

β_2 -Glycoprotein-1 (apolipoprotein H) excretion and renal tubular malfunction in diabetic patients without clinical proteinuria

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Abstract

Aim—To compare the urinary excretion of β_2 -glycoprotein-1 with that of two other markers of early tubular disorder in diabetic patients without clinical proteinuria.

Methods—The urinary excretion of retinol binding protein, β_2 -glycoprotein-1, and N-acetyl- β -D-glucosaminidase was measured in 90 known diabetic patients who had a negative reagent strip test for proteinuria.

Results—Among 43 patients with urinary albumin excretion within the reference range, 23 (53%) had raised urinary N-acetyl- β -D-glucosaminidase activity, five (12%) increased excretion of β_2 -glycoprotein-1, and five (12%) increased loss of retinol binding protein. Among 47 patients with an albumin excretion of 0.9–7.9 mg/mmol creatinine, 42 (89%) had increased urinary N-acetyl- β -D-glucosaminidase, 23 (49%) an increased output of β_2 -glycoprotein-1, and 16 (34%) a raised excretion of retinol binding protein. The excretion of these markers of tubular defects seldom exceeded two and a half times the upper reference limit and the differences between the findings in the insulin dependent and non-insulin dependent patients with similar albumin excretion were small and insignificant.

Conclusions—In diabetic patients with a negative dipstick test for proteinuria: (a) assay of urinary β_2 -glycoprotein-1 may be a more sensitive test for the detection of impaired tubular reabsorption of protein than measurement of retinol-binding protein; (b) assay of N-acetyl- β -D-glucosaminidase can detect tubular injury at a time when protein reabsorption remains normal; and (c) impaired renal tubular function may be present in the absence of evidence of glomerular malfunction.

and fatal renal disease in both insulin dependent and non-insulin dependent diabetes mellitus.¹⁻³ More importantly, it has been shown that improvement of glycaemic control at this stage can reduce the proteinuria and significantly improve the outlook for long term renal function.⁴ The increased albumin excretion is usually considered to be of glomerular origin, but there is evidence to suggest that tubular malfunction may also contribute^{5,6}; in this connection it is of interest that impaired tubular reabsorption of low molecular mass plasma proteins has been reported in diabetic patients with normal albumin excretion.⁷⁻⁹

β_2 -glycoprotein-1 (β_2 G1) is a plasma protein with a molecular mass of about 50 kilodaltons which, because of its high isoelectric point, would be expected to be more easily filtered at the glomerulus than more anionic proteins.¹⁰ Increased urinary excretion of this protein in patients with plasma creatinine concentrations within the reference range and no gross glomerular impairment has recently been identified as a reliable marker of tubular malfunction.¹¹ Its main advantage over low molecular weight markers of tubular malfunction is its stability in acid urine down to pH 4.5.¹⁰

Longer established markers of renal tubular defect include urinary retinol binding protein (RBP) and N-acetyl- β -D-glucosaminidase (NAG). Unbound RBP is a 21 kilodalton protein that is freely filtered at the glomerulus and reabsorbed by the proximal tubule, and most conditions associated with disturbance of tubular function lead to an increase in its excretion.^{11,12} NAG is an enzyme of 140–150 kilodaltons that is shed into the glomerular filtrate from the lysosomes of the proximal renal tubule cells, and increased urinary excretion is found when active tubular injury occurs.¹³

Methods

Specimens of urine were obtained from 90 patients with diabetes mellitus—46 men aged between 18 and 85 years and 44 women aged between 23 and 81 years. The patients were all attending a diabetic clinic at University College Hospital and included many who were born in the Indian subcontinent. They were selected in two stages, the primary selection being on the basis that they did not have a history of renal disease or proteinuria to dipstick testing and that their plasma creatinine concentration was within 2 standard

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Renal impairment is an important feature of diabetes mellitus, causing significant morbidity and mortality, and much effort has been devoted to the detection of this complication at a stage when it is silent and potentially reversible. It has been shown that low level albuminuria, which is not detectable by conventional testing, reliably predicts a high risk of the subsequent development of progressive

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Table 1 Urinary excretion of retino binding-protein (RBP), β_2 -glycoprotein-1 (β_2 G1), and N-acetyl- β -D-glucosaminidase (NAG) among 90 diabetic patients with a negative dipstick test for proteinuria

Urinary constituent	All patients			Patients with increased excretion		
	Mean*	Median*	Range*	Number	(%)	Median excretion
Findings among 20 insulin dependent patients with albumin excretion of <0.9 mg/mmol creatinine:						
RBP	10.0	8.5	0-43.9	2	10	2.0 x URL
β_2 G1	20.2	19.4	5.9-44.2	2	10	1.3 x URL
NAG	36.0	34.8	12.0-109	13	65	1.7 x URL
Findings among 23 non-insulin dependent patients with albumin excretion of <0.9 mg/mmol creatinine:						
RBP	10.5	4.4	0-61.3	3	13	3.5 x URL
β_2 G1	19.1	16.7	0-47.7	3	13	1.2 x URL
NAG	23.3	20.9	6.0-68.2	10	43	1.5 x URL
Findings among 12 insulin dependent patients with albumin excretion of 0.9-7.9 mg/mmol creatinine:						
RBP	17.2	10.0	5.2-42.3	5	42	2.2 x URL
β_2 G1	40.4	43.2	25.6-69.6	7	58	1.4 x URL
NAG	49.9	47.2	28.4-82.2	12	100	2.0 x URL
Findings among 35 non-insulin dependent patients with albumin excretion of 0.9-7.9 mg/mmol creatinine:						
RBP	23.4	13.5	0-362	11	31	1.4 x URL
β_2 G1	33.0	24.2	0-85.7	16	46	1.5 x URL
NAG	49.4	42.2	17.2-136	30	86	1.9 x URL

*Protein values are in μ g and enzyme activity in μ mol of substrate hydrolysed per hour, and all results are expressed per mmol of urinary creatinine. The reference intervals applying, which were determined on morning urine specimens from healthy hospital staff, are as follows: RBP 1-16, β_2 G1 6.9-34.5, and NAG <25. URL = Upper reference limit.

deviations (SD) of the mean expected for a healthy adult of the same age and sex. The secondary selection aimed to provide roughly equal numbers of patients with normal and low level albuminuria. Thirty two of the patients, with a mean age of 46 years, were classified as insulin dependent and had been diabetic for 0-49 years, the average duration being 15 years. Fifty eight, with a mean age of 60 years, were classed as non-insulin dependent and had been diabetic for 0-24 years, the average duration being 6.7 years. Glycated haemoglobin (HbA_{1c}) concentrations, measured on venous blood samples obtained either immediately after the urine specimens were collected for study or within seven days of such, were available from 87 of the patients.

Mid-stream specimens of urine were collected in the morning when the patients arrived at the clinic. The specimens were

Table 2 Statistical analyses of differences in urinary excretion of retinol binding-protein (RBP), β_2 -glycoprotein-1 (β_2 G1), and N-acetyl- β -D-glucosaminidase (NAG) between insulin dependent (ID) and non-insulin dependent (NID) patients

Urinary constituent	Estimate of difference between population medians*	95% Confidence interval*	Significance of difference
ID v NID diabetics with urinary albumin of <0.9 mg/mmol creatinine:			
RBP	3.0	(-1.8 to 6.4)	} NS
β_2 G1	1.7	(-5.9 to 7.9)	
NAG	10.1	(0.0 to 20.4)	
ID v NID diabetics with urinary albumin of 0.9-7.9 mg/mmol creatinine:			
RBP	1.4	(-4.8 to 9.2)	} NS
β_2 G1	12.8	(-6.2 to 25.5)	
NAG	6.0	(-9.8 to 16.3)	
ID diabetics with urinary albumin of <0.9 v ID diabetics with urinary albumin of 0.9-7.9 mg/mmol creatinine:			
RBP	3.0	(-1.2 to 11.4)	NS
β_2 G1	22.1	(11.7 to 30.0)	Significant
NAG	14.4	(4.3 to 28.3)	Significant
NID diabetics with urinary albumin of <0.9 v NID diabetics with urinary albumin of 0.9-7.9 mg/mmol creatinine:			
RBP	5.6	(1.0 to 10.7)	} Significant
β_2 G1	10.4	(2.1 to 20.4)	
NAG	22.8	(13.7 to 34.8)	

*Protein values are in μ g and enzyme activity in μ mol of substrate hydrolysed per hour, and all results are expressed per mmol of urinary creatinine.

immediately tested with an Ames Multistix, centrifuged at 1800 \times g for 10 minutes and the supernatant fluid divided into portions for the different assays. All the aliquots were frozen within four hours of voiding and stored at -20°C without preservative until shortly before analysis.

All the urine samples were assayed for creatinine, albumin, β_2 G1, RBP, and NAG, and all assays except those for creatinine, were performed in duplicate. Results under the detection limit of the method were recorded as zero. To reduce the effects of the variable concentration of the specimens, protein results were expressed per mmol of creatinine.

The significance of the differences in the results among the patient groups was determined using non-parametric statistics by calculating the 95% confidence intervals for the differences of the estimates for the population medians.¹⁴

The creatinine concentration was measured by the Jaffé reaction, using the kinetic method used in the American Monitor Perspective analyser. Albumin was measured by an immunoturbidimetric method with a Cobas-Bio analyser, using the Ames Microalb kit from Miles Laboratories Ltd (Stoke Poges, Slough, SL2 4LY). RBP was measured by the ELISA method of Topping *et al*¹⁵ and β_2 G1 by the sandwich ELISA procedure of Sansom *et al*.¹⁶ NAG activity was measured by a colorimetric procedure, based on the method of Yuen *et al*,¹⁷ using a Cobas-Bio centrifugal analyser and the kit available from Cortecs Diagnostics Ltd (Deeside Industrial Estate, Clywd CH5 2NT). The between-batch coefficients of variation of the methods for albumin, RBP, β_2 G1, and NAG were 4%, 11%, 6%, and 8%, respectively. Blood HbA_{1c} was measured using an automated procedure involving agar-gel electrophoresis followed by scanning densitometry.

Results

The overall findings are summarised in table 1 in which the results have been grouped according to the type of diabetes present and the level of albumin excretion. The significance of the differences in the excretion of the markers among the patient groups are indicated in table 2. Among those with an albumin excretion of <0.9 mg/mmol creatinine, 53% had an increased NAG excretion, 12% a raised output of β_2 G1, and 12% an increased loss of RBP. By comparison, the incidence of increased excretion of NAG, β_2 G1, and RBP among all those with albumin outputs of 0.9-7.9 mg/mmol creatinine, was 89%, 49%, and 34%, respectively. The quantity of NAG, β_2 G1 and RBP output seldom exceeded two and a half times the upper reference limit for healthy subjects. Comparing insulin dependent patients with those who were not, there was no statistically significant difference in the quantities of markers excreted. Comparing patients with low level albuminuria with those with albumin excre-

Table 3 Prevalence of different patterns of excretion of retinol binding protein (RBP), β_2 -glycoprotein-1 (β_2 G1), and N-acetyl- β -D-glucosaminidase (NAG) among 90 diabetic patients with negative dipstick test for proteinuria

	Albumin excretion <0.9 mg/mmol creatinine				Albumin excretion 0.9–7.9 mg/mmol creatinine			
	Insulin dependent diabetics		Non-insulin dependent diabetics		Insulin dependent diabetics		Non-insulin dependent diabetics	
	No	%	No	%	No	%	No	%
Increased NAG, β_2 G1, and RBP	1	5	0	0	4	33	10	29
Increased NAG and β_2 G1	0	0	2	9	3	25	6	17
Increased NAG and RBP	1	5	2	9	1	8	1	3
Increased NAG alone	11	55	6	26	4	33	13	37
Increased β_2 G1 alone	0	0	1	4	0	0	0	0
Increased RBP alone	0	0	1	4	0	0	0	0
Normal NAG, β_2 G1, and RBP	7	35	11	48	0	0	5	14
Totals	20		23		12		35	

tion within the reference range, there was generally a greater excretion of NAG, β_2 G1, and RBP by the former group, the differences in output being significant for all three markers among the non-insulin dependent diabetics but only for NAG and β_2 G1 among the insulin dependent patients.

The prevalences of the different patterns of excretion of the three tubular markers among the patients are shown in table 3. One or more of the markers was present in excess in 78% of those who were insulin dependent and 72% of those who were non-insulin dependent, and in 53% of those who had normal albumin excretion compared with 89% of those who were excreting more than 0.9 mg albumin/mmol creatinine. Increased excretion of all three markers was observed in 16% of the insulin dependent and in 17% of the non-insulin dependent patients, and in only one patient with normal albumin excretion compared with 30% of those with low level albuminuria. Increased excretion of NAG alone was encountered relatively often,

but increased excretion of β_2 G1 or RBP was nearly always accompanied by raised NAG activity, and the prevalence of increased NAG found in combination with raised β_2 G1 or RBP was generally greater among those with increased albumin output. Among all 90 patients the correlation between the quantities of albumin excreted with that of each of the tubular markers was poor, the correlation coefficients varying between 0.30 and 0.40. The correlation between the excretion of β_2 G1 and that of RBP and NAG among all 90 patients is shown in the figure: considerable variation is apparent, the correlation coefficient between the β_2 G1 and RBP results being 0.57, and that between the β_2 G1 and NAG values only 0.51.

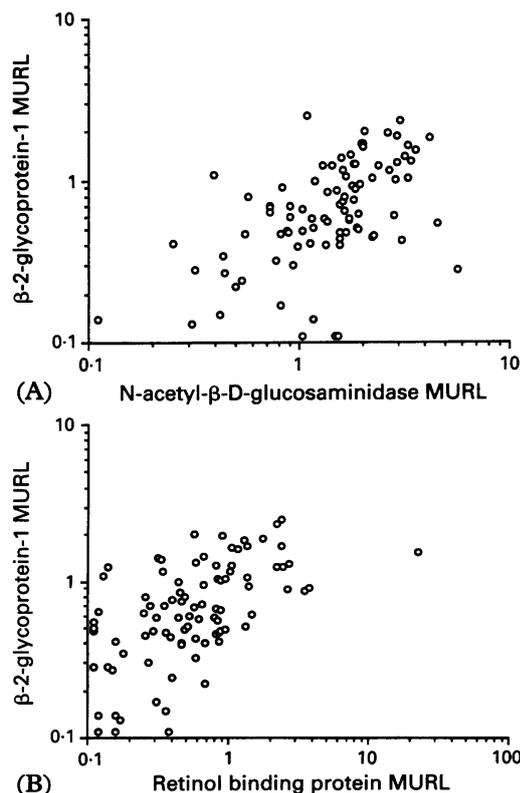
The HbA₁ values in the patients ranged between 6.5 and 15.7% of the total haemoglobin but only 13% of the results fell within the reference range of 5.0–8.0%, high concentrations being encountered more often among the insulin dependent diabetic patients. Among the patients with an albumin excretion of less than 0.9 mg/mmol creatinine the mean (SD) of the results was 10.0 (1.8), and 79% had concentrations above the upper reference limit; among those excreting 0.9–7.9 mg albumin/mmol creatinine the corresponding values were 11.3%, 2.2%, and 96%.

Discussion

It is generally considered^{5,18} that in the evolution of proteinuria in diabetes mellitus the early stage of low level albuminuria is due to an increase in the transglomerular pressure gradient which increases the rate of filtration. Later, depletion or modification of the negative charged polyanion of the glomerular membrane leads to the basement membrane becoming a less effective electrostatic barrier to circulating polyanionic proteins such as albumin. Later still, as the architecture of the basement membrane becomes abnormal, progressive enlargement of the pores occurs and it becomes a less selective sieve, and as larger quantities of albumin enter the filtrate together with higher molecular weight proteins, clinical proteinuria develops.

While Viberti and Keen¹⁸ considered that failure of tubular reabsorption of albumin had no part in producing microalbuminuria,

Plots showing the correlation between the urinary excretion of β_2 -glycoprotein-1 and (A) N-acetyl- β -D-glucosaminidase and (B) retinol binding protein in 90 diabetic patients with a negative dipstick test for proteinuria. Values are plotted as multiples of the upper reference limit (MURL) on logarithmic scales.



Abrass⁵ concluded from her review of published findings that it contributes significantly, high urine flow rate and glycosuria being held responsible. Based on finding no increase in β_2 -microglobulin excretion among insulin dependent diabetic patients with microalbuminuria, Viberti *et al*¹⁹ concluded that there was no tubular resorption defect. But because β_2 -microglobulin is unstable in acid²⁰ or infected urine²¹ and can degrade during the time that the urine is held in the bladder, it cannot be relied on to detect tubular malfunction unless the patients are given adequate amounts of alkali orally before and during the period of urine collection.²² Since then several investigators have shown particularly among insulin dependent diabetic patients, increased excretion of more stable low molecular weight proteins such as RBP,^{6-8 22 23} α_1 -microglobulin,²⁴ lysozyme,^{25 26} and κ light chains.^{9 27} Confirmatory evidence of tubular malfunction at an early stage in the development of diabetic nephropathy comes from studies which have shown increased shedding into the urine of the proximal tubule enzyme NAG,^{6 26 28} and of a tubular brush border antigen.²⁹

The findings reported here add weight to the suggestion that among patients with diabetes mellitus and apparently normal renal function, appreciable tubular injury is often present. Because global reference ranges which did not embrace the elderly were used to interpret the results, however, and because the levels of excretion of the tubular markers seldom exceeded two and a half times the upper reference limit, the figures indicating the prevalence of their increased excretion may be inflated as renal function generally is known to deteriorate with increasing age. Hemmingsen and Skaarup,³⁰ however, have reported that the 24 hour excretion of 10 plasma proteins, including lysozyme and κ and λ free light chains, did not differ significantly among 12 healthy subjects aged 70–83 years and 209 fit subjects aged 10–69 years.

The increased excretion of RBP and β_2 -G1 in the patients studied is very unlikely to be explained by overflow due to high plasma concentrations, as all had a plasma creatinine concentration of less than 124 $\mu\text{mol/l}$,¹¹ and none had the few conditions that have been reported to have an association with raised plasma β_2 -G1 values.³¹ The possibility that an increase in the filtered load of albumin and other proteins resulting from increased glomerular permeability or hyperfiltration, might be responsible for the increase in urinary low molecular weight proteins by increasing the competition for the reabsorption sites, cannot be excluded. The highest ratio of albumin to β_2 -G1 found among the patients with albumin outputs of 0.9–7.9/mmol creatinine, however, was only 542, which is far below the minimum figure of 1000 which typifies primary glomerular disease; and median ratios of 45 and 89 which applied to the insulin dependent and non-insulin dependent patients with low level albuminuria were

close to the maximal ratio of 40 which characterises primary tubular disease.³² The raised NAG excretion found among 89% of the patients with 0.9–7.9 mg albumin/mmol creatinine also points to injury of the tubular cells as being the likely explanation, because NAG with a molecular mass of 140–150 kilodaltons would not be expected to pass from the plasma into the glomerular filtrate in the absence of clinical proteinuria.

Our findings also contribute to the mounting evidence that the pathological process that often develops eventually into diabetic nephropathy may start in the proximal renal tubule rather than the glomerulus. Thus the evidence for tubular injury and malfunction was not confined to those patients who already had evidence of glomerular disturbance but was often found in those with normal albumin excretion; among the latter 19% had increased excretion of RBP or β_2 -G1 and 53% had increased excretion of NAG. Long term studies, however, are needed to assess whether this tubular damage is a precursor of the development of overt diabetic nephropathy.

As the proximal tubule is the most metabolically active part of the nephron it is hardly surprising that it should be affected early in a disease characterised by widespread metabolic disturbance. In this context it is of interest that it has been shown that the urinary excretion of NAG falls after the initiation of treatment and that the level generally correlates with the degree of glycaemic control.^{28 33} This could explain the high incidence of increased NAG excretion found among the patients we studied, as few had normal blood concentrations of HbA_{1c}. As proximal tubular reabsorption of protein is considered normally to be working at near maximal capacity, only a minor degree of tubular impairment could result in increased excretion of RBP and β_2 -G1.

Our results suggest that among diabetic patients with more than 0.9 mg albumin/mmol urinary creatinine, measurement of urinary β_2 -G1 has greater sensitivity than assay of RBP for detecting impairment of the tubular reabsorption of protein, although the magnitude of an increase of β_2 -G1 was on average less than that shown by RBP. The higher sensitivity of β_2 -G1 measurements may be attributed to the greater stability of β_2 -G1 in urine.¹¹ However, it may be more significant that β_2 -G1 has relatively high pI values¹⁰ as a consequence of which, besides being less retarded by the glomerular negative charge barrier, it may normally be reabsorbed more efficiently than more anionic proteins of the same molecular mass.³⁴ In this context it is of interest that κ immunoglobulin light chains, which have similarly high pI values¹⁰ have been reported as being very early markers of renal impairment in diabetes.^{9 27}

The early stages of microalbuminuria often seem to be reversible by strict control of the blood glucose concentration⁴ the use of anti-hypertensive agents or angiotensin converting enzyme inhibitors.³⁵ The possibility of obtain-

ing more certain benefit from still earlier intervention as a result of detecting renal tubular involvement needs to be explored.

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