therefore improve long term outcomes for those patients with kidney impairment including those with diabetic nephropathy. In addition, the American Diabetes Association also recommends the estimation of glomerular filtration rate by e-GFR (mL/min/1.73 m^2) based on the formula proposed by the Cockcroft-Gault (Cockcroft and Gault, 1976) and corrected for body surface area (BSA) and the modification of diet in renal disease (MDRD) formula in patients with diabetes mellitus (Levey, 1999).

In addition, unlike microalbuminuria, GFR increases during the early stages of DM due to hyperglycemia and decreases during later stages of DM reflecting a decline in renal function hence changes in GFR appear much earlier than microalbuminuria in diabetic patients. Previous studies have established the fact that GFR is but one variable of many that predisposes individuals to the likelihood of developing diabetic renal disease along with other complications of DM (Pei *et al.*, 2012).

Although microalbuminuria is accepted as the earliest marker of diabetic nephropathy, large proportion of renal impairment however, occurs in non-albuminuric state (Siddiqi *et al.*, 2017). Therefore, the diagnostic value of microalbuminuria in diabetic nephropathy has been questioned

by a number of researchers worldwide who proposed that other markers are needed for the earlier identification of diabetic renal disease so that measures can be taken to prevent the progression or retard the disease process (Papadopoulou-marketou *et al*., 2017). One of such markers is neutrophil gelatinase associated lipocalin (NGAL).

NGAL also known as human lipocalin-2, siderocalin, oncogene 24p3, or LCN2, is a 25 kDa protein composed of 178 amino acids that belongs to the super family of the lipocalin. The lipocalins are generally proteins that are specialized in binding and transporting small hydrophobic molecules). The family generally shares common molecular organizations that are composed of eight β-strands arranged in a complex β-barrel structure delineating a calyx shape which represents their binding site. NGAL binds to some ligands including the siderophores and interaction with these iron-binding siderophores gave NGAL its characteristic bright red color and also modulates most of its biological activities (Flower *et al.*, 1993; Flower *et al.*, 2009; Devarajan *et al.,* 2010). NGAL is normally expressed in a variety of adult human tissues such as the bone marrow, the uterus, the prostate, the salivary gland, the stomach, the colon, the trachea, the lung, the liver and the kidney (Cowland *et al.*, 1997). NGAL is also highly expressed in the tubular epithelium of the distal nephrons of the kidney and is released from tubular epithelial cells following damage such as that happens in acute kidney injury. NGAL protein exists in three distinct molecular forms i.e. the 25 kDa monomer, the 45 kDa homodimer generated by dimirization of the two identical NGAL monomers and the larger 135 kDa heterodimer generated by association of the monomer with the 92 kDa MM-9, also called gelatinase B (Goetz *et al.,* 2002; Cai *et al.*, 2010).

NGAL is a biomarker of renal tubular injury that is upregulated in the distal tubules and collecting duct. It has extensively been evaluated for early detection of kidney damage

(Bachorzewska-Gajewska *et al.*, 2007). Because of its small molecular size and resistance to degradation, NGAL is readily excreted and detected in the urine both in its free form and in complex with matrix metallopeptidase 9 (MMP-9). Urinary levels correlate with plasma or serum levels regardless of the cause of the increased NGAL production but particularly high urinary levels can be expected when it is released directly into the urine by the kidney tubules or urothelial carcinomas. NGAL appears to be upregulated in cells under "stress", such as from infection, inflammation, ischemia or neoplastic transformation or in tissues undergoing involution. It may have an antibacterial role as shown by its binding of enterobactin and other siderophores depriving the microorganisms of iron (III), an important microbial nutritional requirement (Wagener *et al*., 2006).

1.2 STATEMENT OF THE RESEARCH PROBLEM

Diabetic nephropathy is one of the most common complications of diabetes mellitus. The prevalence of the condition has been on a steady rise since 1998. Diabetic nephropathy develops in approximately 40% of type 2 diabetic patients and nearly 20% of them will likely progress to end stage renal disease (ESRD). As a result, diabetic nephropathy will seemingly continue to account for a large proportion of all cases of chronic kidney disease (CKD) giving this rapid increase in the prevalence of DN. In addition, it will still remained by far the most common underlying cause of hemodialysis treatment in patients with other causes of kidney diseases, a modality that is cost intensive and a burden to the healthcare delivery system hence representing a compelling medico-social issue of interest.

1.3 JUSTIFICATION OF THE STUDY

Neutrophil gelatinase associated lipocalin is increasingly being use for the routine investigations and management of patients with kidney diseases in the developed countries. In the developing countries, there are little if any, studies reported on NGAL amongst patients with kidney diseases despite the fact that NGAL has been reported to be a novel biomarker of early kidney injury. Early detection of kidney diseases especially DN can greatly improve patient's outcome by preventing progression to ESRD especially amongst the population of type 2 diabetic patients

1.4 AIM OF THE STUDY

The aim of the study was to evaluate serum NGAL in type 2 diabetic patients with diabetic nephropathy attending Aminu Kano Teaching hospital, Kano.

1.5 OBJECTIVES OF THE STUDY

The objectives of the study were as follows;

1. To measure anthropometric variables of type 2 diabetic patients with DN and apparently healthy individuals (controls).
2. To measure levels of serum NGAL, FPG, serum albumin, serum creatinine and glycated haemoglobin (HbA1c) in type 2 diabetic patients with DN and apparently healthy individuals.
3. To determine the e-GFR of type 2 diabetic patients with DN and apparently healthy individuals.
4. To determine the relationship if any between NGAL and HbA1c in type 2 diabetic patients with DN.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a condition that is characterized by chronic and impaired carbohydrates, lipid and protein metabolism caused by the total deficiency or insufficiency in the secretion of insulin or action of insulin (Jorge *et al*., 2005). Diabetes mellitus is one of the chronic non-communicable disease (CNCD's) that is associated with long term complications affecting the brain, kidney, and the heart. The condition may be as a result of the destruction of the β-cells of the pancreas causing insulin deficiency. It may also result be as a result of insulin resistance. The classical feature of the disease is hyperglycemia accompanied by polydipsia, polyphagia and polyuria. The condition is accompanied by weight loss, generalized pruritus, neuropathy, retinopathy and nephropathy. Life threatening consequences of uncontrolled diabetes include diabetes-ketoacidosis, lactic acidosis and hyperosmolar non-ketotic state (Jorge *et al*., 2005).

The onset of diabetes mellitus may be preceded by impaired fasting glucose (IPG) resulting also called the pre-diabetic state and may remain asymptomatic for many years leading to irreversible damage to vital organs and putting such individuals at high risk of developing diabetes and its complications (Nathan *et al.,* 2007; Carlos *et al*., 2012). Pre-diabetes is diagnose based on a fasting plasma glucose level of between 6.1 mmol/L – 6.9 mmol/L while a fasting glucose level of 7.0 mmol/L or greater is a confirmation of the diagnosis of diabetes mellitus (ADA, 2012). Both venous and capillary blood samples are regarded as suitable for the diagnosis of diabetes mellitus (ADA, 2019). Globally report has shown that the prevalence of both pre-diabetes and

diabetes mellitus are on the increase so also the burden associated with the two conditions (IDA,2006). International Diabetes Federation (IDF) has also indicated that by the end of 2013 there were more than 382 million (8.3% of adult world population) people diagnosed with diabetes most of whom (about 80%) are living in the low-and-middle-income countries. By 2035, it is projected that the number of people with diabetes will reach 592 million With regards to the sub-Saharan Africa, it was estimated that about 20 million people living in these countries are diabetic with about 62% being undiagnosed and the number is expected to reach about 41.4 million by 2035.

Within the sub-Saharan Africa, Nigeria has the highest number of people with diagnosed diabetes mellitus with an estimated 3.9 million people (or an extrapolated prevalence of 4.99%) of the adult population aged 20 – 79 years (WHO, 2013). On the other hand, Akinkugbe *et al* (1997), reported that the prevalence of diabetes in Nigeria was 2.2% with a male: female ratio of 1:1:1 and a significant increase in the prevalence with age. It is however, noteworthy that the prevalence of diabetes mellitus varies within the country itself for example prevalence has been reported from 0.65% in rural Mangu village, Plateau State to 11.0% in urban Lagos (Akinkugbe *et al.,* 1997). With the projected increase in the prevalence of diabetes mellitus in Africa, it is expected that there will be a reciprocal increase in the burden of diabetes-related complications (Wild 2004). This will undoubtedly pose serious health and economic problem to the countries in the region. This is more so as the disease mostly affects people under the age of 64 years in these countries which are the most productive group in these countries as compared with the developed countries where it affects mostly people above the age of 64 years (Wild, 2004).

2.1.1 Classification of Diabetes Mellitus

The classification of diabetes mellitus is aimed at the consolidation of the etiological views concerning DM. The previous terms of insulin-dependent (IDDM) or non-insulin-dependent (NIDDM) which were proposed by WHO in1980 and 1985 have disappeared and the new terms of new classification system identifies four types of diabetes mellitus: type 1, type 2, "other specific types" and gestational diabetes (WHO, 1999).

2.1.1.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus also referred to the juvenile diabetes is characterized by beta cells destruction caused by an autoimmune process usually leading to absolute insulin deficiency (Kumar and Clark, 2002). The disease is usually characterized by the presence of anti–glutamic acid decarboxylase, islet cell or insulin antibodies which identify the autoimmune processes that lead to beta cell destruction. Eventually, all type 1 diabetic patients will require insulin therapy to maintain normoglycemia (Baynes, 2015).

2.1.1.2 Type 2 Diabetes Mellitus

This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise (Baynes, 2015). It is more common in women, especially those with a history of gestational diabetes, and in blacks, Hispanics and Native Americans. The relative importance of defects in insulin secretion or in the peripheral action of the hormone in the occurrence of type 2 diabetes is the subject of controversy. Type 2 DM comprises of 80% - 90% of all cases of DM (ADA, 2015). Most individuals with Type 2 diabetes exhibit intra-abdominal (visceral) obesity, which is closely related to the presence of

insulin resistance. In addition, hypertension and dyslipidemia (high triglyceride and low HDL-cholesterol levels; postprandial hyperlipidemia) often are associated with the condition.

2.1.1.3 Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is an operational classification (rather than a pathophysiologic condition) identifying women who develop diabetes mellitus during gestation. Women who develop type 1 diabetes mellitus during pregnancy and women with undiagnosed asymptomatic type 2 diabetes mellitus that is discovered during pregnancy are classified with GDM. In most women who develop GDM, the disorder has its onset in the third trimester of pregnancy (WHO, 2006).

2.1.1.4 Other Specific Types (monogenic diabetes)

Types of diabetes mellitus of various known etiologies are grouped together to form the classification called “other specific types”. This group includes persons with genetic defects of beta-cell function (formerly called maturity-onset diabetes of the young (MODY)) or with defects of insulin action, persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis, persons with dysfunction associated with other endocrinopathies (example acromegaly) and persons with pancreatic dysfunction caused by drugs, chemicals or infections and these group of conditions comprise less than 10% of DM cases (Silvio, 2012).

2.1.2 Pathophysiology of Diabetes Mellitus

A direct link between hyperglycemia, physiological and behavioral responses have been reported. Presence of hyperglycemia triggers the brain to elicit message through the nerve to the

impulses and to the pancreas and other organs thereby minimizing its effect (Patidar, 2011). The various classes of DM are identified based on different pathology.

2.1.2.1 Type 1 Diabetes Mellitus

Type 1 DM is characterized by autoimmune destruction of insulin producing cells in the pancreas by CD_{4+} and CD_{8+} T cells and macrophages infiltrating the islets (Al-homsi *et al.*, 1992). Several features characterize type 1 diabetes mellitus as an autoimmune disease (Hussain and Vincent*,* 2007) which include the presence of immuno-competent and accessory cells in the infiltrated pancreatic islets; association of the susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC; human leucocyte antigens HLA)**,** the presence of islet cell specific autoantibodies**,** the alterations of T cell mediated immunoregulation, in particular in the CD_{4+} T cell compartment, the involvement of monokines and TH1 cells producing interleukins in the disease process**,** the response to immunotherapy as well as the frequent occurrence of other organ specific auto- immune diseases in the affected individuals or in their family members.

Approximately 85% of patients have circulating islet cell antibodies and the majorities also have detectable anti-insulin antibodies before receiving insulin therapy. Most islet cell antibodies are directed against glutamic acid decarboxylase (GAD) within the pancreatic B cells (Raju *et al.*, 2010). The autoimmune destruction of the pancreatic β-cells leads to a deficiency of insulin secretion which results in the metabolic derangements associated with type 1 DM. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal with an excessive secretion of glucagons in type 1 DM patients. Normally, hyperglycemia leads to reduced glucagons secretion, however, in patients with type 1 DM, glucagons secretion is not suppressed by hyperglycemia (Cryer, 2006). The resultant inappropriately elevated glucagon levels

exacerbate the metabolic defects due to insulin deficiency. Although insulin deficiency is the primary defect in type 1 DM, there is also a defect in the administration of insulin. Deficiency in insulin leads to uncontrolled lipolysis and elevated levels of free fatty acids in the plasma, which suppresses glucose metabolism in the peripheral tissues such as the skeletal muscle which impairs glucose utilization (Peterson *et al*., 2017). In addition, insulin deficiency also decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in the liver and the GLUT 4 class of glucose transporters in the adipose tissue which explained the fact that the major metabolic derangements resulting from insulin deficiency in type 1 DM are impaired glucose, lipid and protein metabolism (Zhang *et al*., 2019).

2.1.2.2 Type 2 Diabetes Mellitus

In type 2 diabetes the two major mechanisms of insulin secretion and insulin action broke down with the consequence that the two main pathological defects in type 2 diabetes are impaired insulin secretion through a dysfunction of the pancreatic β-cell, and impaired insulin action through insulin resistance (Holt, 2004). In situations where resistance to insulin predominates, the mass of β-cells undergoes a transformation capable of increasing the insulin supply and compensating for the excessive and anomalous demand. In absolute terms, the plasma insulin concentration (both fasting and meal stimulated) usually is increased, although "relative" to the severity of insulin resistance, the plasma insulin concentration is insufficient to maintain normal glucose homeostasis. With the known intimate relationship between the secretion of insulin and the sensitivity of hormone action in the complicated control of glucose homeostasis, it is practically impossible to separate the contribution of each to the etiopathogenesis of type 2 diabetes mellitus (Kumar *et al.*, 2002). Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance (ADA, 2010). With the exception of maturity onset type of diabetes

of the young (MODY), the mode of inheritance of type 2 diabetes mellitus is unclear. MODY is defined as hyperglycemia diagnosed before the age of twenty-five years and treatable for over five years without insulin in cases where islet cell antibodies (ICA) are negative (Saely *et al*., 2004). The condition, is inherited as an autosomal dominant trait, and may result from mutations in glucokinase gene on chromosome 7p.

2.1.3 Diagnosis of Diabetes Mellitus

The identification of patients with diabetes or pre-diabetes by screening allows for earlier intervention, with potential reduction in future complication rates, although randomized trials are lacking to definitively show the benefit of such (Botero *et al*., 2005). About 25% of patients with type 2 DM already have microvascular complications at the time of diagnosis suggesting that they have had the disease for more than 5 years at the time of diagnosis (Harris, 1992; ADA, 2011). As a result, there are different approaches to diagnose diabetes among individuals.

2.1.3.1 Measurement of Serum Random Plasma Glucose

Random plasma glucose test does not require fasting before the procedure. If a value of 11.1 mmol/L or greater of blood glucose is obtained, this may probably indicate diabetes but need to be further confirmed.

2.1.3.2 Measurement of Serum Fasting Plasma Glucose

Fasting plasma glucose test requires an overnight fasting of eight - twelve hours before the procedure. A fasting serum glucose greater than 7.0 mmol/L on two or more occasions performed on different days confirmed a diagnosis of diabetes (Cox and Elelman, 2009).

2.1.3.3 Oral Glucose Tolerance Test

Oral glucose tolerance test (OGTT) evaluates the body's response to glucose oral load. The test requires fasting of at least eight but not more than 16 hours. When the random plasma glucose test falls between 8.9 - 11.1 mmol/L or the fasting plasma test falls within the range of 6.1 - 7.0 mmol/L, then OGTT is required (ADA, 2018). The procedure is performed after a fasting glucose level is first determined and then the individual is given 75 g of glucose or 100 g for pregnant women. Then blood sample is collected and tested for glucose every 30 minutes to one hour for a period of two - three hours. The subject is considered to be normal if the glucose level at two hours is less than 7.8 mmol/L. A fasting level of ≥7.0 mmol/L and a two hour glucose level of 11.1 mmol/L or higher confirmed the diagnosis of diabetes (Cox and Elelman., 2009).

2.1.3.4 Measurement of Glycated Hemoglobin

The life span of hemoglobin in vivo is 90 to120 days. During this time, glycated haemoglobin A is formed which is the ketoamine compound resulting from the combination of hemoglobin A and glucose (WHO, 2011). Several sub-fractions of glycated hemoglobin have been isolated including glycated haemoglobin A fraction (HbA1c) which is of most significant, hence serving as a retrospective indicator of the average glucose concentration. HbA1c is recommended as an essential indicator for the monitoring of blood glucose control. A blood HbA1c ≥ 6.5% is considered as a diagnosis of diabetes (Gillett, 2004).

2.1.3.5 Measurement of Serum Fructosamine

Albumin is the main component of plasma proteins. As albumin also contains free amino groups, non-enzymatic reaction with glucose in plasma occurs. Therefore, glycated albumin can similarly serve as a marker for the monitoring of blood glucose. Glycated albumin is usually

considered to provide a retrospective measure of the average blood glucose concentration over a period of 1 to 3 weeks with a reference interval of 205 – 285 μmol/L (Ayyapan *et al*., 2015).

2.1.3.6 Diagnosis of Gestational Diabetes Mellitus

After at least 6 weeks of delivery, it is advicesable that the woman should receive an oral glucose tolerance test and be reclassified as having diabetes, normal glucose tolerance, impaired glucose tolerance or impaired fasting glucose. Also women at high risk such as those with positive family history of diabetes or history of GDM, marked obesity, or belonging to a high risk ethnic group should be screened as soon as feasible. If the initial screening is negative, they should undergo retesting at 24 - 28 weeks of gestation. A fasting blood glucose level >7.0 mmol/L or a casual plasma glucose >11.1 mmol/L meets the threshold for the diagnosis of diabetes. In the absence of unequivocal hyperglycemia, the diagnosis must be confirmed on a subsequent day. Confirmation of the diagnosis precludes the need for any glucose challenge. In the absence of this degree of hyperglycemia, evaluation for GDM in women with average or high-risk characteristics should follow one of two approaches (ADA, 2010). With either approach, the diagnosis of GDM is based on an OGTT.

Diagnostic criteria for the 100 g OGTT are derived from the original work of O'Sullivan and Mahan (1964) modified by Carpenter and Coustan (1982) are as follows;

Fasting glucose of 5.3 mmol/L or 1 hr glucose of 10.0 mmol/L, a 2 hr glucose of 8.6 mmol/L or a 3 hr glucose of 7.8 mmol/L. Alternatively, the diagnosis can be made using a 75 g glucose load and the glucose threshold values listed for fasting, 1 hr, and 2 hr are of 5.3, 10.0 and 8.6 mmol/L respectively.

2.1.4. Complications of Diabetes Mellitus

Complications of diabetes mellitus include both acute and chronic complications (Baynes, 2015).

2.1.4.1 Acute complications

The acute complications are marked by hyperglycemic crises which include diabetes ketoacidosis (DKA); (which is an acute major complication of diabetes characterized by hyperglycaemia, ketoacidosis and ketonuria) and hyperglycemic hyperosmolar state (HHS); (which is a condition characterized by hyperosmolarity and dehydration without significant ketoacidosis). (Baynes, 2015).

2.1.4.2 Chronic complications

The chronic complications include microvascular complications, diabetic retinopathy, diabetic nephropathy, diabetic neuropathy and macrovascular disease. Other complications and associated conditions may include impaired growth and development, associated autoimmune conditions, hypothyroidism, hyperthyroidism, celiac disease, vitiligo, primary adrenal insufficiency (Addison's disease), lipodystrophy (lipoatrophy and lipohypertrophy), necrobiosis lipoidica diabeticorum, non-alcoholic fatty liver disease, infections seen in patients with diabetes, limited joint mobility, and edema (Zou and Wang 2009).

2.1.5. Diabetic Nephropathy

Diabetic nephropathy (DN) is caused by diabetes mellitus and is one of the major causes of end-stage renal failure worldwide (Zou and Wang 2009). Clinically, microalbuminuria is an important index of assessing the progression of DN (KDOQI, 2007). However, it is not accurate to evaluate the severity or prognosis simply based on the degree of proteinuria. It is now well recognized that not all diabetic patients who developed renal failure have massive albuminuria

(Radclie *et al.*, 2016). Specially, nondiabetic renal disease (NDRD) which might commonly be superimposed with diabetic renal lesions in some patients with type 2 diabetes, could only be confirmed and excluded by biopsy (An and Liu, 2013).

2.5.1.1 Pathophysiology of Diabetic Nephropathy

It is well known that a collaboration of metabolic and hemodynamic alterations and inflammation are involved in the development of DN in patients with diabetes (Cao *et al.*, 2011). However, only in the past few years, studies have provided broad insight into pathogenic mechanisms and the molecular basis of DN. Blood pressure changes within the kidney have been reported to occur early in diabetes and to be critical in the progression of DN. Impairment of glomerular microcirculation and altered intra renal pressure lead to glomerular hyper- trophy and sclerosis. Studies using in vitro model of mechanical stretch have shown that podocytes and mesangial and tubular cells release several molecules when experiencing recurrent episodes of dilatation and relaxation similar to in vivo conditions (Forbes *et al.*, 2007). These molecules are responsible for the functional and structural changes in the glomeruli and include transforming growth factor-β1 (TGF-β1), glomerular capillary remodeling cytokine, capillary pressure regulators angiotensin II (Ang II), angiotensin-converting enzyme (ACE), angiotensin II receptor type 1 (AT1) and type 2 receptor (AT2), vascular endothelial growth factor (VEGF), as well as proinflammatory cytokines, such as interleukin-6 (IL-6), interleukin-18 (IL-18), and monocyte chemo attractant protein-1 (MCP-1) (Navarro-Gonzalez *et al.*, 2008) . It has also been shown that these molecules induce pathogenic changes either via elevating oxidative stress through activation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase or directly by activating cellular remodeling signaling leading to cellular morphological changes and increase synthesis of extracellular matrix (ECM) remodeling (Schena, 2005).

Hyperglycemia generates advanced glycation end products (AGEs) within tissue and plasma. These are generated via non-enzymatic oxidative reaction of amino acids from proteins present in renal tissue and plasma (Daroux *et al*, 2010). AGEs are known to induce renal complications by two pathways. One by remaining irreversibly bound to tissue protein such as matrix proteins (type IV collagen, laminin) and impair their degradation by matrix metalloproteinases which contribute to fibrosis via excess accumulation of ECM proteins (Busch *et al.*, 2010). Second, by interacting with the receptor for AGE (RAGE) expressed by podocytes and endothelial and mesangial cells in the kidney, AGEs also induce specific cellular responses including the release of profibrotic cytokines, such as TGF-β1, connective tissue growth factor (CTGF) and the angiogenic growth factor VEGF (D'Agati and Schmidt, 2010) and (Bohlender *et al.*, 2005). TGF-β1 plays an important role in the progression of DN because it promotes renal cell hypertrophy apart from stimulating ECM accumulation of the two hallmarks of diabetic renal disease (Castro *et al*., 204). Studies considered that TGF-β1 could be useful as a bio- chemical marker to estimate the progression of diabetes to DN (Shakar *et al.*, 2014). A higher level of urinary CTGF has also been shown to correlate with progression of DN, reflecting glomerular damage and fibrosis (Roestenberg *et al*., 2004). Furthermore, ligation of AGEs to RAGE also results in increased expression of NADPH oxidase and mitochondrial-dependent reactive oxygen species (ROS) generation (Coughlan *et al.*, 2008). All these profibrotic factors and their induced oxidative stress lead to glomerular cell proliferation, expansion, or hypertrophy.

Renal inflammation also plays a significant role in DN progression. In diabetic patients, the progression of glomerular structural and functional changes leads to interstitial infiltration of inflammatory cells, particularly macrophages and lymphocytes, attracted by chemoattractant cytokines released from injured renal tissue (Navarro-Gonzalez *et al*., 2011). In turn, these

inflammatory cells worsen the progression of DN via the release of pro inflammatory and tissue remodeling cytokines which also promote oxidative stress through activation of NADPH oxidase subunits, such as tumor necrosis factor-alpha (TNF-α), interferon-γ, and interleukin-1 (IL-1) (Navarro-Gonzalez *et al.*, 2011). Further studies showed that under these conditions of stress, immune cells and renal glomerular and tubular epithelial cells also produce pro inflammatory cytokines including MCP-1, IL-18, and IL-6. Ultimately, the deposition of ECM in the tubular component of the kidney (tubule interstitial fibrosis) is postulated to be the major determinant of the progression of renal disease in diabetes (Rodriguez-Iturbe *et al.*, 2005). The massive entry of proteins into the urinary space results in intense protein reabsorption activity of proximal tubular cells; this event is in turn followed by the formation of proteinaceous casts at distal points that cause tubular dilatation and obstruction (Remuzzi *et al.*, 1990). There is a loss of tubular basement membrane integrity, and the proteins derived from the urinary space are accumulated in an abnormal amount in the interstitium where they trigger the inflammatory reaction (Abbate *et al.*, 1998).

2.5.1.2 Biomarkers of Diabetic Nephropathy

A biomarker is considered as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson *et al.*, 2001). Biomarkers provide a dynamic and powerful approach to understanding the spectrum of a disease from the earliest manifestations to the terminal stage. Significant strides have been made to identify serum or urine biomarkers which can clinically detect early stages of DN and progressive kidney function decline in diabetic patients. In clinical practice, the most commonly used markers of renal disease and progression of DN are serum creatinine, e-GFR, blood urea, proteinuria, and albuminuria. The ideal measure

of renal function is GFR although e-GFR is use because of the ease of use and much easy to calculate despite the fact that, it reflect late functional changes and not early structural alterations of the kidney (Currie *et al*., 2014). The estimation equation uses serum creatinine which is still largely influenced by several factors that influence it thus compromising the estimation of GFR (Botev *et al.,* 2011). On the other hand, microalbuminuria has been recognized as the earliest marker of DN in clinical practice; although large proportion of renal impairment occur in the nonalbuminuric state before the onset of microalbuminuria (Perkins *et al*., 2007). Indeed, several studies have shown that diabetic patients can still develop DN without any change in their urinary albumin levels, and in some instances, microalbuminuria is shown to regressed back to normoalbuminuria in patients with advanced DN (Macisaac *et al*, 2011). In addition, a moderate increase in albumin excretion may be associated with a variety of other conditions, including obesity, exercise, diet, smoking, infection, and inflammation (Sharma, 2009). Taken together, these observations indicates that urinary albumin levels may not necessarily go in parallel with the progression of DN but rather represent an initial reversible phase of kidney damage. Increasing knowledge of the early molecular events in DN will spur the development of new alternative drugs and combined with methods already used in practice, may prevent the onset of disease entirely.

2.5.1.2.1 Glomerular filtration rate

Glomerular filtration rate (GFR) is the best marker of renal excretory function. The current gold standard methods for determining GFR in the research setting are inulin and ^{51}Cr-EDTA plasma clearance. The time consuming and labour intensive nature of these techniques, as well as the requirement of an experienced personnel, however, made it difficult for routine use in clinical practice. The traditional and the most commonly used index for assessment of GFR is serum

creatinine, although its sensitivity is poor in the early stages of renal impairment because by the time an increase in serum level is detected, a significant decline in GFR has already taken place (Perrone *et al*., 1992). The recently developed Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula appears to be more accurate in patients whose GFR is > 90 mL/min per 1.73 m^2 (Levey and Stevens, 2010). However, marked underestimation of GFR in the diabetic patients continues to be evident using this equation when compared to its performance in healthy individuals (Camargo *et al*., 2011). The current Kidney Disease Improving Global Outcomes guidelines staging system classifies chronic kidney disease stages 1 and 2 using e-GFR cut-offs of > 90 mL/min/1.73m^2 and 60 - 89 mL/min/1.73m^2 respectively (Inker *et al*., 2014). Routine clinical tests do not measure this degree of GFR decline accurately, meaning that this potentially critical early stage of renal dysfunction remains undetected (McNamara *et al.*, 2009).

2.5.1.2.2 Neutrophil Gelatinase Associated Lipocalin

Neutrophil gelatinase associated lipocalin (NGAL) is a small molecule of 25 kDa belonging to the lipocalin superfamily. NGAL is stored mainly in the specific granules of neutrophils and also expressed at low levels in several other human tissues (Yang *et al*., 2009). Plasma proteins of low molecular weight are excreted in increased quantities in the urine due to deficient tubular reabsorption or increased secretion by tubular epithelial cells. Similarly, urinary enzymes are thought to be sensitive markers of tubular damage as they are not filtered at the glomerulus due to their high molecular weight (Narita *et al.,* 2006). These proteins play a role in binding and transporting small hydrophobic molecules, apoptosis and immune regulation. NGAL shows significant promise in the diagnostic and clinical settings as a marker of acute kidney injury (Bolignano *et al*., 2008) and is thought to also play a renoprotective role as a mediator of tubular

cell proliferation (Yang *et al*., 2009). Studies have confirmed an association between NGAL and obesity, insulin resistance and hyperglycaemia in human subjects. Urinary NGAL concentration has been found to be increased in diabetic subjects compared with healthy controls and to correlate negatively with e-GFR, and positively with cystatin C (CysC), serum creatinine and urea in patients with type 2 DM (Nielsen *et al*., 2010; Bolignano *et al.*, 2008). Significant increases in urinary NGAL concentration have been demonstrated from normo, micro, to macroalbuminuric groups of patients with type 1 DM (Bolignano *et al*., 2009). Similar results have been published in a study of type 2 diabetic patients (Fu *et al*., 2012). Urinary NGAL correlates positively with glomerular hyperfiltration early in the clinical course of diabetes and higher values have been found to be associated with enhanced decline in e-GFR in type 2 diabetes patients with proteinuria, although this correlation was no longer statistically significant after adjustment for factors including systolic blood pressure, HbA1c and diabetes duration (Nielson *et al*., 2012). However, other prospective studies have not confirmed these associations (Chou *et al*., 2013) and further investigation of the role of urinary NGAL in DN is required.

2.5.1.2.3. Microalbuminuria

Microalbumunuria is the diagnostic marker use to the detection of DN at early stage, important for early intervention to slow the loss of kidney function and reduce adverse outcomes. However, it has been reported that a large proportion of renal impairment occurs even before the appearance of microalbuminuria (Rigallleau et al, 2007). Since albuminuria is an important component of DN, it is important to establish the definition of the different degrees of UAE. Normoalbuminuria refers to UAE of <30 mg/day or 20 µg/min, while microalbuminuria and macroalbumnuria refers to UAE of 30 – 300 mg/day or 20 – 200 µg/min and >300 mg/day or 200 µg/min respectively (Cohen- Bucay and Viswanathan, 2012). Albuminuria has several

confounding factors that are associated with it including exercise, urinary tract infections, acute illness and cardiac failure. Furthermore, it has been reported to occur in the urine of non-diabetic subjects indicating the non-specificity of albumunuria for the accurate prediction of diabetic kidney disorder (Jain et al 2005)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY AREA

This study was conducted at the Endocrinology Unit of Chemical Pathology Department of Aminu Kano Teaching Hospital, a tertiary hospital serving as a referral center that is located along Zaria Road, Kano. Kano is located between latitudes 11 º 25' N to 12 º 47' N and Longitude 8 º 22' E to 8º39'E and 472m above sea level having a population of over three million. Kano state is bordered by Katsina state to the North West, Jigawa state to the North East, Bauchi to the South East and Kaduna state to the south west.

3.2 STUDY POPULATION

The study involved 160 subjects made up of 80 diabetic patients with DN presenting to the endocrinology clinic of Aminu Kano Teaching Hospital, Kano and 80 apparently healthy individual non- diabetic subjects serving as study controls.

3.2.1 Inclusion Criteria

1. Patients diagnosed with type 2 diabetes and positive for microalbuminuria attending the endocrinology unit of Aminu Kano teaching hospital.
2. Patients between the age of 18 and 70 years.

3.2.2 Exclusion Criteria

1. Patients with established kidney disease.
2. Pregnant women,
3. Patients with complication likely to compromise the renal integrity such as inflammation, vigorous exercise, fever.
4. Women in their menstrual circle.

5. Those that declined consent to participate in the study.

3.3 STUDY DESIGN

The study was cross-sectional involving a total of 160 subjects (one hundred and sixty), eighty (80) were patients with diabetes mellitus.

3.4 ETHICAL CONSIDERATION

Ethical approval was sought and granted by the Ethics Committee of the Aminu Kano Teaching Hospital, Kano to conduct this research. The approvals were obtained in accordance with the declaration of Helsinki which established a code of ethics on human experimentation. Informed consent was sought from all subjects before inclusion in the study in accordance with the ethical guidelines of the Ethics Committees of the Aminu Kano Teaching Hospital, Kano. The nature of the study was fully explained to each subject before inclusion in the study and collection of blood sample (appendix II). Subjects that volunteered to take part in the study were instructed to be on their normal diet and observed an overnight fast and abstain from smoking on the day of the sample collection. Questionnaire developed for the study was administered to each subject (appendix III). Medical history was also obtained on social habits, health status and family history of non-communicable diseases.

3.5 SAMPLE SIZE DETERMINATION

The sample size can be calculated using the following formula (Naing *et al*., 2006)

$$n = \frac{Z^2PQ}{e^2}$$

Where n= number of sample

Z = 95% confidence interval

P = prevalence of diabetes mellitus (5.0%), (IDF, 2013).

Q = 1 - P

$$N = (1.96)^2 \times (0.05) \times (1-0.056)/0.0025 = 72.9$$

The minimum sample size is approximately 73. In addition to 10% attrition rate (7), the minimum sample size is 80. Therefore 80 subjects were recruited along with 80 controls.

3.6 SPECIMEN COLLECTION AND PROCESSING

Ten milliliters (10 mL) of blood sample was collected from each subject through the ante-cubital vein after an overnight fast lasting between 10 – 12 hours. The site of collection was aseptically cleaned with alcohol swipe and allowed to dry before blood was collected. Five milliliters (5mL) of the blood collected was transferred into a grey top tube for glucose analysis and the other 5mL was transferred into a red top tube for NGAL and other parameters and left to clot before serum was harvested after centrifugation at 3000 rpm for five minutes. The separated plasma and sera were then stored frozen at -80 ^{o}C prior to analysis. Measurement of anthropometric variables was performed on each subject. Height (m) was measured using a standard hospital scale with the subject barefooted. Body weight (kg) was taken with the subject in light underwear using standard hospital scale. Waist circumference (cm) was measured at the level of the naval with the subject standing and breathing normally. Body mass index (BMI) was calculated as weight (kg)/ height (m^2) and these parameters were recorded.

3.7 ANALYTICAL METHODS

3.7.1 Estimation of Fasting Plasma Glucose

3.7.1.1 Principle

The enzyme glucose oxidase catalyzes the reaction between glucose, water and oxygen to yield gluconic acid and hydrogen peroxide (H_2O_2). Hydrogen peroxide in the presence of peroxidase is broken down and the oxygen released reacts with 4-aminophenazone and phenol to yield a pink color which is proportional to the concentration of the glucose in the sample and the color developed was read at 540 nm (Trinder, 1969).

3.7.1.2. Procedure

In to labeled test tubes, 10 ul of sample, standard and blank were added respectively. The solution was then mixed with 1 ml of the glucose reagent. The tubes were then incubated at 37^{o}C for 10 minutes and the color developed was read at 540 nm (Trinder, 1969).

3.7.2 Estimation of serum Creatinine

3.7.2.1 Principle

The method employed the Jaffe reaction of 1886 between creatinine and picric acid in an alkaline medium forming a complex with absorbance maxima at 520 nm (Jaffe, 1886).

3.7.2.2 Procedure

The samples were run on ABBOTT autoanalyzer after pipetting into the appropriate sample cups and the results obtained were recorded (Abbot, 2009).

3.7.3 Estimation of serum Albumin

3.7.3.1 Principle

The method is based on the specific binding of bromocresol green, an anionic dye to protein at acidic pH producing a color. The color change produced has absorbance maxima at 580 nm and the intensity of the color formed is directly proportional to the albumin concentration in the sample (Gustafsson, 1976).

3.7.3.2 Procedure

Samples were run on the ABBOTT autoanalyzer and the results obtained were recorded (Abbott, 2009).

3.7.4 Estimation of serum Neutrophil Gelatinase Associated Lipocalin

3.7.4.1 Principle

This human NGAL ELISA kit uses a pre-tittered, matched pair of coating and detection of antibodies to achieve sensitive and accurate measurement of NGAL. The assay is a sandwich ELISA performed on a microplate wells coated with a monoclonal antibody against human NGAL. Bound NGAL is detected with another monoclonal antibody labeled with biotin and the assay is developed with horseradish peroxidase (HRP) conjugate streptavidin and a color-forming substrate. The assay is a four step procedure (Stejskal *et al*., 2008).

3.7.4.2 Procedure

Samples, standards, quality control and dilution buffer (blank) were added in the pre-coated appropriate micro wells after dilution. The microplate was then incubated at room temperature for 1 hour while shaking at 300 rpm on an orbital microplate shaker. The wells were washed three times with wash solution (0.35ml per well), after the final wash, it was inverted and tapped against paper towel. Fifty (50µl) of biotinylated antibody solution was added and incubated at

room temperature for 60 minutes while shaking at 300 rpm on an orbital microplate shaker. Streptavidin-horse radish peroxidase was added into each well and incubated at room temperature for 30 minutes while shaking at 300 rpm on an orbital microplate shaker and the plate then washed three times using the wash solution. Substrate solution was added into each well and covered with aluminum foil avoiding exposure to direct sunlight and incubated at room temperature for 10 minutes and stop solution was added to stop the reaction with the color developed measured at 630 nm wavelength (Stejskal *et al*., 2008).

3.7.5 Estimation of Glycated Haemoglobin

3.7.5.1 Principle

The fine care HbA1c rapid quantitative test is based on fluorescence immunoassay technology. It uses a sandwich immunodetection method, when sample is added into the sample well of the test cartridge, the fluorescence-labeled detector HbA1c antibodies and detector Hb antibodies on the sample pad bind to HbA1c antigens and Hb antigens in blood specimen respectively and form immune complexes. As the complexes migrate on the nitrocellulose matrix of test strip by capillary action, the detector antibodies and HbA1c are captured to HbA1c antibodies that have been immobilized on test strip as well as the complexes of detector antibodies and Hb are captured to Hb antibodies that have been immobilized on test strip. Thus the more HbA1c antigens and Hb antigens in blood specimen, the more complexes accumulated on test strip. Signal intensity of fluorescence of detector antibodies reflect the amount of HbA1c and Hb captured and Finecare immunoassay system shows HbA1c ratio in blood specimen (Ejilemele *et al*., 2015)..

3.7.5.2 Procedure

The machine was activated and the ID chip was checked to match the test cartridge as well as the detection buffer. This was then inserted into the finecare machine. The detection buffer was

mixed with 10 µL of whole blood for 1 minute. Into the sample well of the test cartridge, 75 µL of sample mixture was added. This was incubated at room temperature for 5 minutes and inserted into the machine. The 'Test' button was then pressed to initiate the testing procedure. The finecare system scanned the sample-loaded on the test cartridge immediately and results were displayed on main screen and printed (Ejilemele *et al*., 2015).

3.7.6. Estimated Glomerular Filtration Rate

The Modification of Diet in Renal Disease simplified equation of Levey 1999 was used to calculate the estimated GFR (e-GFR) in mL/min/1.73m^2. Renal dysfunction stage 2 was defined as e-GFR 60 - 89 mL/min/1.73m^2 according to the Kidney Disease: Improving Global Outcomes chronic kidney disease definition (Levey *et al*., 1999).

The Modification of Diet in Renal Disease Equation;

GFR (mL/min/1.73m²) = 175 $(S_{cr})^{-1.154}$ x $(Age)^{-0.203}$ x 0.742 (if female) x 1.212 (if African American).

3.8 STATISTICAL ANALYSIS

The data were recorded on an Excel spread sheet and later subjected to statistical analysis using Statistical Package for the Social Sciences (SPSS) software version 20.0. Results were expressed as mean and standard deviation. Differences in socio-demographic variables of subjects were analyzed by one-way analysis of variance (ANOVA) and student *t*-test. The statistical test used for correlation was the Spearman non-parametric 2 tailed correlation test. A p-value of ≤ 0.05 was considered as significant in all statistical comparisons.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

A total of 80 diabetic patients with no established diabetic nephropathy and 80 control subjects were recruited in this study.

4.1.1: Clinical characteristics of Diabetic nephropathy and control group

Results from table 4.1, shows an age distribution among the diabetic nephropathy and control group. There was a significant difference between the two groups in terms of age. There is no significant difference in gender. The glucose level of the control group was found to be within normal while among the 80 DN patients, 41 were having normal FPG level with 51.2% and 39 were having hyperglycaemia with 48.8%. Among the control group, all were having normal serum albumin (100%), while among the 80 DN patients, 22 were having a low serum albumin with 27.5%, 58 were having normal serum albumin with 72.5%. there was a significant difference in serum albumin between the DN group and control group (p=0.000). Mean values of serum creatinine of both groups were all within normal and there is no significant difference between the 2 groups. Among the diabetic nephropathy group, 18 were having normal HbA1c level with 22.5% and the rest were having an elevated HbA1c level from which 51 subjects with 63.8% were having a good glycaemic control, while 11 with 13.8% were having a fair glycaemic control. There is a significant difference between the DN group and the control group which was having a normal HbA1c level.

Table 4.1.1: Clinical characteristics of Diabetic nephropathy and control groups

Variables	Test Groups (%)	Control Group (%)	p – value
Age (Years)			
20 – 29	2 (2.5)	26 (32.5)	0.0000
30 – 39	2 (2.5)	41 (51.2)	
40 – 49	19 (23.8)	7 (8.8)	
50 – 59	31 (38.8)	6 (7.5)	
60 – 69	24 (30.0)	0 (0.0)	
70 – 79	2 (2.5)	0 (0.0)	
Sex			
Male	35 (43.8)	34 (42.5)	1.0000
Female	45 (56.2)	46 (57.5)	
BMI			
Under Weight	3 (3.8)	0 (0.0)	0.0000
Normal Weight	25 (31.2)	47 (58.8)	
Over Weight	26 (32.5)	28 (35.0)	
Obese	26 (32.5)	5 (6.2)	
Glucose Level			
Hypoglycaemia	0 (0.0)	0 (0.0)	0.0000
Normal	41 (51.2)	80 (100.0)	
Hyperglycaemia	39(48.8)		
Serum Albumin			
Low	22 (27.5)	0 (0.0)	0.0000
Normal	58 (72.5)	80 (100.0)	
High	0 (0.0)	0 (0.0)	
Serum Creatinine			
Low	0 (0.0)	0 (0.0)	1.0000
Normal	80 (100.0)	79 (98.8)	
High	0 (0.0)	1 (1.2)	
HbA1c			
Normal	18 (22.5)	80 (100.0)	0.0000
Good Control	51 (63.8)	0 (0.0)	
Fair Control	11 (13.8)	0 (0.0)	
Poor Control	0 (0.0)	0 (0.0)	

BMI=body mass index, HbA1c=glycaeted haemoglobin

4.1.2: Comparison between the Mean and Standard Deviation of FPG, BMI, SA, and SC Levels of Diabetic Nephropathy and Control Group.

Results from table 4.2 showed the mean value of FPG, BMI, SA and SC levels of both the test and control groups. There is a significant difference between the variables of the test and control group *p*-value (<0.005).

Table 4.1.2: Comparison between the Mean and Standard Deviation of FPG, BMI, SA, and SC Levels of Diabetic Nephropathy and Control Group.

Variables	Diabetic nephropathy Group (n = 80) Mean ± SD	Control Group (n = 80) Mean ± SD	*p* – values
DURATION	8.53 ± 4.78	-	-
FPG	7.60 ± 3.46	4.08 ± 0.39	0.0000*
BMI	27.33 ± 5.29	25.08 ± 3.65	0.0012*
SA	35.13 ± 5.62	40.49 ± 1.47	0.0000*
SC	97.83 ± 11.82	78.78 ± 40.02	0.0000*

FPG= Fasting plasma glucose, BMI= Body mass index, SA= serum albumin, SC= serum creatinine.

4.1.3: Comparison of NGAL level, HbA1c and eGFR in both Diabetic Nephropathy and Control Group.

Results from table 4.3 showed a comparison of the level of NGAL, HbA1c, eGFR and FPG in the control and DN group. The mean value of NGAL in the DN group (3.71±2.62µg/ml) was high when compared with that of the control (1.08±0.78µg/ml) with a significance *p*=0.000. The mean value of HbA1c in the DN group (6.73±1.08%) was high when compared with that of the control group (4.71 ± 0.44%) with *p*-value of 0.000. So also the EGFR of DN group and control group is 76.57 ± 11.20 (ml/min/1.73m²) and 118.23 ± 12.11 (ml/min/1.73m²). Fasting plasma glucose of the DN group was high when compared with that of the control group with mean and standard deviation of 7.6 ± 3.46 (mmol/L) and 4.08 ± 0.39 (mmol/L) (p=0.000) respectively.

Table 4.1.3: Comparison of the levels of NGAL, HbA1c and eGFR in both DN and control group.

Variables	DN Group (n = 80) Mean ± SD	Control Group (n = 80) Mean ± SD	*P* – values
NGAL(µg/ml)	3.72 ± 2.62	1.08 ± 0.78	0.000*
HbA1c (%)	6.73 ± 1.08	4.71 ± 0.44	0.000*
eGFR (ml/min/1.73m²)	76.57 ± 11.20	118.23 ± 12.11	0.000*
FPG (mmol/L)	7.60 ± 3.46	4.08 ± 0.39	0.000*

FPG= Fasting plasma glucose, HbA1c= glycated haemoglobin, EGFR= estimated glomerular filteration rate, NGAL= neutrophil gelatinase associated lipocalin.

4.1.4 Comparison of FPG, NGAL HbA1c and eGFR Based on Duration of diagnosis of Diabetes Mellitus Categories within the Diabetic Nephropathy Group

The result show a comparison in the values of NGAL, HbA1c and eGFR within the DN group with different duration of onset of DM. The different duration of onset of DM was categorized into <5, 5-9 and >10years. There is a significant difference in the level of NGAL *P*= 0.000 and shows no significant difference in FPG, eGFR and HbA1c level of all the duration class at *p*=0.414, 0.990 and 0.414 respectively.

Table 4.1.4: Comparison of FPG, NGAL HbA1c and eGFR based on duration of diagnosis Diabetes Mellitus categories within the diabetic nephropathy group.

Variables	<5 years	5-9years	>10years	*p*-value
FPG (mmol/L)	6.48 ± 3.50	7.69 ± 3.87	8.13 ± 2.99	0.414
NGAL(ug/ml)	2.89 ± 1.16	3.81 ± 1.70	4.09 ± 3.57	0.000
HbA1c (%)	6.60 ± 1.06	6.84 ± 0.88	6.84 ±1.38	0.057
eGFR (ml/min/1.73m^2)	80.43 ± 11.08	75.20 ± 11.01	75.66 ± 11.30	0.990

FPG= Fasting plasma glucose, HbA1c= glycated haemoglobin, eGFR= estimated glomerular filteration rate, NGAL= neutrophil gelatinase associated lipocalin

4.1.5: Relationship Between Duration of Diabetes Mellitus, FPG, NGAL, eGFR and Glycaemic Control of the Diabetic Nephropathy Group Categorized into Good and Fair control.

The result show that there is a significant difference in the level of serum NGAL between the DN group with fair and good glycaemic control at p=0.000 and show no significant difference in FPG and eGFR at p=0.062 and p=0.219 respectively.

Table 4.1.5: Relationship Between Duration of Diabetes Mellitus, FPG, NGAL, eGFR and Glycaemic Control of the Diabetic Nephropathy Group Categorized into Good and Fair control.

Variables	Fair control (N=11)	Good control (N=51)	*p*-value
Duration (yrs)	8.7 ± 2.23	8.39 ± 5.07	0.700
FPG(mmol/L)	6.46 ± 1.99	7.92 ±3.59	0.062
NGALμg/ml	5.40 ±5.93	3.54 ±1.34	0.000
eGFR((ml/min/1.73m^2)	79.07 ± 14.34	77.93 ± 10.93	0.219

FPG= Fasting plasma glucose, eGFR= estimated glomerular filteration rate, NGAL= neutrophil gelatinase associated lipocalin.

4.1.6: **Relationship between Glycated Haemoglobin, Duration of Diabetes Mellitus and NGAL within Diabetes Nephropathy Group**.

The result show that there is a correlation between NGAL and duration of diabetes mellitus at *p*-value of 0.011 and also a negative correlation between NGAL and eGFR at p=0.013. But shows no relationship between NGAL and glycated hemoglobin and NGAL and FPG at p=0.061 and 0.734 respectively.

Table 4.1.6: Relationship between glycated haemoglobin, duration of diabetes mellitus and NGAL within diabetes nephropathy group.

NGAL	R	Significant (2-tailed)
DURATION(yrs)	0.283	0.011*
Hb1Ac(%)	0.211	0.061
eGFR((ml/min/1.73m^2	-0.216	0.013*
FPG(mmol/L)	-0.039	0.734

Key: eGFR= estimated glomerular filtration rate, HbA1c= glycated haemoglobin, FPG= Fasting plasma glucose. * Correlation is significant at the 0.05 level (2-tailed)

4.1.7: Relationship between Glycated Haemoglobin, Duration of Diabetes Mellitus and NGAL within Diabetes Nephropathy Group.

Regression analysis show that NGAL has no relationship with eGFR and HbA1c within the DN group with p= 0.214 and 0.349 respectively, but also show that NGAL had a relationship with FPG and duration of diabetes mellitus with p= 0.000 respectively.

Table 4.1.7: Relationship between Glycated haemoglobin, Duration of Diabetes Mellitus and NGAL within Diabetic Nephropathy group.

NGAL	R	Significant (2-tailed)
DURATION(yrs)	0.149	0.000*
Hb1Ac(%)	0.376	0.214
eGFR((ml/min/1.73m^2	0.104	0.349
FPG(mmol/L)	0.094	0.000*

DURATION = duration of diabetes, FBS = fasting plasma glucose, HbA1c = glycaemic control, EGFR = estimated glomerular filteration rate, *Regression is significant at $p < 0.05$.

4.2 DICUSSION

Diabetes nephropathy is a major complication of diabetes mellitus accounting for 20% to 40% of population requiring renal replacement therapy (Kaul *et al*., 2018). Pathologically, it is a diffuse process involving glomerular endothelial cells, and interstitium. It evolves through glomerular hyperfilteration (eGFR ≥90mL/min/1.73m²) (stage1), silent phase (normoalbuminuria) incipient nephropathy (microalbuminuria) with eGFR of 60-89mL/min/1.73m² (stage 2), overt nephropathy (macroalbuminuria) with eGFR 30-59mL/min/1.73m² (stage 3) and established renal failure with GFR <30mL/min/1.73 (Masakazu *et al*., 2015).

In this study, 80 patients with DN as well as 80 apparently healthy non diabetic individuals serving as control group were recruited.

The present study reported a mean value of the BMI of both diabetic nephropathy and control group. It shows a significant difference between the BMI of the two groups in which that of the test was high. This is similar to the findings of Mahfouz *et al.* (2016) who reported a high BMI for type 2 DM patients with microalbumin compared to healthy controls in addition to the positive relationship between higher BMI and diabetes nephropathy. Also in a study carried out by Bays *et al*. (2007), it was reported that a BMI of ≥25kg/m² is associated with the increase prevalence of diabetes mellitus. Natalia *et al*. (2016) also reported a BMI of 25≤BMI≤27.49kg/m² and added that higher than normal BMI was consistently associated with an increased risk of being diagnosed with type 2 diabetes mellitus and subsequently diabetes nephropathy. Another study which supported our findings is that carried out by Mohammedi *et al.* (2018) on associations between body mass index and the risk of renal events with type 2 diabetes, reporting that higher BMI is an independent predictor of major renal events in patients with type 2 diabetes. This means that excess weight and obesity maybe a major contributing

factor to type 2 DM and its complications supporting the fact that overweight and obesity are consistent parameters associated with diabetes mellitus complications and subsequently DN (Bay *et al*., 2007).

This study also reported that fasting plasma glucose as well as glycated haemoglobin were significantly high in the DN group when compared with that of the healthy control group. Sun *et al.* (2013) and Mahfouz *et al.* (2016) has earlier reported similar findings among their subjects. A study by Chen *et al.* (2013) has also corroborated our findings and he noted that hyperglycaemia and HbA1c are the two most significant indicators of diabetic nephropathy. It can therefore be inferred that patients with long standing poorly controlled DN had higher HbA1c and fasting plasma glucose Kaori *et al*. (2013). This in effect means that DN progression begins with long standing poorly controlled blood glucose levels (Schlondorff and Banas, 2009). Therefore, hyperglycaemia resulting from elevation of glucose level in the body and poor diabetic control marked by HbA1c may be the driving force for the development of diabetes complications including diabetic nephropathy (DN) and microangiopathy due to its high affinity to oxygen (Kundu *et al.,* 2013). It also adds to the fact that HbA1c as an indicator of long term glycaemic control, not only provides reliable measure of chronic hyperglycaemia but also correlates with the risk of long term diabetes complication.

Albumin represent plasma protein and is synthesized in the liver and secreted into the vascular space to distribute in all body tissues. It plays a decisive role in the maintenance of homeostasis and makes a balance between hydrostatic and colloid osmotic pressure within vessels (Nicholson *et al.,* 2000). Several studies have reported that, decrease serum albumin and creatinine are associated with renal injury (NKF, 2002; Junlin *et al*., 2019). This study reported a low level of serum albumin amongst the patients. It was established that, low levels of albumin is

significantly associated with adverse renal outcome independent of clinical and histopathological features. Hence subjects with the lowest level of albumin demonstrated a 7.37fold greater risk of ESRD which could serve as a prognostic utility of diabetic nephropathy.

Serum creatinine is significantly elevated in patients with DN in this study compared with the control group. Report by Mahfouz *et al*. (2016), indicates a slight increase in creatinine level in subjects with DN. In kidney impairment, serum creatinine is reported to increase. This increase in serum creatinine may be commonly seen in patients with hyperglycaemia, poor glycaemic control. The increase in the levels of serum creatinine therefore likely indicates progression to DN.

Glomerular filtration rate (GFR) is the ideal measure of kidney function. Decline in GFR appears earlier than changes in urine protein levels and these changes remains throughout the entire course of diabetes mellitus (Alwakeel *et al*., 2011). The estimated GFR in our patients with diabetic nephropathy group was lower when compared with the control group. Similar findings were reported by Mohammad *et al*. (2016) and Kaul *et al*. (2018). However, another study reported an increase in eGFR among type 2 diabetic patients with diabetic nephropathy. The study demonstrated an independent relation between decrease eGFR and urinary albumin excretion among the patients (Unsal *et al*., 2012). Report determining the relationship between eGFR and microvascular complications in type 2 diabetes mellitus patients, indicated significant association between reduced eGFR and diabetic nephropathy hence emphasizing the prognostic role of reduced eGFR in the development of diabetes nephropathy in type 2 diabetes mellitus patient (Prakash *et al*., 2018). The reduced eGFR is the primary clinical abnormality of DN and which suggests that the decrease eGFR observed in this study may be due to the decline in renal function caused by prolonged hyperglycaemia.

Neutrophil gelatinase associated lipocalin (NGAL), initially identified by Allen and Venge in 1989 from human neutrophils, is expressed at low levels in several human tissues including the kidney (Ahmed *et al.,* 2017). The level of the serum NGAL in the diabetiic group was significantly higher as compared to the control group. The finding in this study closely agrees with the finding of Abber *et al.* (2014), who reported higher level of NGAL in diabetic patients with increased urinary albumin when compared with that of the healthy controls (1.57 ± 0.72 versus 0.70 ± 0.58 respectively). It should however be noted that while we with used serum in the NGAL analysis, the other study utilizes urine for the NGAL analysis which may account for the differences in our values. Ferdau e*t al*. (2011) also observed that, urinary levels of markers of kidney damage including NGAL were higher in normoalbuminuric patients with diabetes compared with the control subjects but increase with increasing severity of albuminuria and decreased GFR in diabetic patients. It is well known that hyperglycaemia and other conditions including hyperlipidaemia lead to inflammation and metabolic stress. This results in endothelial dysfunction and tubulointerstitial damage thus leading to an increase in the release of tubular biomarkers (Kaul *et al*., 2018). Therefore, the increase level of NGAL in the DN group in our study may be due to the presence of DN and to its protective role in response to metabolic stress.

On the other hand, a study in animal model by Alter *et al*. (2012), have shown that urinary biomarkers including NGAL were elevated in an established rat model of diabetic nephropathy and concluded that these biomarkers appeared even before the classical biomarkers of diabetic nephropathy such as albuminuria. Neilson *et al.* (2010) also noted that serum NGAL is significantly higher in diabetic nephropathy group compared with the control group.

Mahfouz *et al*. (2016) observed that serum NGAL was higher in type 2 diabetic patients with diabetes nephropathy when compared with the controls, and that serum NGAL showed a positive

correlation with urinary albumin creatinine ratio and negatively with GFR, thus suggesting that NGAL levels changes with the progression of albuminuria from absent to present.

This study also assesses the relationship between NGAL and duration DM. We observed a strong correlation between NGAL and duration of diabetes. Study by Kaul *et al.* (2018) reported a strong relationship between NGAL and duration of diagnosis of DM. A positive correlation between NGAL and duration of diagnosis of DM was also reported by Papadopoulou *et al*. (2017), who noted that NGAL is an early predictive marker of DN. Correlation between NGAL and duration of DM diagnosis was also observed by Georgia *et al*. (2019).

This study also carried out correlation analysis between NGAL and eGFR, a strong negative relationship was found. The finding agreed with most previous published studies that reported a similar correlation between NGAL and eGFR. NGAL is significantly elevated in patients with type 2 diabetes with decreased GFR well before the appearance of diabetic nephropathy. To support this, Wang *et al*. (2007) in a study in Hong Kong and El-Mesallamy *et al*. (2013) in Egypt reported similar trends. In addition, Papadopoulou-Marketou *et al*. (2017) also reported similar finding in a study of NGAL as an early predictive marker of diabetic nephropathy in Children and Young Adults with type 1 diabetes mellitus.

Correlation was also reported between NGAL and cystatin C by Marcelo *et al*. (2017) who observed that there was a positive correlation between NGAL and worse eGFR corroborating the findings of Khalid *et al*. (2017) who observed NGAL to be significant increase in parallel with the deterioration of eGFR. The above findings underpin the value of NGAL as a biomarker of early renal damage because of its positive correlation with duration of diagnosis of diabetes mellitus and reduced eGFR. This may reflect a progress of the early renal structural damage occuring during the disease. However, the regression model that include there was association

between NGAL and HbA1c and duration of diagnosis but no such association was observed between NGAL and eGFR and HbA1c. This study indicates that NGAL cannot be a predictive marker of diabetes nephropathy in terms of estimated GFR and HbA1c.

However, this study did not observe any significant correlation between NGAL and glycaemic control. Similar finding by Elkhidir *et al*. (2017) supported our finding, where correlations were not significant between controlled and uncontrolled diabetes patients. El-Mesallamy *et al*. (2013) in Egypt also observed the same correlations in a study on effect of obesity and glycaemic control on NGAL and Insulin like growth factor axis in type 2 diabetic patients. However, the finding contradicts a study from China were a strong positive correlation between NGAL and glycaemic control was reported (Wang *et al*., 2007).

However, the regression model indicates that NGAL can be a predictive marker of diabetes nephropathy when compared with duration of diagnosis of diabetes and fasting plasma glucose but cannot be a predictive marker of diabetes nephropathy in terms of eGFR and HbA1c.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 SUMMARY

NGAL is one of the excellent emerging biomarkers in the urine and blood for the early detection of acute and chronic kidney injury as well as DN. This study compares diabetic nephropathy group with control group. It sorts the relationship between NGAL and DM parameters and reported a negative correlation between NGAL and e-GFR in type 2 diabetic patient with DN. It also reported no correlation between NGAL and glycated haemoglobin in the same group.

5.2 CONCLUSION

It was concluded from the findings of this study that serum NGAL was elevated in type 2 diabetic patients with diabetic nephropathy compared with healthy individuals. A strong correlation between NGAL and eGFR was also observed, this may routinely suggest the use of NGAL as biomarker to diagnose DN and also as a prognostic tool for the staging and progression of DN.

5.3 RECOMMENDATIONS

It was recommended from the findings of this study that:

1. Serum NGAL should be incorporated into the routine monitoring of diabetes mellitus patients that are predisposed to DN.
2. A reference range for the NGAL levels should be established for the population to facilitate routine referencing.

3. Further studies on NGAL should be carried out on other disease conditions such as hypertension and CVD to determine the utility of NGAL in such patients as an earlier predictor of renal impairment.

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APPENDIX I

AMINU KANO TEACHING HOSPITAL

P.M.B. 3452, ZARIA ROAD, KANO.

(☎07068297399)www.akth.info/www.akth gov.ng, email: equiries@akth.info/akthkano@yahoo.com

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NHREC/21/08/2008/AKTH/EC/2483

AKTH/MAC/SUB/12A/P-3/VI/2583 **29th April, 2019**

Aisha Abubakar Jibril
Department of Med. Lab. Science
BUK, Kano.

Ufs:

The Head of Department
Med. Lab Science
BUK, Kano.

ETHICS APPROVAL

Further to your application in respect of your research proposal titled "Evaluation of Neotrophil Gelatinase Associated Lipocalin (NGAL) among Type 2 Diabetic Patients with Diabetic Nepropathy Attending Aminu Kano Teaching Hospital, Kano", The Committee reviewed the proposal and noted same as a prospective study.

In view of the above, Ethics approval is hereby granted to conduct the research.

However, the approval is subject to periodic reporting of the progress of the study and its completion to the Research Ethics Committee.

Regards,

Abubakar S. Mahmud
Secretary, Research Ethics Committee
For: Chairman

APPENDIX II

CONSENT FORM

Good Morning,

My name is Aisha Abubakar Jibril, Msc. Medical Laboratory Science student from Bayero University Kano. This questionnaire is designed for a research work titled "Evaluation of Neutrophil Gelatinase Associated Lipocalin in Type 2 Diabetic Patients with Diabetic Nephropathy". Your response to the questionnaire will be strictly confidential and blood sample will be collected from you for the purpose of the study. The outcome of the study will be communicated to you via short message (sms), email or to place in your Hospital folder in case of the need for further investigations and treatment.

I ……. give consent to participant in this study. I have been fully informed on the nature of the study. I understand that I can withdraw from this study at any time without giving reason and without any penalty involved.

…… ……..

Participant signature / Date Investigator signature / Date

APPENDIX III

RESEARCH QUESTIONNAIRE

My name is Aisha Abubakar Jibril, Msc. a Medical Laboratory Science student from Bayero University Kano. This questionnaire is designed for a research work titled "Evaluation of Neutrophil Gelatinase Associated Lipocalin in Type 2 Diabetic Patients with Diabetic Nephropathy" supervised by Mal. Ahmad Mohammed Bello and Mal. Saleh Idris Tudunwada.

1. Age: ----------

2. Gender: Male [ ], Female [ ]

3. Phone number ……..

4. Education- formal; Primary [ ], Secondary [ ], Tertiary [ ], Non formal [ ].

5. Ethnic group: Hausa [ ], Fulani [ ], Igbo [ ], Yoruba [ ], others [ ] -------specify

6. Medical history;

i. Height……..

ii. Weight……

iii. BMI……..

iv. Duration of diagnosis of diabetes mellitus; < 5years [ ], 5-10years [ ], >10years [ ].

v. Are you currently on Insulin or any other medication; YES ( ) NO ( ) Sprcify……..

vi. History of smoking; YES ( ) NO ( )

vii. History of high blood pressure (HBP); YES ( ) NO ( )

viii. History of microalbumin. YES ( ) NO ( )

ix. History of urinary tract infection

x. Are you currently on your menstrual period; YES ( ) NO ( )

xii. Do you have any other disease apart from diabetes mellitus; YES ( ) NO ( )

Thank you for your precious time!