ORIGINAL ARTICLE

Urine Protein Excretion Among Chinese Patients With Type 2 Diabetes Mellitus

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Summary

Background: Urinary excretion of low molecular weight proteins such as β_2-microglobulin and retinol binding protein (RBP), and enzymes such as N-acetyl-β-D-glucosaminidase (NAG), may be useful as indicators of renal tubular dysfunction in diabetes mellitus.

Objective: To describe the profile of urinary protein and enzyme excretion in 240 Chinese patients with type 2 diabetes mellitus in Singapore.

Materials and Methodology: Cross-sectional study of consecutive patients presenting for follow-up at a Government primary care clinic. Information was obtained from interview, physical examination and laboratory analysis. Data analysis included descriptive statistics on urinary protein and enzyme excretion, comparison of unadjusted and adjusted means of these among patient subgroups, as well as correlation with control of diabetes and other clinical parameters.

Results: Albuminuria correlated with urine β_2-microglobulin ($r=0.34$, $p<0.01$) and RBP ($r=0.46$, $p<0.01$). Hypertensive patients had significantly higher mean urine albumin (geometric mean 15.13mg/gCr) and β_2-microglobulin (363.18μg/gCr) levels compared to patients without hypertension (7.07mg/gCr; 219.20μg/gCr; $p<0.05$). Patients with complications of diabetes also had higher albumin (15.55 vs 6.20mg/gCr), β_2-microglobulin (344.47 vs 288.83μg/gCr) and RBP excretion (152.02 vs 94.54mg/gCr). Two-hour postprandial sugar correlated with β_2-microglobulin ($r=0.33$, $p<0.01$), RBP ($r=0.35$, $p<0.01$) and NAG ($r=0.28$, $p<0.01$). Urinary protein excretion did not correlate with HbA1c, fasting blood sugar, age of patient or duration since diagnosis.

Conclusion: These results among 240 Chinese patients in Singapore were consistent with reports from other study populations.

Key Words: Albumin, β_2-microglobulin, Retinol binding protein, N-acetyl-β-D-glucosaminidase, Type 2 diabetes, Chinese

Introduction

Microalbuminuria is an established marker of nephropathy in patients with type 2 diabetes mellitus (non-insulin-dependent diabetes)[1], and its presence has been attributed to glomerular dysfunction, possibly by impairment in charge permselectivity of the glomerulus[2,3]. In recent years, studies on the excretion of low molecular weight proteins such as β_2-microglobulin[4], retinol binding protein (RBP)[5], and renal tubular enzymes such as N-acetyl-β-D-glucosaminidase (NAG)[6,7], have indicated that these markers of tubular dysfunction may be earlier indicators of renal impairment, as they are detected even in the absence of microalbuminuria[6].

Most of the studies on urinary protein excretion in patients with type 2 diabetes were conducted on Caucasian or Japanese patients. Yu *et al*[8] from the Peoples' Republic of China reported that β_2-microglobulin excretion was increased in Chinese diabetic patients (not specified if type 1 or type 2 diabetes) compared with controls, especially patients with nephropathy. Chan *et al*[9] looked at albuminuria in 164 Chinese patients with type 2-diabetes in Taiwan, and concluded that patients with albuminuria had higher mean arterial pressure and other cardiovascular risk factors compared to those without. In Singapore, Wei *et al*[10] studied the relationship between urinary protein excretion and renal function in patients with type II diabetes, and concluded that patients with abnormal renal function (serum creatinine >141μmol/L) had significantly higher amounts of high molecular weight proteins (molecular weight > 90 Kilo Daltons or KD), and low molecular weight proteins (<69 KD) in the urine, compared to those with normal renal function.

While it is not established that any ethnic differences exist with regard to urinary protein excretion, a study in Chinese subjects resident in Singapore would contribute towards knowledge in this area. This preliminary report, therefore, aims to describe the profile of urinary excretion of albumin, β_2-microglobulin, RBP and NAG, and their associations with various clinical and biochemical parameters, among 240 Chinese type 2 diabetes patients attending a primary care clinic in Singapore.

Materials and Methods

Study population

Two hundred and forty Chinese patients with type 2 diabetes who were registered with a Government Primary Care Clinic were selected to be study subjects on a consecutive basis, as they attended follow-up sessions for their diabetes. Inclusion criteria included documented diagnosis of type 2 diabetes, and regular follow-up at two-monthly intervals. Patients who were on insulin due to secondary drug failure were also included. Patients with known history of renal failure due to causes other than diabetes, e.g. glomerulonephritis, were excluded.

Clinical protocol

Information was obtained from direct interview with the patient, documentation in the case records, physical examination of the patient, and laboratory investigations.

Information obtained from interview and case records included known duration of diabetes, presence of associated hypertension, complications of diabetes, and past history of renal disease, as well as current treatment. Presence of ischaemic heart disease was based on the diagnosis by a cardiologist, upon referral, as documented in the case notes. The following were noted on physical examination: blood pressure, the presence of peripheral vascular disease (absent dorsalis pedis and/or posterial tibial pulses, amputation) and peripheral neuropathy (absent ankle tendon reflexes, decreased pin prick sensation, absent position or vibration senses). Retinopathy was ascertained from documented reports of diabetic retinal photography, as described by Yeo *et al*[11] conducted within one year from the time of the examination.

Blood samples were collected on the day of examination for blood glucose estimation (fasting or 2-hour postprandial). Glycosylated haemoglobin (HbA1c) and serum creatinine levels were determined within 3 months from the day of examination.

Urine samples were taken at the time of the interview for the estimation of urinary protein excretion. All samples were collected in the morning to minimise the effect of diurnal variation. Each sample was divided into

2 aliquots. The first aliquot was buffered with 0.5M TrisHCl (pH 7.2), while the second aliquot was left unbuffered. Urine samples were stored at -20°C. They were thawed and centrifuged before analysis. The unbuffered samples were analysed for urine creatinine, albumin, RBP and NAG. The buffered samples were analysed for β_2-microglobulin.

Laboratory analysis

Blood chemistry estimations were conducted by the laboratory located within the clinic. Urine creatinine, protein and enzyme levels were estimated at the Department of Community, Occupational and Family Medicine laboratory. Blood sugar estimations were made using the glucose oxidase method[12]. HbA1c levels were determined by high-performance liquid chromatography (HPLC)[12]. Serum and urine creatinine levels were measured using Jaffe's method[12]. All urinary proteins were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available polyclonal antibodies. Urinary NAG activity was determined according to Noto's Method[13], using a commercial test kit from Boehringer Mannheim. The isoenzymes of NAG were separated by heat treatment according to Tan *et al*[14].

Statistical analysis

All statistical analysis was performed using the SPSS for Windows statistical package. All urinary parameters were adjusted for variability in urine flow by the use of the formula:

$$\frac{(\text{albumin or } \beta_2\text{-microglobulin or RBP or NAG}) \times 100}{\text{urine creatinine in mg/dl}}$$

and expressed as per gram creatinine (/gCr). Urine creatinine in mg/dl was converted to mmol/L by multiplying with the conversion factor 0.0885.

Laboratory normal cut-off values for tubular proteins and enzymes were: 300µg/gCr for β_2-microglobulin, 150µg/gCr for RBP, 3.5ηmol/min/gCr for total NAG and 0.7ηmol/min/gCr for NAG isoenzyme B[15]. Logarithmic transformation ($\log_e$) of corrected urine protein values was done to normalise the distribution. Geometric means were obtained by taking the anti-log values of the corresponding mean values. Means were adjusted for age and gender using the general linear model (GLM). Pearson's correlation coefficient (r) was used to demonstrate the degree of correlation between the log values of the urine proteins or enzymes.

Patients were divided into subgroups according to the amount of albumin excreted in the urine, namely: normoalbuminuria (<2mg/mmol creatinine), microalbuminuria (2 - 20mg/mmol creatinine), and macroalbuminuria (>20mg/mmol creatinine), according to Mutti *et al*[16]. Diabetic complications were grouped into macrovascular (presence of ischaemic heart disease or peripheral vascular disease, or history of stroke) and microvascular (presence of retinopathy or neuropathy).

Statistical tests of significance used for differences in mean values included the following: Students' t tests for differences between 2 groups; analysis of variance (ANOVA) for differences among 3 groups, trend x^2 test for graded differences in proportions among 3 groups.

Results

Tables I and II show the clinical and biochemical profiles of study subjects. The male : female ratio was equal. About half of all patients had associated hypertension. Of these, 59% had blood pressure readings of 160/90mmHg or higher on the day of examination. In patients without a history of hypertension, 31% had blood pressure readings of 160/90mmHg or higher. Twelve per cent of all patients were on dietary control alone, and less than 4% on insulin. As for complications, peripheral neuropathy was common (23%), followed by ischaemic heart disease (18%) and peripheral vascular disease (14%). About one quarter of patients (28%) had microalbuminuria, while 6% had macroalbuminuria, and less than 3% of patients had raised serum creatinine levels of >141mmol/L. Diabetic control was satisfactory in 78% of patients (HbA1c <9%).

Forty one per cent of all patients had urine β_2-microglobulin levels above the laboratory normal of 300µg/gCr. Fifty percent had RBP levels of higher than 150µg/gCr and NAG isoenzyme B levels of higher than 0.7ηmol/min/gCr respectively, and 56% had raised total NAG levels of higher than 3.5ηmol/min/gCr.

Table I
Clinical Profile of Study Subjects (N=240)

Variable		Number (%) or Median (range)
Age (years)	median (range)	63.5 (31.0 - 86.0)
Gender	Male	121 (50.4)
Duration since diagnosis (years)	median (range)	8.5 (0.5 - 34.0)
History of hypertension	Present	122 (50.8)
Treatment	Dietary control	29 (12.1)
	Oral medications	202 (84.2)
	Insulin	8 (3.3)
	Insulin and oral medications	1 (0.4)
Diabetic complications		
* Macrovascular		
Ischaemic Heart Dis.	Present	42 (17.5)
Stroke	Present	13 (5.4)
Peripheral Vasc. Dis.	Present	34 (14.2)
* Microvascular		
Retinopathy	Nil	133 (55.4)
	Background	14 (5.8)
	Proliferative and beyond	13 (5.4)
Neuropathy	Peripheral neuropathy	54 (22.5)
Nephropathy		
* Albuminuria	Microalbuminuria#	66 (27.5)
	Macroalbuminuria#	14 (5.8)
(mg/gCr)	Arithmetic mean	36.1
	95% CI for mean	25.5 - 46.8
* Serum creatinine	>141mmol/L	6 (2.5)
(mmol/L)	Arithmetic mean	84.7
	95% CI for mean	81.1 - 88.3

SD Standard Deviation
CI Confidence Interval
 # Microalbuminuria: urine albumin 2-20 mg/mmolCr
Macroalbuminuria: urine albumin >20 mg/mmolCr

Urine albumin excretion correlated weakly with β_2-microglobulin (r=0.34, p<0.01), RBP (r=0.46, p<0.01) and total NAG (r=0.27, p<0.01). Among the markers of tubular dysfunction, urine β_2-microglobulin correlated moderately with urine RBP (r=0.68, p<0.01), but correlated weakly with total NAG (r=0.32, p=0.02) and its B isoenzyme (r=0.31, p=0.01).

Among patient subgroups, a dose-effect relationship was noted between albuminuria and tubular protein and enzyme excretion. The urinary excretion of tubular proteins (β_2-microglobulin and RBP) increased progressively from normoalbuminuria to macroalbuminuria. The same pattern was noted for NAG activity (Table III). In addition, there was also a

Table II
Biochemical Profile of Study Subjects (N=240)

Variable		Number (%) Mean (95% CI)
Parameters of control		
Fasting blood sugar (mmol/L)	FBS ≥ 7.8 mmol/L	69 (28.8)
	Arithmetic mean	8.7
	95% CI for mean	(8.2 - 9.3)
2-hour post-prandial sugar (mmol/L)	2Hr PPS ≥ 11.1 mmol/L	78 (32.5)
	Arithmetic mean	11.6
	95% CI for mean	(11.0 - 12.3)
HbA1c (%)	≥ 9.0%	52 (21.7)
	Arithmetic mean	8.1
	95% CI for mean	(7.8 - 8.4)
Tubular Protein and Enzymes		
Retinol binding protein (µg/gCr)	>= 150 µg/gCr	119 (49.6)
	Arithmetic mean	323.9
	95% CI for mean	(254.7 - 393.1)
β_2 microglobulin (µg/gCr)	>= 300 µg/gCr	98 (40.8)
	Arithmetic mean	863.3
	95% CI for mean	(624.3 - 1102.2)
NAG total (ηmol/min/gCr)	>= 3.5 nmol/min/gCr	134 (55.8)
	Arithmetic mean	5.9
	95% CI for mean	(8.2 - 6.6)
NAG-B (ηmol/min/gCr)	>= 0.7 nmol/min/gCr	119 (50.4)
	Arithmetic mean	1.4
	95% CI for mean	(1.1 - 1.8)

CI Confidence Interval

significant dose-response relationship. The proportion of patients with β_2-microglobulin, RBP, and NAG increased progressively from normoalbuminuria to macroalbuminuria, though the difference is most marked between normoalbuminuria and microalbuminuria.

When compared to patients without a history of hypertension, patients with associated hypertension had higher mean urine albumin (geometric means: 15.13, 7.07mg/gCr) and β_2-microglobulin excretion (geometric means: 363.18, 219.20µg/gCr), both of which were statistically significant at the 5% level. Patients with hypertension also had higher urinary excretion of RBP (geometric means 122.60, 89.97µg/gCr), and higher NAG activity compared to those without, though these differences were not statistically significant.

Patients with complications of diabetes, whether macrovascular or microvascular, had higher levels of urinary albumin, RBP and β_2-microglobulin excretion (Table IV). There was, however, no difference in NAG activity between patients with and without complications.

As for the association of urinary proteins with parameters of diabetic control, it was noted that the correlation was strongest with 2-hour postprandial

Table III
Mean Urinary Protein Levels in Patients with Normoalbuminuria, Microalbuminuria and Macroalbuminuria#

Urinary Protein / Enzyme 1. geometric means 2. % abnormal		Normo-albuminuria (N=160)	Micro-albuminuria (N=66)	Macro-albuminuria (N=14)
Tubular proteins				
Retinol binding protein	Unadjusted	215.23	441.20*	813.71*
1. in µg/gCr	Adjusted•	212.25	438.07*	797.21
2. % abnormal	% >= 150 µg/gCr	34.4+	81.8+	71.4+
β2 microglobulin	Unadjusted	66.02	224.08*	620.79*
1. in µg/gCr	Adjusted•	70.77	217.97*	595.31
2. % abnormal	% >= 300 µg/gCr	33.8+	51.5+	71.4+
Tubular enzyme				
N-acetyl-β-D-glucosaminidase				
• NAG total	Unadjusted	2.73	3.67	6.81
1. in ηmol/min/gCr	Adjusted•	2.66	3.87*	6.88
2. % abnormal	% >= 3.5 µmol/min/gCr	50.0+	66.7+	71.4+
• NAG-B	Unadjusted	0.37	0.51	0.68
1. in ηmol/min/gCr	Adjusted•	0.39	0.49	0.67
2. % abnormal	% >= 0.7 µmol/min/gCr	44.4+	62.1+	64.3+

 # Normoalbuminuria: urine albumin <2mg/mmolCr
Microalbuminuria: urine albumin 2 - 20 mg/mmolCr
Macroalbuminuria: urine albumin >20 mg/mmolCr
** Difference statistically significant at p<0.05, compared with normoalbuminuria, by ANOVA, post-hoc test: Bonferroni's*
+ Trend x^2 statistically significant difference in proportions at p<0.05
• Adjusted for age and gender by general linear model (GLM)

sugar, but not with fasting blood sugar or HbA1c. Duration of diabetes did not correlate with urine protein excretion and tubular enzyme activity. Age correlated weakly with RBP and β_2-microglobulin excretion. Systolic blood pressure values correlated weakly with all urine protein and enzyme values, whereas diastolic blood pressure correlated weakly with albumin excretion only (Table V).

Discussion

This was a cross-sectional study aimed mainly to describe the profile of urine protein excretion in Chinese patients with type 2 diabetes. The main limitations of this study relate to the setting in which it was conducted. As the study was built into the follow-up sessions of diabetic patients, some of the information was obtained from case records, with the inherent biases

Table IV
Mean Urinary Protein Levels in Patients with and without Complications

Urinary Protein Levels (Geometric Means)		Macrovascular# No (N=166)	Macrovascular# Yes (N=74)	Microvascular# No (N=106)	Microvascular# Yes (N=76)	All Complications No (N=83)	All Complications Yes (N=153)
Albumin (mg/gCr)	Unadjusted	8.25	17.55*	7.29	16.64*	5.99	16.09*
	Adjusted+	8.50	17.08*	7.46	16.25*	6.20	15.55*
RBP (µg/gCr)	Unadjusted	87.36	159.97*	93.22	202.35*	87.97	163.53*
	Adjusted+	96.16	145.33	98.69	191.14*	94.54	152.02
β2-microglobulin (µg/gCr)	Unadjusted	243.23	399.41*	249.39	367.23	213.79	369.08*
	Adjusted+	259.56	373.90	264.81	346.19	228.83	344.47
NAGtotal (ηmol/min/gCr)	Unadjusted	3.16	3.06	2.61	4.24	2.43	3.58
	Adjusted+	3.22	3.00	2.61	4.24	2.47	3.52
NAG-B (ηmol/min/gCr)	Unadjusted	0.43	0.41	0.40	0.48	0.39	0.42
	Adjusted+	0.43	0.39	0.40	0.48	0.40	0.42

 # Macrovascular: Ischaemic heart disease or peripheral vascular disease or stroke
Microvascular: Retinopathy or neuropathy

RBP: retinol binding protein
NAG: N-acetyl-β-D-glucosaminidase
β-NAG: β-isoenzyme of N-acetyl-β-D-glucosaminidase

+ Adjusted for age, gender by GLM
** Statistically significant at p<0.0 using Students' t test between patients with and without complications*

Table V
Association of Urinary Protein and Enzyme Excretion with Clinical Parameters

	CORRELATION COEFFICIENTS (r) Urinary Proteins and Enzymes				
	Albumin (mg/gCr)	**RBP (µg/gCr)**	**β2 microglo-bulin (µg/gCr)**	**NAG total (ηmol/min/gCr)**	**β-NAG (ηmol/min/gCr)**
Parameters of Diabetic Control					
* FBS (mmol/L)	-0.16	-0.10	-0.20	0.18	0.03
* 2-Hr PPS (mmol/L)	0.07	0.35**	0.33**	0.28**	0.07
* HbA1c (%)	-0.03	0.03	-0.13	0.10	0.08
Age	0.09	0.22**	0.18**	0.10	0.08
Duration of Diabetes	0.04	0.09	0.07	0.02	0.02
Blood Pressure					
* Systolic	0.19**	0.16*	0.16*	0.17*	0.14*
* Diastolic	0.16**	0.11	0.05	0.09	-0.09

* *Statistically significant at p<0.05*
** *Statistically significant at p<0.01*
RBP: Retinol binding protein
NAG: N-acetyl-β-D- glucosaminidase

associated with secondary data. Retinal photography was conducted by appointment, and though the photographs were sent for reporting by trained individuals, inter-observer variation is likely to be present. In addition, investigations or procedures deemed not to be necessary for the follow-up care of these patients could not be carried out. Hence, HbA1c, for example, if last done within 3 months of the interview, was not repeated at the time the fasting or 2 hour postprandial blood for glucose was taken, and had to be scheduled for the next follow-up visit, which is normally two months later. This, however, is acceptable, as HbA1c levels reflect the degree of diabetic control in the preceding 3 months.

In our study, it was not surprising to find that β_2-microglobulin correlated moderately with RBP, as both are markers of tubular dysfunction. This was reported in patients with IDDM as early as in 1989 by Watts *et al*[17]. The same was reported in type 2 diabetes patients by Shimuzu *et al*[18] who noted in addition an association with NAG, as was also seen in our patients.

It was interesting to note that urinary albumin excretion, a marker of glomerular dysfunction, correlated weakly with β_2-microglobulin and RBP excretion, both being markers of tubular function impairment. Kanauchi *et al*[19] found no difference in the urinary levels of β_2-microglobulin between type 2 diabetes patients with microalbuminuria and those without. Among patients with IDDM, however, the reports were conflicting[17,20,21]. This discrepancy could be due to the instability of β_2-microglobulin with varying urinary pH.

In contrast to β_2-microglobulin, RBP is stable in acid pH, and at varying temperatures[22]. As was noted in our study, Shimuzu *et al*[18] and Holm *et al*[23] also reported an association between RBP excretion and albumin/creatinine levels in patients with type 2 diabetes. It is still not clear why this is so. One possible explanation is that both glomerular and tubular dysfunction coexist in these patients. Another postulate, as demonstrated in animal studies[24], is that a high urinary concentration of albumin interferes with tubular resorption of low molecular weight proteins like RBP, resulting in an increased concentration in the urine.

In type 2 diabetes patients without overt proteinuria, urinary β_2-microglobulin excretion has been reported to decrease significantly with short-term glycaemic control[25]. Similarly NAG is known to be associated with glycaemic control[26,27] and has been reported to decrease with short-term improvement in blood sugar levels[27,28]. In our study, of the three parameters of diabetic control, 2-hour postprandial blood sugar correlated best with tubular protein (β_2-microglobulin and RBP) and NAG levels, though not with urinary albumin excretion. However, none emerged as strong predictors of urinary protein or enzyme excretion from stepwise regression analysis. Koh *et al* also did not find any significant difference between urinary β_2-microglobulin, RBP and NAG levels and HbA1c levels[29].

In conclusion, therefore, there is much similarity between the pattern of urinary protein and enzyme excretion in Chinese type 2 diabetes patients compared to type 2 diabetes patients of other ethnic groups as reported in the literature.

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