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Plasma Cystatin C Concentration in Non-Insulin-Dependent Diabetes Mellitus: Relation with Nephrophathy

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Abstract. We determined plasma concentrations of cystatin C, β_2-microglobulin – β_2-MG (low molecular mass protein markers of glomerular filtration rate – GFR), creatinine (marker of GFR) and urinary N-acetyl-β-D-glucosaminidase (NAG) excretion (marker of glomerular and tubular dysfunction) in 41 non-insulin-dependent diabetic patients. A significant increase of all the measured parameters ($p<0.001$, $p<0.05$, $p<0.05$ and $p<0.001$, respectively) in comparison to the control group, was observed. In the patients with microalbuminuria, only plasma cystatin C concentration and urinary NAG excretion increased significantly in comparison to patients with normoalbuminuria. At a cut-off level of 1.74 mg/l for cystatin C and 1.81 U/g creatinine for NAG (95% percentile of the normoalbuminuric group), the sensitivity of the tests for detecting microalbuminuria was 82% for cystatin C and 86% for NAG. The specificities were 88 and 92%, respectively. The present study demonstrated that determination of plasma cystatin C might be useful in the detection of incipient diabetic nephropathy and is a potentially better marker than creatinine or β_2-MG. No correlation between parameters measured in plasma or urine and glycated hemoglobin was found.

Key words: cystatin C; β_2-microglobulin; N-acetyl-β-D-glucosaminidase; diabetic nephropathy.

Introduction

Diabetic nephropathy is the most important long-term complication of diabetes mellitus. Recognized as a major cause of the end-stage renal disease followed by premature morbidity and mortality, is characterised by albuminuria, subsequent proteinuria, declining glomerular filtration rate and elevated blood pressure[2]. When renal function decreases, the serum concentrations of many low molecular weight proteins increase. As a consequence, the blood level of some small proteins, such as lysozyme, α_1-microglobulin, β_2-microglobulin (β_2-MG) and cystatin C, have been proposed as indices of renal function[8, 9, 12]. Especially the latter one has been intensively studied in the last few years.

Human cystatin C, a member of the cystatin superfamily (inhibitors of cysteine proteinases), has a molecular mass of about 13 000 kDa. Produced by nucleated cells, is present in low concentrations in serum, urine, cerebrospinal fluid, saliva, semen, and colostrum. Cystatin C is mainly removed from circulating blood by renal glomerular filtration, followed by tubular reabsorption and final degradation. The low molecular mass of cystatin C, in combination with its stable production

Abbreviations used: HMF – 5-hydroxymethylfurfural, β_2-MG – β_2-microglobulin, NAG – N-acetyl-β-D-glucosaminidase, GFR – glomerular filtration rate.

rate, indicates that the plasma concentration of cystatin C is almost exclusively determined by the glomerular filtration rate (GFR), what makes cystatin C an excellent indicator of GFR[9, 13, 14]. According to TIAN et al.[22], combined measurements of cystatin C in serum and urine may be useful, especially to detect mild reduction of GFR and renal tubular damage, but in the case of urine a sensitive ELISA test should be used.

In the present study, we evaluated the clinical usefulness of measuring plasma cystatin C levels compared with other parameters related to kidney dysfunction in non-insulin-dependent diabetes mellitus, such as plasma β_2-MG and plasma creatinine, urinary excretion of albumin and urinary activity of N-acetyl-β-D-glucosaminidase (NAG).

Materials and Methods

Patients treated at the Clinic of Angiology of Wrocław University Medical School, in 1997–1998 were studied. The group with non-insulin-dependent diabetes mellitus (type II) included 41 cases. A control group of 12 healthy adults without inflammatory states nor abnormalities in lipids and carbohydrate metabolism, in routine medical check-ups, was also examined. Biological and physical characterization of the populations studied is given in Table 1. Heparinized blood samples were obtained from fasting subjects of both groups. Blood was centrifuged in order to obtain plasma. From red cells hemolysates were prepared. Urine of the same patients was taken of the 24 h sampling. Plasma, hemolysates and urine were immediately frozen and stored at –80°C until determination.

For definition of the glycemic condition of the patients, fasting blood glucose in plasma (short-term control) and concentration of glycated hemoglobin (GHb) in hemolysates (long-term control) were determined. Blood glucose was measured by the commercial test using glucose oxidase (Analco, Poland). GHb was estimated by the method of EROSS et al.[7], using 5-hydroxymethylfurfural (HMF) as standard and total Hb by the Drabkin's method. Results were given in nmol HMF/mg Hb. Plasma concentrations of cystatin C and β_2-MG were determined by particle-enhanced turbidimetry using a commercial kit of the Dako Company (Denmark). Both assays were performed on a COBAS MIRA instrument. For simultaneous determination of creatinine in serum and urine the routine Jaffe method was used. Urine activity of NAG (E. C. 3.2.1.30) was estimated by fluorimetry[15] using 4-methylumbelliferyl--N-acetyl-β-D-glucosaminide as substrate. The concentration of albumin in urine was determined according to SCHOSINSKY et al.[20]

Patients were divided into 3 groups according to albumin excretion rate[5]: with normoalbuminuria (albumin excretion below 30 mg/24 h), with microalbuminuria (albumin excretion between 30 and 300 mg/24 h) and the group with macroalbuminuria (albumin excretion over 300 mg/24 h).

Results are expressed as median values and ranges (minimum and maximum). Statistical comparisons of the groups were made using the U Mann-Whitney's

Table 1. Clinical characteristics of healthy subjects and non-insulin-dependent diabetic patients

	Healthy subjects n = 12	Diabetic patients n = 41
Sex (M/F)	5/7	8/33
Age (years)	54 (51–72)	67* (42–78)
Disease duration (years)	– –	8 (0.5–34)
Plasma glucose (mmol/l)	4.90 (4.10–6.10)	9.50** (4.50–17.00)
Glycated hemoglobin (nM HMF/mgHb)	1.01 (0.74–1.32)	2.50** (1.00–7.38)
Blood pressure (systolic/diastolic) (mmHg)	120/75 (100/70–130/85)	150/85* (110/70–200/120)
Urinary albumin excretion rate (mg/24 h)	21.10 (7.80–30.00)	117.60*** (14.70–1560.00)
Creatinine clearance (ml/min)	96.32 (75.49–152.85)	72.90** (38.80–154.95)

Median and range (parentheses) are given.
Statistical significance in relation to control subjects: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
HMF – 5-hydroxymethylfurfural, Hb – hemoglobin.

and/or the Kolmogorov-Smirnov's tests. Correlation analysis was performed using Spearman's test. A p value of less than 0.05 was considered statistically significant.

Results

The concentrations of cystatin C, β_2-MG and creatinine in plasma as well as NAG activities in urine of the control group and patients are shown in Table 2. Cystatin C was increased in diabetic patients about 2.5-times and β_2-MG was elevated about 1.8-times with respect to the controls. A more significant increase ($p<0.001$) was observed in cystatin C than in β_2-MG ($p<0.05$). Creatinine was elevated only about 18% ($p<0.05$). Urinary NAG excretion increased about 4.3--times ($p<0.001$).

We examined the influence of albuminuria on the levels of the analyzed parameters. As shown in Table 3, plasma cystatin C concentration and urinary NAG excretion increased significantly in patients with microalbuminuria in comparison to patients with normoalbuminuria ($p<0.01$). Additionally, the levels of almost all the examined parameters were significantly higher in the patients with macroalbuminuria in comparison to patients with microalbuminuria. In the latter group plasma cystatin C concentration correlated more tightly with albumin excretion rate ($r = 0.50$, $p<0.05$) than β_2-MG ($r = 0.35$).

Calculations of sensitivity, specificity and diagnostic efficiency of the plasma cystatin C, creatinine and β_2-MG and urinary NAG are shown in Table 4. For each parameter, only cystatin C and NAG showed similar, high values. Using a cut-off value of 1.74 mg/l for cystatin C and 1.81 U/g creatinine for NAG, we found sensitivities of 82 and 86%, respectively. The sensitivity of creatinine (upper cut-off level 1.31 mg/dl) and β_2-MG (upper cut-off level 2.54 mg/l) was lower. A high diagnostic efficiency was found for NAG (0.793) and cystatin C (0.727).

Plasma cystatin C concentration correlated slightly with blood pressure of all diabetic patients ($r = 0.19$) and more tightly in the subgroups with microalbuminuria ($r = 0.26$) and macroalbuminuria ($r = 0.35$).

No significant correlation was found between the measured parameters in plasma and urine of the patients and glycated hemoglobin concentrtion in hemolysates.

Table 2. Analytical data of control subjects and diabetic patients in plasma (P) and urine (U)

	Control subjects n = 12	Diabetic patients n = 41
P – cystatin C (mg/l)	0.87 (0.41–1.74)	2.14** (0.69–3.93)
P – β_2-MG (mg/l)	1.68 (0.98–2.36)	2.97* (0.86–11.15)
P – creatinine (mg/dl)	1.08 (0.88–1.28)	1.28* (0.88–2.25)
U – NAG (U/g creatinine)	1.08 (0.58–2.04)	4,67** (0.53–46.88)

Median and range (parentheses) are given.
Statistical significance in relation to control subjects: * $p<0.05$, ** $p<0.001$

Table 3. Parameters in plasma (P) and urine (U) were clustered on the basis of urinary albumin excretion rate

	Normo-albuminuria n = 5	Micro-albuminuria n = 21	Macro-albuminuria n = 11
P – cystatin C (mg/l)	1.02 (0.69–1.74)	2.14[a]** (0.80–2.95)	2.75[a]**, [b]* (1.56–3.93)
P – β_2-MG (mg/l)	1.49 (1.25–2.09)	2.18 (0.86–8.56)	4.67[a]*, [b]* (1.09–11.15)
P – creatinine (mg/dl)	1.04 (0.88–1.28)	1.20 (0.97–1.30)	1.44[a]* (1.04–2.25)
U – NAG (U/g creatinine)	1.40 (0.58–5.76)	4.51[a]** (0.53–20.44)	10.96[a]**, [b]* (2.64–46.88)

Median and range (parentheses) are given.
[a] Significance in relation to the normoalbuminuria group.
[b] Significance in relation to the microalbuminuria group.
* $p<0.05$, ** $p<0.01$.

Table 4. Diagnostic validity of cystatin C compared to creatinine, β_2-microglobulin (β_2-MG) and N-acetyl-β-D-glucosaminidase (NAG) for the detection of microalbuminuria

Parameter	Sensitivity (%)	Specificity (%)	Diagnostic efficiency
P – cystatin C	82.4	88.2	0.727
P – creatinine	48.2	85.7	0.413
P – β_2-MG	66.7	83.3	0.556
U – NAG	86.5	92.3	0.793

The cut-off lower and upper limit of 30–300 mg/24 h of albumin in urine was selected as indicator of the developing nephropathy and was used for calculations of sensitivity and specificity. For the other parameters, the following limits were used as cut-off limits: cystatin C 1.74 mg/l, creatinine 1.31 mg/dl, β_2–MG 2.54 mg/l, NAG 1.81 U/g creatinine. Sensitivity, proportion of patients with positive values among those with microalbuminuria; specificity, proportion of patients with negative values among those with normoalbuminuria; diagnostic efficiency, sensitivity × specificity.

Discussion

It is very important in managing diabetes mellitus to detect diabetic nephropathy as early as possible and to prevent its development. In recent years, several

papers have confirmed the usefulness of cystatin C determination as a marker of early deterioration of GFR, being more sensitive than serum creatinine[1, 11, 16, 17, 21]. However, these papers analyzed cystatin C behavior in various renal diseases, but did not relate it to evolutionary states of diabetic nephropathy.

To test the hypothesis that cystatin C may be a suitable marker of diabetic nephropathy, we studied the level of plasma cystatin C in patients with the non-insulin-dependent type of the disease and analyzed the results in view of parameters routinely used in clinical practice such as albuminuria and plasma creatinine. For comparison, plasma β_2-MG level and urinary NAG activity were also determined. NAG is synthesized in the renal proximal tubular cells, but its excretion increased not only with advanced nephropathy, but in the incipient stage as well[10, 18].

Plasma (or serum) cystatin C level proves to be independent of gender, weight, infections, dietary factors and liver diseases, but some authors suggest its relation with age. It was reported that an increase in cystatin C level is accentuated at 50 years of age. Therefore, from the point of view of clinical practice, the use of this age looked suitable for dichotomizing the cystatin C reference values[16].

The plasma cystatin C concentration of our control group (median 0.87 mg/l, range 0.41–1.74) was similar to that obtained by ERLANDSEN et al.[6] for the serum of blood donors (median 0.85, range 0.42–1.39 mg/l).

Cystatin C showed a significant increase in diabetic patients with microalbuminuria, while β_2-MG only increased in patients with macroalbuminuria in comparison to normoalbuminuric patients. Both parameters increased as the degree of renal injury advanced, probably as a result of tubular damage. Plasma concentration of creatinine was higher only in the subgroup with macroalbuminuria.

Urinary NAG excretion increased in micro- and macroalbuminuric patients, which is in accordance with the observations of other authors[10, 18]. NAG activity elevation in the early stage of diabetic nephropathy may result from glomerular alteration, but at the advanced stage of the disease from tubular damage[18]. It is known that, as a consequence of abnormalities in glomerular barrier function, plasma proteins reach the tubular fluid epithelium. They are endocytosed via clathrin-coated pits by a receptor-mediated or constitutional pathway and then degraded by lysosomes into the constituent amino acids. Proximal tubular cell reserve capacity for this process is limited and increasing the protein load leads to organelle congestion, lysosomal swelling and rupture, eventually exposing the cell cytoplasm and renal interstitium to the injurious effect of lysosomal enzymes[19]. Therefore, an increase in filtered protein with advanced renal disease may accelerate the turnover rate of the lysosomal system and increase urinary NAG excretion.

Our results, which show lack of significant correlation between the examined parameters in plasma or urine and glycated hemoglobin concentration in hemolysates, are consistent with previous studies[2, 3]. The Diabetes Control and Complication Trial demonstrated that metabolic control in the prealbuminuric phase was effective in reducing the incidence of microalbuminuria, even if it was unable to reduced the incidence of overt proteinuria in patients with type I diabetes and established proteinuria. On the other hand, in type II diabetes the studies of the impact of a good metabolic control at the onset and progression of diabetic nephropathy show conflicting results[2, 3, 4]. In general, levels of glycated hemoglobin of less than 7.5–8%, seem to protect patients from developing nephropathy. The other major risk factors are hypertension, smoking habits and hyperlipidemia[3, 4]. Recently, SECO et al.[21] proposed serum cystatin C as a premature marker of reduced GFR in patients with essential hypertension.

Our results suggest that the determination of the plasma concentration of cystatin C may be useful in the detection of incipient nephropathy in patients with non-insulin-dependent diabetes mellitus and is a potentially better marker than creatinine and β_2-MG. The latter parameter was advocated 18 years ago as an index of GFR. It has now been abandoned because its production increased at several malignant and infection diseases, particularly lymphoproliferative disorders[17]. The diagnostic efficiency of plasma cystatin C determination for the detection of microalbuminuria is comparable to that of urine NAG excretion.

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