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

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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.
Aim: The aim of this study was to evaluate serum neutrophil gelatinase associated lipocalin (NGAL) in type 2 DM with diabetic nephropathy.
Methods: 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.
Result: 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.
Conclusion: Serum NGAL could therefore be used as a biomarker to diagnose DN even earlier to incipient nephropathy.
NGAL, Diabetes nephropathy, eGFR, Microalbuminuria, Glycated haemoglobin

INTRODUCTION

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) (Pradeep, 2010).

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) (Flower *et al.*, 1993; Flower *et al.*, 2009; Devarajan *et al.,* 2010). 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 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).

The aim of the study was to evaluate serum NGAL in type 2 diabetic patients with diabetic nephropathy attending Aminu Kano Teaching hospital, Kano.The primary objectiveof the study wastodetermine the relationship if any between NGAL and HbA1c in type 2 diabetic patients with DN

MATERIALS AND METHODS

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.

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.

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.

Study Design

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

Ethical Consideration

Ethical approval was sought and granted by the Ethics Committee of the Aminu Kano Teaching Hospital, Kano to conduct this research. Letter reference number NHREC/21/08/2008/AKTH/EC/2483 dated 29th April, 2019.

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.

Analytical Methods

Estimation of Fasting Plasma Glucose, creatinine and albumin

Fasting plasma glucose was estimated using the glucose oxidase method established by(Trinder, 1969). **The albumin and creatinine were run on**the ABBOTT autoanalyzer using the bromocresol green method (Gustafsson, 1976) and the Jaffe reaction(Jaffe, 1886) respectively.

Neutrophil Gelatinase Associated Lipocalin was analyze using the human NGAL ELISA kit(Stejskal *et al.*, 2008).**Estimation of Glycated Haemoglobin** was analyzed using the fine care equipment for HbA1c rapid quantitative test which is based on fluorescence immunoassay technology(Ejilemele*et al.*,2015).

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).

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.

RESULTS AND DISCUSSION

RESULTS

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

Table 1 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.

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 2 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/1.73 m^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)

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 3. Relationship between Glycated Haemoglobin, Duration of Diabetes Mellitus and NGAL within Diabetes 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 $p < 0.05$.

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.

DISCUSSION

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.

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 in this study, while serum is used 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 occurring 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.

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. There was no correlation between NGAL and glycated haemoglobin reported in this study. A strong correlation between NGAL and eGFR was also observed, this may suggest the routine use of NGAL as biomarker to diagnose DN and also as a prognostic tool for the staging and progression of DN.

RECOMMENDATIONS

It is recommended from the findings of this study that serum NGAL should be incorporated into the routine monitoring of diabetes mellitus patients that are predisposed to DN and a reference range for the NGAL levels should be established for the population to facilitate routine referencing. 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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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
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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