CORRELATIONS BETWEEN GLOMERULAR EXTRACELLULAR BASEMENT MEMBRANE PROTEINS AND GLOMERULAR FILTRATION RATE AMONG PATIENTS WITH TYPE 2 DIABETICS

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ABSTRACT

Background

Diabetic nephropathy is an irreversible complication that occurs in about 40% of type 2 diabetic mellitus (DM) patients. It is characterized by a rapid decline in glomerular filtration rate. Although, microalbuminuria is considered to be first early sign of diabetic nephropathy, other glomerular basement membrane proteins like urinary fibronectin and laminin have been shown to appear in urine before the development of microalbuminuria.

Aim

The aim of this study was to determine the correlation between urinary fibronectin, laminin, microalbuminuria and glomerular filtration rate (GFR) among type 2 diabetes.

Materials And Methods

This is a hospital-based cross-sectional study involving 120 type 2 DM patients without any known complications. Microalbuminuria was measured by an immunoturbidmetric assay while urinary fibronectin and laminin were measured by ELISA technique. The glomerular filtration rate was estimated by the Cockcroft–Gault equation.

Results

A significant negative correlation was found between microalbuminuria, urinary fibronectin, urinary laminin and GFR.

Conclusion

Urinary fibronectin and laminin may be important biomarkers in early diabetic nephropathy.

KEYWORDS: eGFR, Cockcroft-Gault, Urinary Laminin, Urinary Fibronectin.

INTRODUCTION

Diabetic nephropathy is a common micro-vascular complication affecting patients suffering from diabetes mellitus. It is a clinical syndrome characterized by an increasing and continuous excretion of urinary albumin, a persistent fall in glomerular filtration rate (GFR), intra-renal hypertension and increased relative mortality from cardiovascular diseases.[1] About 40% of diabetics develop clinical diabetic nephropathy, leading subsequently to renal failure.[2] The incidence of diabetic nephropathy is higher after a mean duration of diabetes mellitus of 25 years.[3] Once, diabetic nephropathy develops in diabetic patients, it is an irreversible process and the progression cannot be halted.[4] For this reason recent studies are directing their attention on the search of early markers of renal damage that may identify this subset of diabetics that are at risk of diabetic nephropathy.[5] Glomerular extracellular basement membrane proteins like urinary fibronectin and urinary laminin have been investigated to evaluate diabetic nephropathy.[6] These markers have been shown to appear in the urine before the development of microalbuminuria, which is traditionally regarded as the first clinical sign of diabetic nephropathy.[7] Microalbuminuria is the increased urinary excretion of albumin in apparently healthy adults that is greater than normal but is not usually detected by the crude laboratory dipstick method for total protein.[8] It is the stage that precedes overt proteinuria. Microalbuminuria has many limitations chief amongst them is that it occurs when significant renal failure has already

occured.[9]

At the time of initial diagnosis of diabetic nephropathy, there are no significant renal structural abnormalities. Renal plasma flow (RPF) and glomerular filtration rate (GFR), i.e the amount of blood reaching the kidneys and it's ability to sieve metabolic waste products are elevated in about 3 years after diagnosis, and by this time structural changes of diabetic nephropathy are now evident. In the subsequent, 10-15 years, there is progressive renal structural damage but renal hyperfiltration persists and albuminuria is detected in the urine. This is a dangerous sign and marks the beginning of progressive renal insufficiency. At this stage no treatment has been shown to stop the rate of fall of GFR. After about 5 years of detecting albuminuria, about 50% of the patients would have experienced a 50% reduction in the GFR and a doubling of their serum creatinine. The elevated glomerular filtration rate that occurs in 25 to 40 per cent of diabetics have been suggested as having a role in the initiation and evolution of diabetic nephropathy.[10] Studies in laboratory animals have shown that manipulations that evoke glomerular hyperfiltration and hyperfunction fastens and worsens glomerular lesions of diabetes[11] and preliminary evidence in humans suggests that diabetic patients with hyperfiltration lost glomerular function at a much faster rate than in a control group with a normal filtration rate[12].

The series of stages in the development of diabetic nephropathy are as follows: stage 1 (early hypertrophy and hyper-function), stage 2 (glomerular lesion without clinical disease), stage 3 (incipient diabetic nephropathy/microalbuminuria), stage 4 (overt diabetic nephropathy/macrolbuminuria), stage 5 (end stage renal disease).

These five stages correlate well with the classification of chronic kidney diseases in the USA by the National foundation- kidney disease outcome quality initiative (NKF-K/DOQ1)[13] as follows: stage 1(kidney damage with normal or increased GFR; GFR >90 ml/min), stage 2 (mildly decreased GFR; GFR = 60-89 ml/min), stage 3 (moderately decreased GFR ; GFR = 30-59),stage 4 (severely reduced GFR; GFR = 15-29 ml/min), stage 5 (end stage renal failure; GFR < 15ml/min).

Glomerular filtration rate (GFR) is the most suitable measurement used to assess kidney function since it accounts for age, sex and body mass index (BMI). GFR assesses the ability of the one million glomeruli in each kidney to filter blood, process it and excrete metabolic waste products. When the kidneys are damaged by chronic kidney disease, the GFR gradually declines and the amount of kidney function can be estimated or calculated by the GFR. The normal value for an apparently healthy normal sized person is 90-125 ml/min. Currently the two most common methods for determining GFR are creatinine clearance and estimated GFR (eGFR).[14]

A 24 hour urine sample will be required for creatinine clearance. A blood sample is collected anytime during the 24 hour period and then creatinine clearance can be calculated. Creatinine is secreted by the renal tubules and in fact, it is known that creatinine clearance usually overestimates the GFR especially in patients with advanced kidney failure.[15] Creatinine clearance is affected by muscle mass, age and weight.

The formula- derived eGFR is now widely used in clinical practice. In the UK, the National Service Framework for Renal Services recommends the adoption of formula-derived eGFR in the annual evaluation of all patients with diabetes.[16] Similarly the American Diabetes Association recommends estimating the GFR using the Cockcroft-Gault formula[17] corrected for body surface area and the modification of Diet in Renal Disease (MDRD) [18]equation in all patients with diabetes. It is expected that this processes will help to identify patients with diabetic nephropathy early. The Cockcroft–Gault formula[17] for calculating GFR is as follows below.

$$\frac{140 - \text{Age(years)} \times \text{weight (kg)}}{72 \times \text{plasma creatinine (mg/dl)}} \times 0.85 \text{ (if female)}$$

Note: 1 μmol/L creatinine = 0.011mg/dl creatinine

Apart from albuminuria and hypertension, one of the hallmarks of clinical nephropathy is the relentless decline in GFR. It has been suggested that diagnosis of diabetic nephropathy should be attempted before the microalbuminuric stage[5] when it might already be too late. Urinary fibronectin and

laminin which has been shown to appear before the development of microlbuminuria might be helpful in this aspect[6]. The aim of this study was to evaluate the correlation of glomerular extracellular basement membrane proteins (fibronectin and laminin) and GFR among type 2 DM patients.

URINARY ALBUMIN

Albumin which has a molecular weight of 65 KDa is the most abundant plasma protein in the body. The function includes maintenance of oncotic pressure and transportation of many substances in the blood.[19] In healthy people , albumin is too large to cross the glomerular basement membrane, the small amount that manages to pass through the glomerulus is almost completely reabsorbed meaning that urine contains little or no albumin.[20] Elevated urine albumin excretion rate (UAER) in considered a well established marker of glomerualar damage. The UAER in considered normal when it is less than 30 mg/day or 20 μg/min, and classified as microalbuminuria when it is between 30-300 mg/day or 20-20 μg/min and macroalbuminuria when it is above 300 mg/lay or 200 μg/min.

URINARY FIBRONECTIN

Fibronectin is a glycoprotein with a high molecular weight of 440 KDa. It is synthesized by the liver and is a major component of the glomerular extracellular matrix.[21] Fibronectin has many functions including cell adhesion, growth, migration, differentiation, coagulation and tissue repair.[22] Urinary fibronectin levels correlate with progression of biopsy proven glomerular diffuse lesion.[23] The excretion of the products of urinary fibronectin correlates with UAER [24] and more often than not, urinary fibronectin excretion has been shown to have a weak negative correlation with creatinine clearance in patients will overt protinuria[24].

URINARY LAMININ

Laminin is a high molecular weight (≈900 kDa) glycoprotein of the basement membrane; a protein network foundation for most cells and organs.[25] Laminin as a protein contains and α-chain, β-chain and a γ-chian.[26] Due to its, high molecular weight, serum laminin cannot be filtered in the normal glomerulus, and the urinary laminin is derived from the kidneys.[27] Immunohistochemistry, has shown that laminin in found in the mesangial expansion and thickened basement membranes characteristic and diabetic nephropathy.[28] The urinary laminin excretion was also found to correlate with type IV collagen, which is the main constituent of the glomerular basement membrane.[29]

MATERIALS AND METHODS

Study population

This study was a hospital-based cross sectional study conducted at University of Port Harcourt Teaching Hospital, Port Harcourt, Rivers State, Nigeria. The subjects were type 2 diabetic patients without any known complications attending the diabetic clinic of this hospital. 120 of them were recruited using the formula[30] below.

$$N = \frac{Z^2 pq^2}{d^2}$$

Where n = minimum sample size
z= 95% confidence interval= 1.96
P= Prevalence of the target population which is 6.8%[31]
q= 1-p = 0.932
d= degree of accuracy desired (at 0.05)
n= 97.4
The final sample size was adjusted for a 10% non-response rate,[32] giving a projection of 120 subjects. After fulfilling the inclusion and exclusion criteria, we took a detailed case summary and treatment history of the subjects.

Inclusion criteria: diagnosed type 2 diabetics patients of both sexes without any known complications.

Exclusion criteria:

1. Type 1 diabetics
2. Type 2 diabetics with complications
3. Type 2 diabetics on angiotensin converting enzyme inhibitors or angiotensin receptor blockers.
4. Diabetics with overt proteinuria,

All subjects gave their informed written consent and ethical approval for this study was granted by the ethical research committee of the University of Port Harcourt Teaching Hospital.

Materials

We obtained about 20 millilitres fresh early morning urine samples from asymptomatic type 2

diabetic patients for the measurement of urinary albumin, fibronectin, laminin and creatinine. Venous blood samples were also collected for measurements of plasma creatinine. The blood specimen was centrifuged at 1000xg for 15 minutes and plasma obtained for creatinine was stored in the refrigerator for analysis in batches every other day. Urine samples from a -20mls sterile bottles were aliquoted in plain specimen bottles for urinary laminin and fibronectin and centrifuged at 800xg for 10 minutes and stored at – 20^{c} for weekly batch analyses while the rest was used daily for microalbuminuria and urine creatinine measurements. Plasma and urine creatinine was determined using the modified Jaffe method[33] urinary albumin using immunoturbidmetric assay,[34] urinary fibronectin[35] and laminin[36] using ELISA kits. eGFR was calculated using the Cockcroft- Gault equation.[17]

Statistical analysis

We used the statistical package for social sciences (SPPS) version 16.0 for statistical analysis. Data were expressed as mean values and variables were evaluated by Pearson's correlation. A p-value of $\leq$ 0.05 was considered significant.

RESULTS

A total of 120 type 2 diabetic patients without any known complication were recruited for this study. Fifty seven (47.5%) were males while sixty three (52.5%) were females giving a male to female ratio of 1:1.1. The age of the subjects ranged from 24-74 years, mean age was 51.30$\pm$11.06. Majority of the subjects were within 41-50 years age group. Age and sex distribution of subjects is given in table 1.

Table 1: Sex and age distribution of subjects

	Type 2DM (n=120)
Sex	
Male	57(47.5%)
Female	63 (52.5%)
Age group in years	
21-30	4 (3.3%)
31-40	14 (11.7%)
41-50	42 (35.0%)
51-60	34 (28.3%)
61-70	23 (19.2%)
> 70	3 (2.5%)
Mean age (years) 51.03$\pm$ 11.06	DM= Diabetes mellitus

The eGFR in type DM patients was calculated using the Cockcroft-Gault equation. Fifty three (44.2%) of type 2 DM patients had normal/increased GFR >90 ml/min). Forty seven (38.2%) of the type 2 DM patients had mildly decreased GFR 60-89 ml/min) and twenty (16.7%) of the patients had moderately decreased GFR (30-59 ml/min). No patient in stage 4 (severely reduced GFR) or stage 5 (end stage renal failure) was detected in the study. The results are shown in table 2.

Table 2. eGFR in type 2 DM patients

Stage	Description	GFR (ml/min)	Type 2 DM(n=120)
1	Normal/increased GFR	> 90	53 (44.2%)
2.	Mildly decreased GFR	60-89	47 (39.2%)
3	Moderately decreased GFR	30-59	20 (16.7%)
4	Severely reduced	15-29	ND
5	End stage renal failure	< 15	ND

GFR= Glomerular Filtration rate DM = Diabetes Mellitus, ND= Not detected.

(a) Correlation between urinary albumin (microalbuminuria) and GFR in type 2 DM.

We evaluated the correlation between GFR and excretion of urinary albumin (microalbuminuria) by calculating the estimated GFR using Cockcroft-Gault equation. There was a significant negative correlation between the estimated microalbuminuria and GFR (r = - 0.183; P= 0.046) (figure 1)

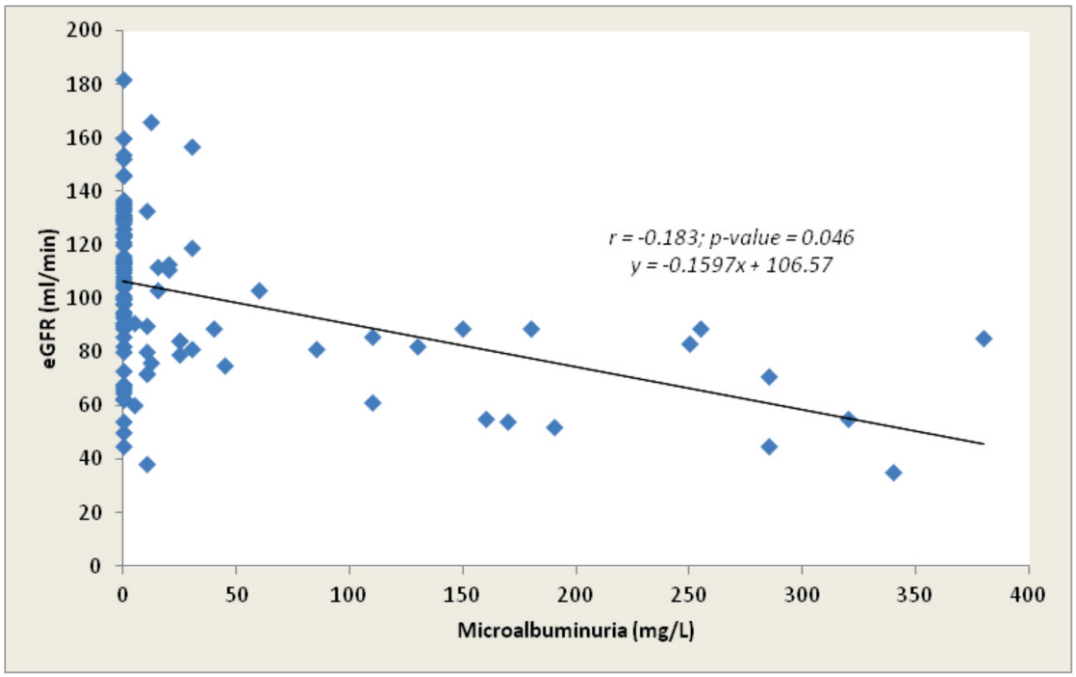

Figure1.Correlation between microalbuminria and GFR in type 2 DM patients (r = - 0.183 ; p = 0.046)

(b) **Correlation between urinary fibronectin and GFR in type 2 DM patients**.

Similarly there was also a negative correlation between eGFR calculated by the Cockcroft-Gault equation and urinary fibronectin (r = - 0.090; p= 0.329 (figure 2). However, this correlation was not significant.

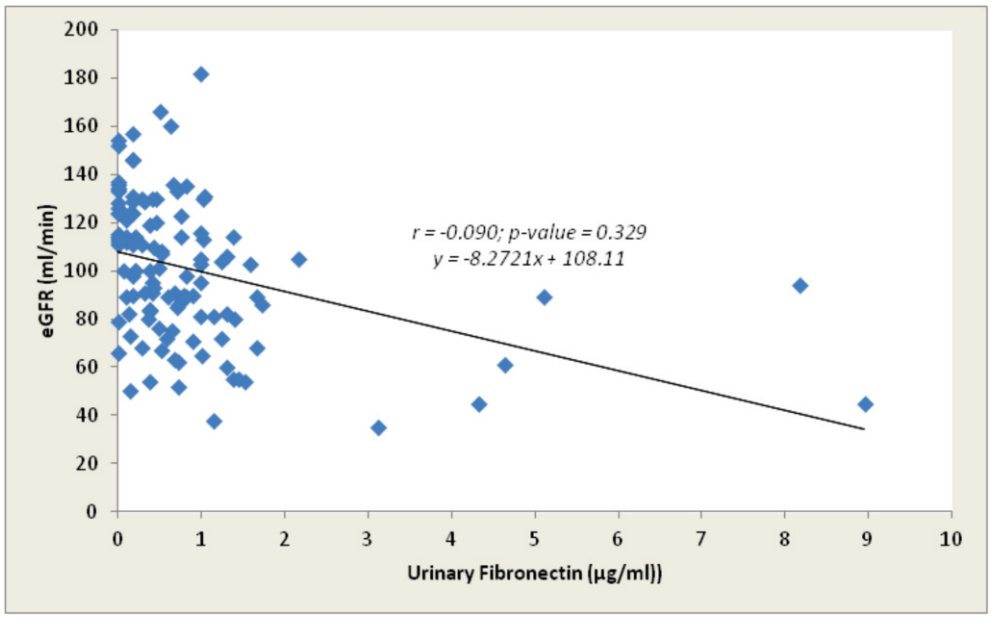

Figure 2: Correlation between urinary fibronectin and eGFR in type 2 DM patients

(c) Correlation between urinary laminin and GFR in type 2 DM patients

We also evaluated the correlation between urinary laminin and GFR and also found that there was a significant negative correlation between eGFR, calculated by Cockcroft-Gault equation and urinary laminin(r= -0.183; p= 0.045).(figure 3)

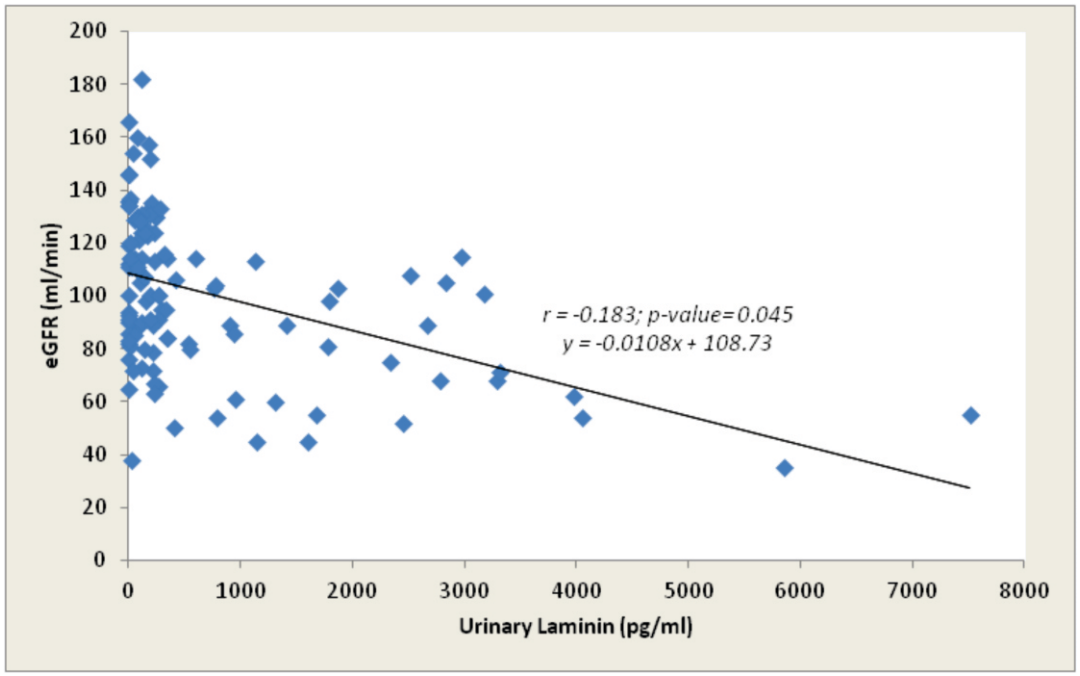

Figure 3: Correlation between urinary laminin and eGFR in type 2 DM patients

DISCUSSION

The importance of calculating the eGFR as recommended by the American Diabetes Association and the UK, National Services Framework for Renal Services in the annual evaluation of all diabetics cannot be over emphasized . The above recommendation is buttressed by the fact that in this study, in which the study subjects were type 2 DM without any known complications, about 39.2% of them already had stage 2 chronic kidney disease while 16.70% had stage 3 chronic kidney disease.

Several methods can be used to evaluate the kidney function, the estimation of GFR by the Cockcroft-Gault equation[17] is one of the most common methods. GFR is the measure of the clearance of exogenous substances (inulin, iohexol, Cr-ethylenediaminetetraacetic acid) and also it is a measure of the clearance of endogenous substances

(creatinine, urea, β_2-microglobulin). In clinical

medicine, serum creatinine is commonly used because it is freely filtered through the glomerulus and it is not reabsorbed by the renal tubules; and also it is comparatively cheap.[37] In this study, the creatinine clearance is represented by serum creatinine measurement, and evaluated by the Cockcroft-Gault equation, which also takes into account the age, weight, and sex of the patient.[17]

The decrease of GFR indicates glomerular injury and the increased excretion of glomerular extracellular matrix proteins such as fibronectin and laminin can be used to predict the GFR and hence the deterioration of kidney function. Early detection of diabetic nephropathy will help to prevent end stage renal failure and generally improve long term outcomes in these patients.

Microalbuminuria is a well recognized predictor of end stage renal disease (ESRD) and is one of the important triad of the components of clinical nephropathy; the others are hypertension and declining GFR.[1] However, declining GFR does not become evident until kidney damage becomes very advanced.[38] In this study, there was significant negative correlation between the GFR and microalbuminuria. This is consistent with findings of Saha[39] *et al* on the correlation between GFR and microalbuminuria.

In our study, we measured the level of urinary fibronectin in type 2 DM patients without any known complications, and evaluated the correlation between urinary fibronectin and GFR. We found that that as the excretion of urinary fibronectin is increased, the GFR correspondingly decreased, albeit not significantly. Our results were disimilar to a study by Kanauchi[40] *et al* in Japan which found a weak inverse correlation between the urinary level of fibronectin and GFR. Urinary fibronectin has been investigated in diabetic nephropathy and has been shown to appear in urine before the development microalbuminuria[6]; the world acclaimed early marker of diabetic nephropathy.

Concerning urinary laminin, we also measured the levels in type 2 diabetics without any known complications, and evaluated the correlation between urinary laminin and GFR. We also found that as the excretion of urinary laminin was increasing, the GFR was decreasing and there was a significant negative correlation. This is in agreement with a study by Jianlin[41] *et al* which found that the level of serum and urinary laminin had significant negative correlation with GFR. Studies has also shown that urinary laminin appears in the urine before the microalbuminuric stage[6]. The correlation results of this study between GFR and urinary laminin is almost identical to that GFR and microalbuminuria, implying that urinary laminin is an important biomarker in the detection of early diabetic nephropathy.

Even though, the correlation between urinary laminin and eGFR is weak, it is nonetheless significant; however urinary fibronectin showed an insignificant inverse association. This could be attributed to the fact that, the study population in this study were type 2 DM patients without any known complications unlike the other studies[39,40,41] that comprised of diabetics with complications.

CONCLUSION

Based on the findings of our study, we believe that urinary fibronectin and laminin has the potential for use as a predictor of GFR and in the assessment of the function of the kidneys in type 2 DM patients. They are thus important biomarkers in early diabetic nephropathy. This will help in early

detection of diabetic nephropathy. We also suggest further prospective studies, with larger patient population size especially in Nigeria as there is none yet.

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