International Journal of Research in Medical Sciences
Sasikala T et al. Int J Res Med Sci. 2019 Jun;7(6):2015-2021
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012
DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20192475

Original Research Article

Urinary brush border enzymes for early diagnosis of tubular dysfunction in patients with type 2 diabetes mellitus

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Received: 16 March 2019
Accepted: 03 May 2019

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ABSTRACT

Background: Diabetic nephropathy is a major cause of premature morbidity and mortality in type 1 and type 2 diabetes mellitus (T2DM) and hence new markers with better sensitivities are being investigated. The study was taken up to investigate whether urinary activities of N-acetyl-β-D-glycosaminidase (NAG), alkaline phosphatase (ALP), lactate dehydrogenase LDH) and Gamma glutamyl transferase (γ-GT) can be used as screening markers of renal dysfunction in patients suffering from T2DM.
Methods: One hundred and four patients with T2DM along with 30 age- and gender-matched healthy individuals were included in the study. Patients were divided into three groups based on their u-MA levels i.e. normoalbuminuric (group1), micro albuminuric (group 2) and macroalbuminuric (group 3).
Results: Urinary enzymes activity was significantly higher in patients with T2DM compared to controls ($p<0.05$). NAG, ALP, LDH, and GGT were significantly higher in group 3 compared to group1 and group 2 ($p<0.0001$). NAG, ALP, LDH and GGT showed significant positive correlation with MA ($p=0.0001$, $r=0.308$; $p=0.0001$, $r=0.369$; $p=0.002$, $r=0.304$, $p=0.044$, $r=0.202$ respectively). GGT and LDH showed highest sensitivity (86.21%, 84.00% respectively) and specificity (78.57%,53.49% respectively) for diagnosing renal dysfunction in patients with normoalbuminuria.
Conclusions: The study suggests that u-GGT and LDH can be useful markers for assessing renal dysfunction in T2DM patients even before microalbuminuria manifests.

Keywords: Alkaline phosphatase, Diabetic nephropathy, Gamma glutamyl transferase, Lactate dehydrogenase, N-Acetyl-β-D-glucosaminidase

INTRODUCTION

Diabetic nephropathy (DN) is one of the micro vascular complications of diabetes mellitus (DM) and a major cause of premature morbidity and mortality in type1 and T2DM.[1] Several glomerular and tubular biomarkers predicting onset or progression of DN have been identified and are becoming increasingly important in clinical diagnostics. Commonly used markers for diabetic nephropathy are blood urea, serum creatinine and urinary micro protein.[2] These markers are insensitive and there is a time delay between renal injury and detection. Glomerular filtration rate (GFR) calculated using the modification of diet in renal disease (MDRD) equation is an indicator of impaired renal function. However, MDRD formula tends to underestimate GFR at levels greater than 60 mL/min.[3] Microalbuminuria (MA) is an established marker of DN and it has been used for many years as a predictor of incipient DN.[4,5] However, it has been reported that a large proportion of renal impairment occurs even before appearance of microalbuminuria.[6] Hence more sensitive biomarkers which can detect renal

impairment at an early stage are required. Changes in the renal tubules, which may be termed diabetic tubulopathy, are increasingly being implicated in the development of progressive diabetic kidney disease.[7,8] It has been reported that, in addition to the glomeruli, the renal tubules are heavily involved in the pathogenesis of DN.[9,10] Some studies have demonstrated that tubular damage markers increase in patients with diabetes even before diagnosis of microalbuminuria.[11-13] Serum cystatin C and some of the potential urinary biomarkers of renal tubular injury include brush border enzymes alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), and lysosomal enzymes N-acetyl beta D-glucosaminidase (NAG) and other markers including Neutrophil gelatinase associated lipocalin and β2-microglobulin.[4,14,15] Urinary enzymes are released following tubular epithelial cell damage and excreted in the ultra-filtrate and thus cause the enzyme activities to increase in urine.[5] These can thus enable detection of subclinical tubular injury.[16] More sensitive urinary biomarkers which could be used to detect nephrotoxicity at early stages on various parts of nephron are being investigated. It has been shown that early renal tubular damage is characterized by increased proximal tubular enzymuria.[17-22] However, contradictory findings were noted.[15,23] Hence it needs to be further studied. Thus, the study was taken up to investigate whether urinary activities of NAG, ALP, LDH, and GGT can be used as early predictors for tubular dysfunction and screening biomarkers of renal dysfunction in patients suffering from T2DM and their associations with T2DM and severity of microalbuminuria.

METHODS

The study included 104 patients with T2DM according to World Health Organization criteria attending the Endocrinology outpatient department of Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India, during the period August 2012 to April 2013.[24] Patients were divided into three groups based on their u-MA levels i.e. normoalbuminuric (group 1), microalbuminuric (group 2) and macroalbuminuric (group 3). Thirty age-and sex-matched healthy individuals from among the patient's relatives and hospital staff was taken as controls. Patients suffering from hypertension, urinary tract infections, cerebrovascular diseases, and renal parenchymal diseases, use of nephrotoxic drugs, smoking, and patients not willing to participate in the study were excluded from the study. The study was approved by the institutional ethics committee. Subjects satisfying the inclusion criteria were included after obtaining a written informed consent. A sample size calculation was performed based on previous studies. A sample size of 30 was obtained at p value 0.05 and CP power 90.[25]

Four ml of peripheral venous blood was collected in a fasting state from both cases and controls. Of this 1 ml blood was transferred into fluoride bulb and the remaining was transferred into a plain vial. A spot urine sample was also obtained at this time point. Oral glucose tolerance test was performed in the control subjects to rule out type 2 DM. Blood and urine samples were centrifuged at 2000rpm for 15min. The separated plasma, serum and urine were stored at -80ºC until analysis.

Plasma glucose (Coral diagnostics, Goa, India), Creatinine, u-GGT (Randox, UK), urinary MA, ALP and LDH (Beckman system packs, CA, USA) were analyzed on Synchron CX9 fully automated analyzer (Beckman-coulter, CA, USA). Creatinine was measured by Jaffe's rate method with calibration traceable to isotope dilution mass spectrometry (IDMS) reference method using the National institute of standards and technology (NIST) Standard reference material 967. [26] HbA1c was estimated by high performance liquid chromatography (HPLC) method (Bio-Rad). u-NAG was estimated by spectrophotometry stop rate reaction using 4-nitrophenyl N-acetyl β-D glucosaminide (N9376) (Sigma-Aldrich, Co; St. Louis, MO, USA) as a substrate by using Lambda 25 UV-visual double beam spectrophotometer (Perkin Elmer, Singapore). eGFR was calculated for all the patients according to the Cockcroft-Gault formula (CG) (eGFR (mL/min/1.73m^2) =(140-age) x weight (kg) 72 x serum creatinine (mg/dL) x (0.85 if female).[26]

Statistical analysis

Data distribution was studied by using Kolmogorov Smirnov test. Data obtained was expressed as mean± SD for data showing a normal distributed, median inter quartile range for data which showed a non-normal distribution. Urinary analytes were corrected for creatinine to nullify the effect of urine volume changes over time which can influence their interpretation. Differences in all biochemical parameters studied among study and control groups were tested using parametric independent samples T test. Data for urinary enzymes was normalized by logarithmic transformation for comparisons. Difference in markers among the groups were tested using analysis of variance (ANOVA) followed by post hoc test for pair wise comparisons. Pearson's correlation or Spearman rank correlation analysis was done to study the correlations among the parameters as appropriate. Receiver operative characteristic curve (ROC) analysis was performed to study diagnostic utility of markers studied. Statistical analysis was performed using Microsoft Excel Spread Sheet (Microsoft Redmond, USA), Med Calc (version 13.2.2, Belgium) and SPSS for windows version 16.0 (SPSS Inc, Chicago, IL, USA). A 'p' value of <0.05 was considered as statistically significant.

RESULTS

Table 1 shows the baseline characteristics of the groups studied. Patients with T2DM had significantly higher levels of plasma glucose, serum creatinine and urinary microalbumin compared to the control group (p=0.001,

p=0.025, p=0.0001 respectively). Urinary creatinine levels were significantly lower (p=0.008) in patients with T2DM when compared to the control group.

Comparison of urinary enzymes between the patients with T2DM and control group

Table 2 shows the activity of urinary enzymes in the groups studied. Activity of all the enzymes studied i.e. u-NAG, GGT, ALP and LDH corrected for urinary creatinine were significantly higher in patients with T2DM compared to the control group (p<0.001).

Table 1: Baseline characteristics of the control group and T2DM patients.

Parameters	Controls (n=30)	T2DM patients (n=104)	p value
Age (years)	50.00±6.95	55.5±11.4	0.525
Sex M/F	17/13	55/49	1.000
FBG (mg/dL)	92.3±8.69	140.0±48.2	0.001†
PPBG (mg/ dL)	118.33±12.12	199.5±65.7	0.001†
S.Cr (mg/dL)	0.66±0.15	1.62±2.80	0.025†
U. Creatinine(mg/dL)	98.6±33.4	65.1±33.7	0.008†
U.Microalbumin (mg/g Cr)	4.6±3.3	26.7*(9.6-116.9)	0.0001†

Data presented as Mean ± Standard deviation for normally distributed parameters and * median (95% CI) for skewed data; n= number; M = male; F= female; FBG =fasting blood glucose; PPBG= postprandial blood glucose; S. Cr=serum creatinine; U. Cr=urinary creatinine; U. microalbumin=urinary microalbumin; † statistically significant.

Table 2: Urinary enzymes in the control group and T2DM patients.

Parameters	Controls (n=30)	T2DM patients (n=104)	p value
U. NAG (U/g Cr)	4.7 ± 4.5	7.9* (4.82- 12.95)	0.0001†
U. GGT (U/g Cr)	14.8 ± 11.5	27.50*(20.0- 37.10)	0.0001†
U. ALP (U/g Cr)	3.32±2.6	5.48* (3.84-9.29)	0.0001†
U.LDH (U/g Cr)	18.52± 12.1	33.60* (21.47-57.1)	0.0001†

Data presented as Mean ± Standard deviation for normally distributed parameters and * median (95% CI) for skewed data; n= number; U.NAG= Urinary N-acetyl-β-D-glucosaminidase; U.ALP= Urinary alkaline phosphatase; U.LDH= Urinary lactate dehydrogenase; U.GGT= Urinary gamma glutamyl transferase; † statistically significant.

Table 3: Urinary enzymes in the T2DM group classified based upon the levels of urinary microalbumin.

		T2DM patients			p value
Parameters	**Controls(n=30)**	**Group 1 (n=56)**	**Group (n=30)**	**Group 3(n=18)**	
U. Microalbumin (mg/g Cr)	4.6±3.3	11.19* (5.6-17.6)	39.51*(38.8-133.0)	998.18*(482-2040.3)	0.0001†
U. NAG (U/g Cr)	4.7±4.5	6.05* (4.37-8.81)	11.24* (6.67-19.76)	13.19*(7.37-27.75)	0.0001†
U. GGT (U/g Cr)	14.8±11.5	26.12*(13.6334.70)	30.92*(22.59-34.96)	47.77*(21.50-68.21)	0.0001†
U. ALP (U/g Cr)	3.32±2.6	4.79*(2.66-7.07)	6.27*(4.37-10.0)	12.5*(7.5-42.38)	0.0001†
U. LDH (U/g Cr)	18.52±12.1	30.4*(17.68-47.19)	33.84*(21.13-54.41)	51.55*(34.52-153.51	0.0001†

Urinary enzymes were log transformed before analysis. Data presented as Mean ± Standard deviation for normally distributed parameters and * median (95% CI) for skewed data; n= number; Group1=Normoalbuminuria (<30mg/g Cr); Group 2= Microalbuminuria (30-300 mg/g Cr); Group 3= Macroalbuminuria >300 mg/g Cr); U.NAG= Urinary N-acetyl-β-D-glucosaminidase; U.ALP=Urinary alkaline phosphatase; U.LDH= Urinary lactate dehydrogenase; U.GGT=Urinary Gamma Glutamyl Transferase; U. Microalbumin=urinary microalbumin; † statistically significant.

Further, the excretion of the urinary enzymes in the T2DM was compared after classifying the patients based on their u-MA levels into three groups i.e. normoalbuminuric (group 1), microalbuminuric (group 2) and macroalbuminuric (group 3).

Table 3 shows the activity of the urinary enzymes in these three groups. (Group 3) i.e. patients with macroalbuminuria had significantly higher excretion of NAG and ALP compared to group 1 i.e. patients with normoalbuminuria and group 2 i.e. patients with microalbuminuria (p=0.0001). Excretion of NAG and ALP paralleled MA excretion. Excretion of GGT increased across these groups being highest in group 3 i.e. patients with macroalbuminuria than in group 1 i.e. patients with normoalbuminuria and group 2 i.e. patients with microalbuminuria (p=0.0001). Excretion of LDH was found to be higher in group 3 compared to group 1 and group 2 (p=0.0001).

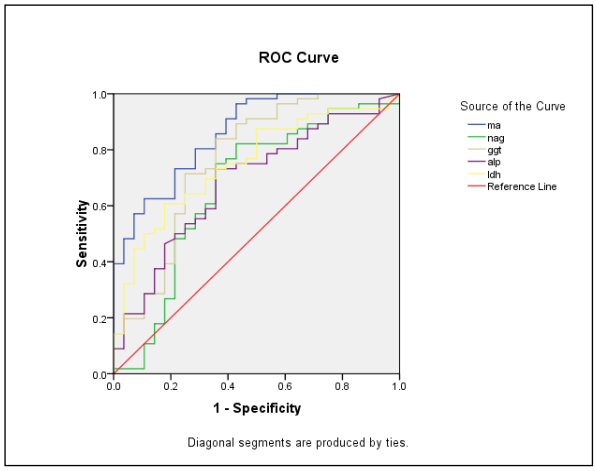
Table 4: Spearman's rank correlation analysis of urinary biomarkers in T2DM patients.

Parameters	Parameters	r value	p value
Duration of diabetes	MA	0.164	0.100
	U.NAG	0.254**	0.010**
	U.GGT	0.130	0.190
	U.ALP	0.055	0.583
	U.LDH	0.142	0.994
HbA1c	MA	0.182	0.065
	U.NAG	0.114	0.251
	U.GGT	0.177	0.074
	U.ALP	0.122	0.217
	U.LDH	0.043	0.668
MA	U.NAG	0.308**	0.0001**
	U.GGT	0.202*	0.044*
	U.ALP	0.369**	0.0001**
	U.LDH	0.304**	0.002**

**Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed); r = spearman correlation coefficient; U.NAG= Urinary N-acetyl-β-D-glucosaminidase; U.ALP: Urinary alkaline phosphatase; U.LDH= Urinary lactate dehydrogenase; U.GGT=Urinary gamma glutamyl transferase; MA= Microalbumin.

Table 4 shows the Spearman's rank correlation analysis of urinary biomarkers in patients with T2DM with duration of diabetes, glycemic control and u-MA. u-NAG showed a significant positive correlation with duration of diabetes (p=0.010, r=0.254). No correlation was found with other markers. u-MA showed a significant positive correlation with u-NAG, ALP, LDH and GGT (p= 0.0001, r=0.308; p= 0.0001, r=0.0.369; p=0.002, r=0.304, p=0.044, r=0.202 respectively).

To investigate the diagnostic utility of urinary enzymes in patients with normoalbuminuria, ROC curve analysis was performed (Figure 1).



NAG= Urinary N-acetyl-β-D-glucosaminidase; ALP=Urinary alkaline phosphatase; LDH= Urinary lactate dehydrogenase; GGT= Urinary gamma glutamyl transferase.

Figure 1: Receiver operating characteristic curve analysis of urinary biomarkers in patients with T2DM with normoalbuminuria.

The control group was taken as the reference group. All the markers studied had significant area under the curve (AUC) with u-MA having the highest AUC. The performance of all the markers derived from ROC analysis in terms of sensitivity and specificity is tabulated in (Table 5).

Table 5: Sensitivity and specificity of urinary biomarkers in T2DM with normoalbuminuria.

Parameter	AUC	Sensitivity	Specificity	Significance	95% confidence intervals
U.MA	0.860	96.4	57.1	0.0001	0.761-0.926
U.NAG	0.675	75.0	66.7	0.0087	0.565-0.772
U.GGT	0.813	86.2	78.5	0.0001	0.735-0.876
U.ALP	0.681	73.2	63.3	0.0029	0.571-0.777
U.LDH	0.758	84.0	53.4	0.0001	0.654-0.844

U.NAG= Urinary N-acetyl-β-D-glucosaminidase; U.ALP: Urinary alkaline phosphatase; U.LDH= Urinary lactate dehydrogenase; U.GGT= Urinary gamma glutamyl transferase; MA= Microalbumin.

As shown in Table 5, the diagnostic performance of u-GGT was the best (AUC 0.813) followed by u-LDH, u-ALP and u-NAG (AUC 0.758, 0.681, 0.675 respectively).

Calculation of cut-off value of GGT and LDH for the detection of tubular dysfunction: The ROC-curve for calculating the optimal cut-off value of GGT and LDH for detection of tubular dysfunction is shown in Figure 1. At a cut-off value of GGT greater than 12.32, the sensitivity and specificity were 86.21 [95% confidence intervals (CI), (74.62-93.85) and 78.57 (95% CI, 59.05-91.70), respectively. At a cut-off value of LDH greater than 25.44, the sensitivity and specificity were 84.00 [95% confidence intervals (CI), 69.30 - 93.19) and 53.49 (95% CI, 37.65 - 68.82), respectively.

DISCUSSION

The findings of the present study show that patients with T2DM have significantly higher excretion of tubular enzymes compared to the control group and these

enzymes are increased even before microalbuminuria sets in suggesting early tubular dysfunction.

Urinary excretion of enzymes (NAG, GGT, ALP and LDH) was significantly higher in the patients with T2DM compared to the control group. This is in agreement with previous reports.[4,16,17,23,27] Oxidative stress has been considered a common pathogenic factor in T2DM and its complications. Hyperglycemia leads to enhanced reactive oxygen species production and as a result tubular cell damage and urinary enzyme excretion develops.[28,29]

Microalbuminuria, a marker of glomerular dysfunction was found to be higher in patients with T2DM compared to controls (p=0.0001). It has been proposed that the damage to renal tubules differs in relation to the presence or absence of proteins in patient's urine and that the quantity of proteins in glomerular filtrate reflects the severity of damage.[27] Hence the excretion of urinary enzymes was further assessed in relation to the degree of proteinuria in patients with T2DM. Excretion of u-NAG was found to progressively increase in patients with T2DM from normoalbuminria to macroalbuminuria i.e patients with macroalbuminuria excreted significantly higher compared to patients with micro and normoalbuminuria.

u-NAG is present in lysosomes of the proximal tubular epithelial cells, which metabolizes glycoproteins. As a consequence of proximal tubule lesions there is release of these enzymes in the urine.[28] Similarly, other authors have reported increased excretion of u-NAG in normolabuminuric diabetic patients.[9,30-32] Studies have shown that u-NAG excretion increases even before microalbuminuria commences.[32,33] However in the present study, u-NAG activity was found to increase in parallel with MA, which is evident from significant positive correlation between NAG and MA. Our finding is in agreement with that reported by Cohen-Bucay A et al.[34]

The location and function of the brush-border membrane make it a good target for the primary involvement in the pathogenesis of diabetic renal complications. GGT is a brush-border enzyme, which reflects damage to proximal tubules. In the present study, GGT activity increased across the diabetic group being highest in macroalbuminuria than in micro and normoalbuminuria groups. The present study found a significant positive correlation between GGT and MA. This finding is in agreement with previous studies who reported increased urinary GGT excretion in both normo and microalbuminuria diabetic patients compared to controls.[16,17] This indicates the usefulness of urinary GGT as a better marker for prediction of diabetic nephropathy. ROC analysis also supports our finding (Figure 1).

Excretion of ALP and LDH, other brush-border enzymes in urine also reflect damage to proximal tubules. In the present study increased excretion of ALP and LDH was seen in macroalbuminuria group compared to normo and microalbuminuria group. This is in agreement with a previous study.[28]

The findings of increased excretion of GGT and LDH in the present study even in the normoalbuminuria group suggests that tubular damage most likely precedes glomerular damage and therefore reinforcing observations that urinary enzyme excretion can be used as early markers of renal damage in patients with T2DM.[25] In the present study, MA showed significant positive correlation with urinary enzymes especially NAG, ALP, LDH and GGT. Duration of diabetes and retinopathy has been reported to be the major risk factors for microalbuminuria.[35] Intensive blood-glucose control decreases the risk of micro vascular complications in patients with T2DM.

The findings of the present study show that urinary enzyme excretion occurs early even before microalbuminuria sets in. Thus, urinary enzymes released from the brush border can be used to assess tubular dysfunction in patients with T2DM. These markers are easy to quantify and cost-effective and hence can be used as screening markers even in small centers where the facility for measuring microalbumin may not be present. Authors propose the use of u-GGT and LDH to assess tubular dysfunction in patients with T2DM.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Sasikala T, Bitla AR, Sachan A. Urinary brush border enzymes for early diagnosis of tubular dysfunction in patients with type 2 diabetes mellitus. Int J Res Med Sci 2019;7:2015-21.