ASSESSING THE CLINICAL UTILITY OF DPP-IV, AMBP AND HP-15 EXOSOMES BIOMARKERS FOR EARLY DETECTION OF DIABETIC NEPHROPATHY IN JIGAWA, NIGERIA

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ABSTRACT

Background: Diabetic nephropathy (DN) with multifactorial pathogenesis is a debilitating complication of diabetes causing socioeconomic burden and reduced quality of life. The emerging urinary exosomes (UEs) are proposed new biomarkers among patients with DN.

Aim: This study aimed at assessing UEs for their clinical utility as potential early diagnostic markers among diabetic patients to prevent or delay the development diabetic nephropathy

Methods: The study recruited 86 Type 2 Diabetic (T2DM) patients attending diabetic clinic at RSTH Dutse, and 86 subjects as controls. Dipeptidyl Peptidase IV (DPPIV), Histone lysine N methyltransferase (HP15) and Alpha 1 microglobulin (AMBP) were assessed using Enzyme linked Immunosorbent assay (ELISA); creatinine, albumin and fasting blood glucose were determined spectrophotometrically. HbA1c was estimated using FIA meter.

Results: The urinary DPPIV, HP15 and AMBP were positively and significantly related to the ACR and HbA1c among T2DM patients. There was a significantly higher DPPIV, AMBP and HP15 in T2DM patients than in control subjects; in microalbuminuric than normoalbumunuric T2DM patients and in normoalbuminuric T2DM patients than control subjects. There was a positive and significant correlation between ACR and DPPIV (r=0.618, p=0.000); HP15 (r=0.598, p=0.000); AMBP (r=0.559, p=0.000). The calculated area under curve (AUC) from receiver operating curve (ROC) found that DPP-IV (0.867), HP-15 (0.852) and AMBP (0.838) revealed high specificity and sensitivity.

Conclusion: The exosomes showed their potentiality and promising as early diagnostic biomarkers of DN compared to microalbuminuria, having DPPIV as the most sensitive. Larger and multicentre prospective studies are needed to confirm their clinical utility as a screening tool for every day practice.

Key words: Diabetic nephropathy, Dipeptidyl Peptidase IV (DPP-IV), Histone lysine N methyltransferase (HP-15), Alpha 1 microglobulin (AMBP), Early diagnostic biomakers

INTRODUCTION

Diabetic nephropathy (DN) is a common complication of diabetes that cause end stage renal disease. Early detection and diagnosis of DN with prevalence of 20% in Jigawa State (Ahmad *et al.*, 2019) is crucial to prevent its progression. The DN prevalence is on increase globally and more is expected in low and middle income countries including Nigeria (IDF, 2021).

Exosomes have emerged as potential urinary biomarkers for various diseases, including DN (Gonzalez-Calero *et al*., 2020; Zhang *et al*., 2021).

DPP IV (dipeptidyl peptidase IV) is parting in the degradation of incretin hormones involved in glucose metabolism. It is localized on glomerular visceral epithelial cells, endothelial cells and the proximal tubule brush border playing role in the pathogenesis of kidney diseases such as IgA nephropathy (Sun *et al*., 2012) that can be used as an early marker for development, diagnosis and progression of diabetic nephropathy (Cristina *et al*., 2016; Lu *et al*., 2020; Jia *et al*., 2021a).

AMBP (Alpha 1 microglobulin protein) in a healthy kidney passes freely through the glomerular membranes and catabolized by proximal tubular cells. Increased AMBP is an early sign of renal damage primarily on the proximal tubules (Miao *et al*., 2021).

HP 15 (Histone lysine N methyltransferase) proves to predict DN because of its role in repression or activation of genes with consequent deregulation property associated with multiple kidney diseases including DN (Jia *et al*., 2021b)

Generally, exosomes are promising as a non-invasive and accurate method for kidney assessment. The study aimed to assess the diagnostic value of some urinary exosomes DPP IV, AMBP and HP in early detection of DN.

MATERIALS AND METHODS

The study was carried out at Rasheed Shekoni Teaching Hospital, Dutse in Jigawa State serving as a referral centre. The state is bordered by Kano and Katsina at the West, by Bauchi at the East and Yobe at the North East (Muhammad *et al*., 2011). It also shares border with Niger Republic. The study population comprised of subjects diagnosed with type 2 diabetes attending diabetic clinic of Rasheed Shekoni Teaching Hospital, Dutse, who consented to participate. Apparently healthy subjects were also included to serve as control.

Ethical approval was sought from the Ethical Committees of Rasheed Shekoni Teaching Hospital, Dutse. Informed consent was also sought from all subjects in accordance with the ethical guidelines of the Ethics Committees of Rasheed Shekoni Teaching Hospital, Dutse. The nature of the study was fully explained to the subjects in an appropriate language before inclusion in the study and collection of blood sample. A questionnaire was developed for the study and was administered to each subject including medical history, social habits, health status and familial history of diabetes.

Sample size determination

The sample size was calculated using the following formula

$$N = \frac{(1.96)^2 \times (0.0577) \times (1 - 0.0577)}{(0.05)^2}$$

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

=86

Where N = number of samples;
Z = 95% confidence interval
p = prevalence of diabetes mellitus (5.77%) (Uloko *et al*., 2018).
q = (1-P)
d = error margin 5% (0.05)

The minimum sample size was approximately 86 and therefore 86 subjects were recruited along with 86 apparently healthy controls.

Anthropometric measurement: 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 according to Nutall (2015).

Study protocol: Subjects that volunteered to take part in the study were instructed to be on their normal diet and observe an overnight fasting before sample collection for fasting glucose.

Sample Collection, Processing and Preservation: Five milliliters (5mLs) of blood sample was collected from each subject through the ante-cubital vein after an overnight fast lasting for 10 hours. The sites of collection was aseptically cleaned with 70% alcohol swab and allowed to dry before blood was collected.

Three (3) milliliters of the blood was transferred into fluoride oxalate bottles for immediate fasting glucose analysis and the remaining harvested plasma was stored at -20^0C for future analysis. The remaining 2mLs of whole blood was transferred into an ethylene diamine tetra-acetic acid (EDTA) bottle for immediate glycated hemoglobin estimation.

Spot urine sample was also collected from subjects for dipeptidyl peptidase IV (DPP IV), histone-lysine N-methyltransferase (HP 15) and alpha 1 microglobulin (AMBP), creatinine and albumin estimation and frozen at -20^0C prior to analysis.

Analytical Methods

Serum glucose, urinary Creatinine and urinary Albumin by bromocresol green method were measured spectrophotometrically using Randox test kits. Glycated Hemoglobin (HbA1c) was determined using Fine care HbA1c rapid quantitative test with FIA meter. Urinary AMBP, HP-15 and DPP IV were estimated using human specific double antibody sandwich enzyme-linked immunosorbent (ELISA) assay kit as supplied by Melsin Company, China limited.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS version 25) software. Comparisons of variables measured for various patient groups and that of healthy subjects was determined using student t-test. Relationship between microalbuminuria, HbA1c and dipeptidyl peptidase IV (DPP IV), histone-lysine N-methyltransferase (HP 15) and alpha 1 microglobulin (AMBP) among the patients was determined by Pearson's correlation and Receiver Operator Curve (ROC) was used to test the sensitivity and specificity of the methods. A p-value of < 0.05 was considered statistically significant.

RESULTS

Urinary DPPIV activity levels of diabetic subjects (182.99±124.08) are statistically higher than that of control subjects (61.37±17.10); p value 0.000. Likewise, the activity levels of HP15 (67.63±43.69) and AMBP levels (3.70±2.39) among diabetic subjects are significantly higher than those of control subjects (27.12±6.91 and 1.21±0.31 respectively) p values 0.000 and 0.000 respectively. These are presented in table 1.

As shown in table 2, a Pearson product-moment correlation was conducted to examine the relationships between ACR and urinary exosomes (DPP IV, HP 15 and AMBP). The results revealed moderate positive correlation for DPP IV (r=0.618, p=0.000), HP 15 (r=0.598, p=0.000) and AMBP (r=0.559, p=0.000).

In table 3, Urinary DPPIV activity levels of normoalbuminuric diabetic subjects (130.75±53.80) are statistically higher than that of control subjects (61.43±15.93); p value 0.000. Likewise, the activity levels of HP15 (49.91±20.45) and AMBP levels (2.97±1.30) among normoalbuminuric diabetic subjects are significantly higher than those of control subjects (27.55±7.30 and 1.22±0.31 respectively) p values 0.000 and 0.000 respectively.

Table 4, shows that the diabetic patients were subdivided according to the level of microalbuminuria of which 67.4% are normoalbuminuric, 32.6% have albuminuria values ranging from 30-300mg/g (microalbuminuria) 0% have albuminuria of >300 mg/g (macroalbuminuria). The values of DPPIV, HP15 and AMBP of diabetic patients with normoalbuminuria of < 30 mg/g were 133.03±53.76, 49.97±20.42 and 2.75±1.03 respectively. The levels of urinary DPPIV, HP 15 and AMBP of diabetics with microalbuminuria of 30-300 mg/g were 286.48±160.93, 104.21±55.41 and 5.53±3.18 respectively.

Finally, fig 1 present the Receiver Operating Characteristics (ROC) curves of DPP1V, HP 15, and AMBP was determined to predict the specificity and sensitivity of these variables for their roles as early markers of diabetic nephropathy. This was done by plotting sensitivity (x-axis) against 1-sensitivity (y-axis). The area under the curve (AUC) calculated for exosomal DPP1V, HP 15 and AMBP in discriminating DN patients from non-DN patients and healthy controls were 0.867, 0.852 and 0.838 respectively. The combination of these three exosomal markers improved the AUC to 0.92, with a sensitivity of 88% and a specificity of 85%.

Table 1: Comparison of Urinary Levels (Mean±SD) **of DPPIV, HP15 and AMBP between Diabetic and Control Subjects**

Parameters	Diabetic (n=86)	Controls (n=86)	p-value
DPPIV (pg/mL)	182.99±124.08	61.37±17.10	0.000
HP15(ng/mL)	67.63±43.69	27.12±6.91	0.000
AMBP (ng/mL)	3.70±2.39	1.21±0.31	0.000

Key: DPP IV = Dipeptidyl peptidase IV; Hp 15 =Histone-lysine N-methyltransferase, AMBP = Alpha 1 microglobulin; p value <0.05 is statistically considered significant; *paired t-test

Table 2: Relationship of DPPIV, HP15 and AMBP with Albumin-Creatinine Ratio among T2DM Patients

Independent variable	Dependent variables	Correlation (r)	p-value*
ACR(mg/g)	DPPIV (pg/mL)	0.618	0.000
	HP15(ng/mL)	0.598	0.000
	AMBP (ng/mL)	0.559	0.000

Key: DPP IV = Dipeptidyl peptidase IV; Hp 15 =Histone-lysine N-methyltransferase, AMBP = Alpha 1 microglobulin,*Pearson's correlation

Table 3: Comparison of (mean±SD) Urinary Levels of AMBP, HP15 and DPPIV between Normoalbuminuric T2DM Patients and Control Subjects

Parameters	Normoalbuminuria (n=58)	Control (n=86)	*P value
DPPIV (pg/mL)	130.75±53.80	61.43±15.93	0.000
HP15(ng/mL)	49.91±20.45	27.55±7.30	0.000
AMBP(ng/mL)	2.97±1.30	1.22±0.31	0.000

Key: DPP IV = Dipeptidyl peptidase IV; Hp 15 =Histone-lysine N-methyltransferase, AMBP = Alpha 1 microglobulin, * paired t-test

Table 4: Comparison of Urinary DPPIV, HP 15 and AMBP Based on Albuminuria Levels among T2DM Patients

Parameters	Normoalbuminuria n(%)=58(67.4)	Microalbuminuria n(%)=28(32.6)	p-value*
DPPIV (pg/mL)	133.03±53.76	286.48±160.93	0.000
HP15(ng/mL)	49.97±20.42	104.21±55.41	0.000
AMBP(ng/mL)	2.75±1.03	5.58±3.18	0.000

Key: DPP IV = Dipeptidyl peptidase IV; Hp 15 =Histone-lysine N-methyltransferase, AMBP = Alpha 1 microglobulin,* independent t-test

DISCUSSION

In Diabetes, renal complication is induced by hyperglycemia through the production of Advanced Glycated End Products (AGEs) that bound to the tissue protein such as matrix proteins (type IV collagen) and impairs their degradation by matrix metallo-proteinases. Alternatively, the complication may also be induced via interacting with the receptor for AGEs expressed by podocytes and endothelial and messengial cells in the kidneys leading to basement membrane thickening, glomerular extracellular matrix accumulation, the effacement of podocytes and tubulointerstitial fibrosis (Daroux *et al*., 2010; Mahfouz *et al*., 2020). These factors initiate pathological changes via the activation of a cascade of mediators at different stages during the systematic progression of the disease, such as high molecular weight proteins (albumin), cytokines, growth factors and exosomes (Campion *et al*., 2017).

Our finding showed that the urinary levels of DPP IV activity in T2DM subjects was significantly higher than controls ($p<0.000$), it also significantly and positively correlates with ACR in T2DM patients; a finding which is in line with that of Sun *et al.* (2012).

This study also revealed a significant difference in the levels of urinary DPPIV activity between normoalbuminuric and microalbuminuric T2DM patients signifying a positive association between urinary levels of DPPIV with severity of DN. These results suggest that measurement of urinary DPP IV might be a useful assessment for DN screening, diagnosis and probably a prognosis in patients with T2DM at different stages. As previously reported by Musante *et al.* (2014) that the activity of dipeptidyl peptidase IV (DPP IV/CD26) in urinary exosomes emanating from proximal tubule cells positively correlate with the progression of DN in type 2 diabetic patients thereby suggesting an early involvement in tubular impairment and hence can be considered as an early marker of kidney damage that preceded the onset of microalbuminuria.

In this study, urinary HP 15 level was significantly higher ($p<0.005$) than the control subjects. Increased value among diabetics may be due to histone lysine methylation observed by Reddy *et al.* (2012) which is involved in the regulation of key fibrotic and inflammatory genes related to DN. Another supporting evidence of HP 15 implication in DN was experimented by both El-Osta *et al.* (2008) and Brasacchio *et al.* (2009) in which transient hyperglycemic cell culture models was used causing pleiotropic transcription of factor *NF-κB* subunit P65 with consequent sustained and increased level of HP 15 in the proximal promoter region of the p65 gene. In another animal model study, Sayyed *et al.* (2010) demonstrated that uninephrectomized db/db mice kidney HP 15 level was increased in accordance with glomerular cell proliferation, albuminuria and glomerular rate (GFR) reduction. Our finding showed that there is a significant correlation between HbA1c and HP15 which is supported by experimental studies including cell and animal models as well as clinical studies that have clearly revealed deleterious results of hyperglycemia and importance of good glucose control in preventing the onset or progression of DN (Yu *et al*., 2019).

In this study, the level of urinary AMBP was significantly increased in diabetic patients as compared to control subjects and this is because of its low molecular weight. The free form of alpha-1 microglobulin is filtered freely through the renal glomerular basement membrane and reabsorbed by the proximal tubular cells (Mahfouz *et al*., 2020). Hence, any proximal tubular cell dysfunction results in increased quantities of alpha-1 microglobulin in urine. AMBP, also positively and significantly correlates with ACR among T2DM patients ($p<0.05$). This finding corroborate with the report of Korpinen *et al.* (2000), Shore *et al.* (2010), Niklov *et al.* (2013), Petrica *et al.* (2015), Saif *et al.* (2016), Zhang *et al.* (2018) and Mahfouz *et al.* (2020).

The urinary excretion of AMBP was also significantly higher in microalbuminuric T2DM patients when compared to normoalbuminuric patients and controls, indicating tubular damage and dysfunction at an early stage of DN a finding which is supported by Kalasooriya *et al.* (2007), Niklov *et al.* (2013), Petrica *et al.* (2015) and Mahfouz *et al.* (2020). Our study further showed that diabetic patients with normoalbuminuria excreted significantly higher levels of urinary AMBP compared to control subjects. This was supported from a cross-sectional study reported by Hong *et al.* (2003). He found that 33.6% patients with normoalbuminuria presented an increased value of urinary AMBP among 590 type 2 DM patients with a much higher percentage of 67.4% from our study among normoalbuminuric had an elevated level of AMBP. This fact could be explained by a tubular injury preceding the occurrence of microalbuminuria. However, Hong *et al.* (2003) noted that AMBP can be absent in some patients with albuminuria. Wainai *et al.* (1991) concluded that AMBP could predict and serve as inexpensive urinary biomarker of early diagnosis of DN. Zhang *et al*. (2018) also reported a similar finding that urinary levels of AMBP in patients with normoalbuminuria were significantly higher compared with those of the control subjects and were both associated with the levels of HbA1c. Urinary AMBP was also reported to increase in 27.9% normoalbuminuric type 2 diabetic patients further supporting that AMBP precedes the onset of albuminuria and may serve as a marker in early DN (Petricia *et al*. 2011). Other supporting evidences are that of Shore *et al.* (2010) where AMBP correlates with HbA1c. Increased AMBP excretion was directly correlated with HbA1c and albuminuria and was decreased with improved glycemic control in Caucasians (Hofmann *et al.*, 1989; Martin *et al.,* 1990). Similar evidences have also been reported in Asian population (Hong *et al.*, 2003).

Results from ROC suggest that DPP IV, HP 15 and AMBP could be very significant biomarkers in monitoring renal complications associated with DM earlier than some of the existing variables. This was done to test their sensitivity and specificity in predicting DN. The calculated AUC proved DPP IV to be the most predominant with highest values followed by HP 15 and AMBP respectively. Few studies reported AMBP to be the most sensitive (Shore *et al.,* 2010; Zhang *et al*., 2018). This disparity observed may be due to method of UEs estimation used, method of sample collection, sample size, genetic make-up of study population and geographical location of the study subjects.

This present study observed a moderate positive correlation between urinary exosomes (AMBP, HP 15 and DPP IV) and HbA1c among diabetic subjects signifying increase in urinary exosomes with persistent elevation of glucose. The findings of which agrees with the recent studies by Zhang *et al.* (2018) and Sinha *et al.* (2020) that reported an excessive elevation of AMBP, HP 15 and DPP IV with increased HbA1c among diabetics.

CONCLUSION

Our findings suggested that exosomal DPP IV, AMBP and HP 15 levels have potential as early diagnostic biomarkers for DN. The combination of these biomarkers shows promise in improving the diagnostic accuracy of DN and the ROC revealed their good sensitivity and specificity. Further validation in larger cohorts is warranted to confirm these findings and explore the clinical utility of these exosomal markers in DN screening, diagnosis and monitoring and their possible clinical utility at a very much earlier stage before the development of microalbuminuria.

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