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Free pentosidine and neopterin as markers of progression rate in diabetic nephropathy

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Free pentosidine and neopterin as markers of progression rate in diabetic nephropathy.

Background. Patients with diabetic nephropathy experience a progressive and usually inexorable decline in renal function. The presence of the structurally defined advanced glycation end product (AGE) pentosidine on tissue and circulating proteins has been correlated with the severity of diabetic complications.

Methods. To delineate a role for this AGE in the progression of diabetic nephropathy, glycohemoglobin and free and protein-bound pentosidine were measured in baseline stored serum and urine from a subgroup of patients with diabetes mellitus and proteinuria originally followed by the Collaborative Study Group Trial. To delineate a potential role for an immune-activation response to AGEs, the inflammatory markers, interleukin-6 (IL-6), C-reactive protein (CRP), and the monocyte activation marker neopterin were also measured at baseline. The patients chosen represented 67 subjects whose creatinine levels had "doubled" over the course of the study whether or not they later were treated with captopril, and 67 paired "non-doublers."

Results. Baseline disease activity, as manifested by glycohemoglobin, serum creatinine and degree of proteinuria was equal in the two groups, as was protein-bound pentosidine and the immune-markers IL-6 and CRP. At baseline the "doublers" as compared to the "non-doublers" had elevated serum levels of free pentosidine and neopterin. Baseline increases in these two parameters were also associated with an increased rate of "doubling" of serum creatinine by the proportional hazards method.

Conclusion. Differences in individual responsiveness to AGEs, as manifested by either the production of free pentosidine or its release from a protein-bound form, and by evidence of monocyte/macrophage activation, are associated with progression of diabetic nephropathy.

Renal disease is one of the most serious long-term complications that can develop in patients with diabetes mellitus. In the United States, about 30% of the patients treated for end-stage renal failure have diabetic nephropathy [1], which develops in up to 45% of patients with diabetes [2, 3]. The clinical onset of nephropathy is marked by microalbuminuria (an albumin excretion rate of more than 30 mg/day but less than 300 mg/day). Over a period of one to five years following the onset of microalbuminuria, frank proteinuria usually develops, frequently with associated hypertension [4]. Patients with diabetic nephropathy experience a progressive and usually inexorable decline in renal function, although the rate of deterioration can be significantly slowed by the treatment of associated hypertension. The special role of angiotensin converting enzyme (ACE) inhibitors in slowing progression of diabetic nephropathy independent of its effect on blood pressure has been emphasized in a number of recent studies [5–7].

Another factor associated with the progression of diabetic nephropathy is the degree of control of hyperglycemia, as demonstrated recently in the Diabetes Complications and Control Trial [8]. The patient group treated with strict glycemic control experienced a marked reduction in the development of microalbuminuria. The mechanisms by which hyperglycemia results in nephropathy and other

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diabetic complications are not well defined. A growing literature has associated the accumulation of advanced glycation end products (AGEs) on proteins with the pathogenesis of diabetic complications. The nonenzymatic reaction of a reducing sugar (such as glucose) and the amine group of a protein is termed the Maillard or "browning" reaction. The first step in this reaction leads to the formation of Amadori products such as glycohemoglobin. The Amadori reaction is reversible, and these products do not normally accumulate. However, Amadori products are the precursors of covalently bound structures on proteins called advanced glycation end products [9, 10]. In patients with diabetes, markedly elevated levels of AGEs accumulate on tissue and circulating proteins [11]. In animal models, injecting AGEs into the circulation can induce physiologic and morphologic changes typical of diabetes [12].

The structurally defined adduct pentosidine was one of the first AGEs to be isolated from tissue, and has been demonstrated in tissue and plasma proteins of patients with diabetes [13]. Skin pentosidine levels have been correlated with the severity of diabetic complications [14]. Further, pentosidine in glomerular basement membranes may play a pathophysiologic role in diabetic nephropathy [15, 16]. Plasma levels of both protein-bound and -free pentosidine increase with loss of renal function [17, 18], and both forms can be recovered from the urine [19, 20]. In a group of patients with renal insufficiency unrelated to diabetes, we have demonstrated that serum levels of pentosidine are most positively correlated with the monocyte activation marker neopterin, compared with other markers of immune function [21]. We interpreted this finding as evidence that monocyte activation may play a significant role in the physiologic response to AGE accumulation. To delineate a role for these pathways in the progression of diabetic nephropathy, we measured an Amadori precursor (glycosylated hemoglobin), both the protein-bound and -free form of the AGE pentosidine, and the immune markers (IL-6, C-reactive protein and neopterin). These measurements were performed in the baseline stored serum and urine from a subgroup of patients enrolled in the Collaborative Study Group's Trial of captopril in type 1 diabetic nephropathy [5]. The main endpoint of the Collaborative Study Group Trial was doubling of baseline serum creatinine. The availability of prospectively collected baseline samples from this trial provided an opportunity to compare patients with a poor outcome ("doublers") to those with a better outcome ("non-doublers"), whether or not the patients were subsequently treated with the ACE-inhibitor captopril.

METHODS

Sample selection

The original study was a prospective, double-blind, randomized clinical trial performed in 30 centers to assess the effect of angiotensin-converting enzyme inhibition (captopril) in patients with insulin-dependent diabetes mellitus (IDDM) and diabetic nephropathy [5]. In addition to a clear effect of therapy, this four year study identified two independent baseline predictors of progression of renal failure: serum creatinine and total urinary protein [22]. Of the 409 patients who participated in the original trial, a subgroup of 67 individuals were identified who demonstrated doubling of the serum creatinine (whether treated with captopril or not) within the study period. These "doublers" were then matched for treatment, initial serum creatinine and 24-hour urine protein with a subgroup of "non-doublers." Samples of urine and serum were stored at −70°C at the initiation of the Collaborative Study Group Trial before treatment with captopril or placebo. An additional 10 sets of samples were obtained at the time of doubling of serum creatinine, whether or not the patients were treated by captopril. These samples were matched with 10 sets of samples from "non-doublers" obtained at paired times. All samples were coded at Rush-Presbyterian-St. Luke's Medical Center and assayed in a blinded fashion by the laboratory at Case Western Reserve University (CWRU).

Clinical laboratory data

Measurements of glycosylated hemoglobin, urine and serum creatinine, and urine protein were performed in the central clinical laboratory at Rush-Presbyterian-St. Luke's Medical Center using standard clinical laboratory techniques.

Pentosidine

Protein in serum was assayed in a microassay modification of the Bradford method using Coomassie brilliant blue G250 obtained from Bio-Rad Laboratories (Melville, NY, USA). Protein in urine was measured in the clinical laboratory as described above. Protein (1 mg) from serum and urine was precipitated on ice with 10% trichloroacetic acid from Fisher Scientific (Pittsburgh, PA, USA). The pellets were washed twice with 5% cold trichloroacetic acid and acid hydrolyzed in 2 ml 6 N HCl for 16 hours at 110°C in borosilicate tubes with Teflon coated screw caps. Acid was removed by vacuum centrifugation (Savant, Farmington, NY, USA). The hydrolyzed pellet was dissolved in 250 μl of water/0.01 M heptafluorobutyric acid purchased from Sigma (St. Louis, MO, USA). The hydrolysate was filtered with 0.45 μm nylon microfilterfuge tube obtained from Rainin (Woburn, MA, USA). The equivalent of 0.4 mg of serum protein was injected onto a Waters HPLC system (Waters Division of Millipore, Marlborough, MA, USA). The column used was a 25 $\times$ 0.46 cm C-18 Vydac type 218TP (10 μm) column purchased from Separations Group (Hesperia, CA, USA). The HPLC was programmed with a linear gradient from 0 to 35 minutes of 10 to 17% acetonitrile (HPLC grade obtained from Fisher) in HPLC water and

0.1% heptafluorobutyric acid as a counter ion. Pentosidine eluted at ~30 minutes as monitored by fluorescence excitation at 335 nm and emission at 385 nm. Pentosidine prepared according to published procedure was used as a standard [13]. Results were calculated per mg protein. The intraassay coefficient of variation for peak area with a pentosidine standard of 3.75 pmol was 1.9% (range in 6 assays 1.4 to 2.7%). The interassay coefficient of variation for a sample of urine processed and assayed in each of six assays was 6.32% (mean 18.62 ± 1.18 pmol/mg protein). The detection limit of the assay is 0.2 pmol.

Free pentosidine

The processing of serum or urine in order to measure free (not bound to protein) pentosidine was modified from the methods of Takahashi et al [19, 20]. Serum or urine were applied to a size-selective filter with 10,000 molecular weight cut-off, Microcon 10 (Amicon, Beverly, MA, USA), and centrifuged at 2500 × g for 90 minutes at 4°C. The low molecular weight fraction (0.250 ml) was added to 15 ml of water and applied to a tC18 SepPak column from Waters Division of Millipore (Marlborough, MA, USA). The 15 ml volume passed through the column was then dried under a vacuum and resuspended in 300 μl of 0.01 M heptafluorobutyric acid (HFBA) in water purchased from Sigma (St. Louis, MO, USA) for injection onto the HPLC. Results are expressed per ml of initial fluid volume.

Separation of serum and urine by size-selective centrifugation was directly compared to the TCA precipitation method of Takahashi et al in four separate assays. Duplicate samples of serum (0.25 ml) were subjected to TCA precipitation with 0.75 ml of 10% trichloroacetic acid on ice followed by centrifugation at 5000 × g for 15 minutes, or to filtration using a Microcon 10 as described above. Assays 1 to 3 examined free pentosidine levels in serum from patients with diabetes and mild renal insufficiency (Correlation value for 3 assays only, $\rho = 0.92$, $P < 0.0001$). Assay #4 examined free pentosidine levels in serum samples from patients with end-stage renal failure on dialysis. There was an excellent correlation between the two methods (TCA precipitation and filtration using a 10,000 molecular wt cut-off filter) for determining free pentosidine (Fig. 1, correlation value for the four assays together, $\rho = 0.99$, $P < 0.0001$). Therefore, because of greater ease in processing, filtration was used to prepare all the serum samples for the determination of free pentosidine.

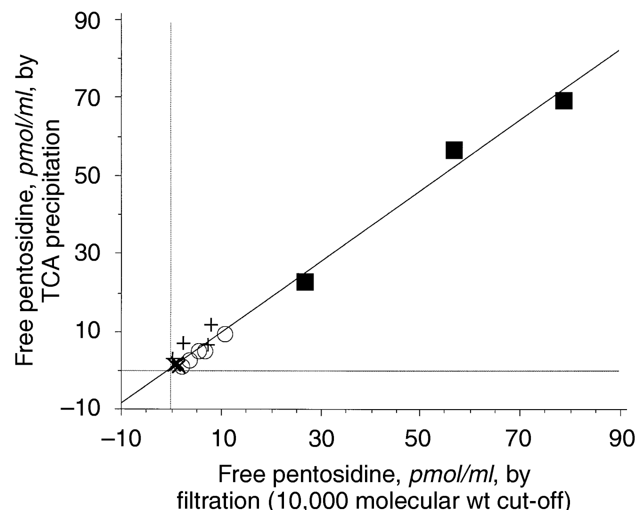

Fig. 1. Comparison of two methods of determining the free pentosidine concentration of serum. TCA precipitation and filtration over a 10,000 molecular weight filter were compared in the same samples. There was no significant difference between the two methods (correlation value, $\rho = 0.92$ for the first 3 assays, $P < 0.0001$; $\rho = 0.99$, $P < 0.0001$ for the 4 assays). Symbols are: (+, X, ○) assays 1–3; (■) assay 4. Assay #4 examined free pentosidine levels in serum samples from patients with end-stage renal failure.

Interleukin-6

Interleukin-6 (IL-6) was measured in a commercially available ELISA kit (Pelikine Compact human IL-6 ELISA; marketed in the United States by Research Diagnostics, Inc., Flanders, NJ, USA). This assay is sensitive to 0.5 to 1 pg/ml. In order to determine the validity of this assay in frozen (−70°C) stored samples, 20 sera that had been assayed by this ELISA in a previous and unrelated study were thawed and assayed more than two years later. There was a small, but insignificant, decrease in the IL-6 signal in the stored samples (mean 5.3 ± 0.6 compared 4.9 ± 0.9, representing a change of 6.25%). The correlation between the samples assayed before and after two years of storage at −70° was $\rho = 0.846$, $P < 0.0001$, by Pearson regression analysis. Therefore this assay was deemed valid for use in stored serum from the Cooperative Trial.

C-reactive protein

C-reactive protein (CRP) was measured using a commercially available enzyme immunoassay (Hemagen Diagnostics, Inc, Waltham, MA, USA). The assay has high reproducibility and accuracy compared to World Health Organization standards. It has tested validity in stored samples. The normal range for this assay is 0.6 to 1.9 μg/ml.

Neopterin

Neopterin was measured using a sensitive (to 0.2 ng/ml) and specific commercially available ELISA, (ICN Pharmaceuticals, Inc, Costa Mesa, CA, USA). The normal range of this assay is 0.3 to 3.0 ng/ml.

Calculations and statistical methods

Creatinine clearance was calculated by the standard formula UV/P. Excretion of protein-bound pentosidine was calculated by multiplying the 24-hour urine protein excretion (in g/total volume) by the pentosidine content of the protein (pmol/mg protein). Excretion of free pentosidine

Table 1. Demographic and clinical characteristics of full cohort of study subjects

	"Doubler"	"Non-doubler"
Age *years*	36.5 ± 0.9	34.8 ± 0.9
Range	21–57	20–49
Sex, *M/F*	39/28	39/28
Race, *C/AA/O*	53/13/1	60/7/0
Baseline serum creatinine *mg/dl*	1.63 ± 0.06	1.53 ± 0.05
End-point[a] serum creatinine *mg/dl*	3.34 ± 0.11	2.69 ± 0.23[b]
Baseline creatinine clearance *ml/min*	59.2 ± 3.4	68.1 ± 4.0
End-point[a] creatinine clearance *ml/min*	27.0 ± 1.7	40.8 ± 3.8[c]
Baseline protein excretion *g/24 hr*	4.9 ± 0.4	4.1 ± 0.3

Plus/minus values are means ± SE. Race is: C, Caucasian; AA, African American; O, Oriental. Although there were more AA among the "doublers," there was no effect of race or sex in the multivariate proportional hazards model, see text.
[a] At time of doubling
[b] $P < 0.05$, [c] $P < 0.01$, comparing "doublers" with "non-doublers" by *t*-test

Table 2. Demographic and clinical characteristics of subset of study subjects examined at time of doubling

	"Doubler"	"Non-doubler"
Age *years*	38.7 ± 2.9	35.2 ± 2.2
Range	26–57	26–46
Sex, *M/F*	5/5	5/5
Race, *C/AA/O*	9/1/0	8/2/0
Baseline[b] serum creatinine *mg/dl*	1.76 ± 0.19	1.61 ± 0.15
End-point[a] serum creatinine *mg/dl*	4.05 ± 0.44	1.89 ± 0.30[d]
Baseline[b] creatinine clearance *ml/min*	48.3 ± 10.0	60.8 ± 8.0
End-point[a] creatinine clearance *ml/min*	25.4 ± 5.2	45.7 ± 14.0[c]
Baseline[b] protein excretion *g/24 hr*	3.9 ± 0.7	4.1 ± 0.8
End point[a] protein excretion *g/24 hr*	5.0 ± 1.1	2.4 ± 0.5[c]

Plus/minus values are means ± SE. Race is: C, Caucasian; AA, African American; O, Oriental.
[a] At time of doubling
[b] At study initiation
Comparisons by *t*-test, [c] $P < 0.01$, [d] $P < 0.001$, comparing "doublers" with "non-doublers"

was calculated by multiplying the 24-hour urine volume by the pentosidine concentration (pmol/ml).

Pearson's correlation was used to compare methods for determining free pentosidine, and to compare results of IL-6 assays on stored samples. Differences between baseline laboratory values for "doublers" and "non-doublers" were tested using *t*-tests without correction for multiple comparisons. Univariate proportional hazards models that calculate the unadjusted hazard ratio for doubling (taking into account time to doubling and censoring) were used to assess the importance of baseline serum neopterin and free pentosidine. Multivariate proportional hazards models were used to assess the independent relationship between neopterin and serum free pentosidine after adjustment for age, baseline serum creatinine, baseline proteinuria, and treatment (that is, captopril or placebo). Optimal prognostic cutpoints for neopterin and free pentosidine were identified using Receiver Operating Characteristics curves and rounded to the nearest sensible value using clinical judgment, specifically for neopterin, a positive test was defined as >3.0 ng/ml; for free pentosidine, a positive test was defined as 1.0 pmol/ml. Sensitivity and specificity were calculated for these definitions using the following formulas: sensitivity = all positives among "doublers"/all "doublers"; specificity = all negatives among "non-doublers"/all who did not double. Pairwise Pearson's correlations were also used to assess important relationships among clinical characteristics. These data analyses was performed using Statview 4.5 (Abacus Concepts, Berkeley, CA, USA) on a Macintosh Centris 650, and Stata 6.01(Stat Corp. College Station, TX, USA).

RESULTS

Clinical characteristics of "doublers" and "nondoublers" are shown in Table 1. Because the subjects were matched at baseline, there were no differences in serum creatinine, creatinine clearance and 24-hour protein excretion in the groups of subjects before treatment. To demonstrate the degree of progression of renal failure over the course of the study, the end point serum creatinine and creatinine clearances are also included in Table 1. All subjects showed a progression of renal failure, but the "non-doublers" did not reach twice baseline by the end of the study period. There were no baseline differences in clinical characteristics or results between those who were later treated with captopril or placebo (data not shown for these parameters). The clinical characteristics of the 10 study subjects at the time of doubling, and the matched "non-doublers" are summarized in Table 2. In this subgroup the "doublers" had significantly greater 24-hour protein excretion at the end point than the "non-doublers," as well as marked differences in end-point serum creatinine (Fig. 3) and creatinine clearances.

Baseline levels of glycohemoglobin, serum protein-bound and -free pentosidine, and urinary excretion of protein-bound and -free pentosidine are shown in Table 3. The baseline levels of glycohemoglobin (12.5 ± 0.4 vs. 12.4 ± 0.3%, $P = 0.82$), protein-bound pentosidine in serum (2.2 ± 0.2 vs. 2.1 ± 0.2 pmol/mg protein, $P = 0.64$) and protein-bound pentosidine urinary excretion (9.8 ± 0.9 vs. 8.4 ± 1.0, $P = 0.29$) did not differ between the "doublers" and "non-doublers" or by treatment group (glycohemoglobin, $P = 0.20$; serum protein-bound pentosidine, $P = 0.30$; urine protein-bound pentosidine, $P = 0.69$). In contrast, serum free pentosidine (1.4 ± 0.2 vs. 0.9 ± 0.1 pmol/ml, $P < 0.007$), and 24-hour free pentosidine excretion (39.6 ± 3.8 vs. 27.1 ± 3.0 nmol/total volume, $P < 0.01$) differed significantly at baseline between the "doublers" and "non-doublers." When the data were analyzed without regard to subsequent "doubling" or "non-doubling," there were no baseline differences between those subjects who were treated with captopril or placebo for any of the parameters, including serum-free pentosidine

Table 3. Baseline glycosylated hemoglobin and advanced glycation end product levels by outcome and treatment group

Assigned to treatment group	"Doublers"		"Non-doublers"	
	ACE-inhibitor	Placebo	ACE-inhibitor	Placebo
Number	25	42	25	42
Glycohemoglobin *%*	13.1 ± 0.6	12.2 ± 0.5	12.8 ± 0.5	12.2 ± 0.4
Serum protein-bound pentosidine *pmol/mg protein*	2.5 ± 0.4	2.1 ± 0.3	2.3 ± 0.4	2.0 ± 0.3
Serum free pentosidine *pmol/ml*	1.56 ± 0.31	1.27 ± 0.15	0.93 ± 0.11[a]	0.92 ± 0.10[a]
24-Hour excretion protein-bound pentosidine *nmol/TV*	9.7 ± 1.7	9.9 ± 1.2	7.8 ± 2.0	8.6 ± 1.2
24 Hour excretion free pentosidine *nmol/TV*	33.4 ± 5.4	42.9 ± 5.1	24.1 ± 4.9	28.8 ± 3.9[a]

Plus/minus values are means ± SE. Comparisons are by 2 way ANOVA.
[a] $P < 0.01$ comparing "doublers" with "non-doublers"

(1.1 ± 0.8 vs. 1.2 ± 0.2, $P = 0.45$), and 24-hour free pentosidine excretion (35 ± 3 vs. 29 ± 4, $P = 0.17$). Although there was no difference between baseline serum creatinine in the "doublers" and "non-doublers" (Tables 1 and 2), Pearson regression analysis showed creatinine to be highly correlated with baseline levels of protein-bound ($\rho = 0.4$, $P < 0.0001$) and free ($\rho = 0.6$, $P < 0.0001$) pentosidine in serum across the group as a whole, as well as in the subgroup at baseline and follow-up (Fig. 2).

Baseline serum levels of the monocyte activation marker neopterin were significantly different between "doublers," and "non-doublers" (3.8 ± 0.3 vs. 2.7 ± 0.2 ng/ml, $P < 0.001$). No such difference was found for the immune markers IL-6 (2.3 ± 0.1 vs. 2.1 ± 0.1 pg/ml, $P < 0.15$) or CRP (3.7 ± 0.6 vs. 3.2 ± 0.6 ng/ml, $P < 0.001$; Table 4). When the data were analyzed based on eventual treatment with captopril or placebo (and without regard for subsequent "doubling" or "non-doubling" status), no differences in baseline neopterin (3.3 ± 0.2 vs. 3.2 ± 0.3, $P = 0.75$), IL-6 (2.1 ± 0.1 vs. 2.2 ± 0.2, $P = 0.48$) or CRP (3.4 ± 0.6 vs. 3.6 ± 0.7, $P = 0.75$) were found. Using Pearson regression analysis, the monocyte activation marker neopterin was highly correlated with baseline levels of serum free ($\rho = 0.5$, $P < 0.0001$) and protein-bound ($\rho = 0.6$, $P < 0.0001$) pentosidine. No such correlation was found for IL-6 or CRP.

There was a significant correlation between serum creatinine and serum levels of protein-bound ($\rho = 0.5$, $P < 0.002$) and free pentosidine ($\rho = 0.8$, $P < 0.0001$) in the subgroup of 20 patients at baseline and at the time of doubling (Fig. 2). The "doublers" showed a highly significant increase in serum creatinine, which was also greater than the increase shown by the "non-doublers" (Fig. 3A). Although protein-bound pentosidine levels tended to increase with follow-up, the differences between "doublers" and "non-doublers" were not significant (Fig. 3B). The "doublers" demonstrated a marked increase in free pentosidine in the follow-up period (Fig. 3C). Baseline levels of free pentosidine were lower in the "non-doublers" (as also

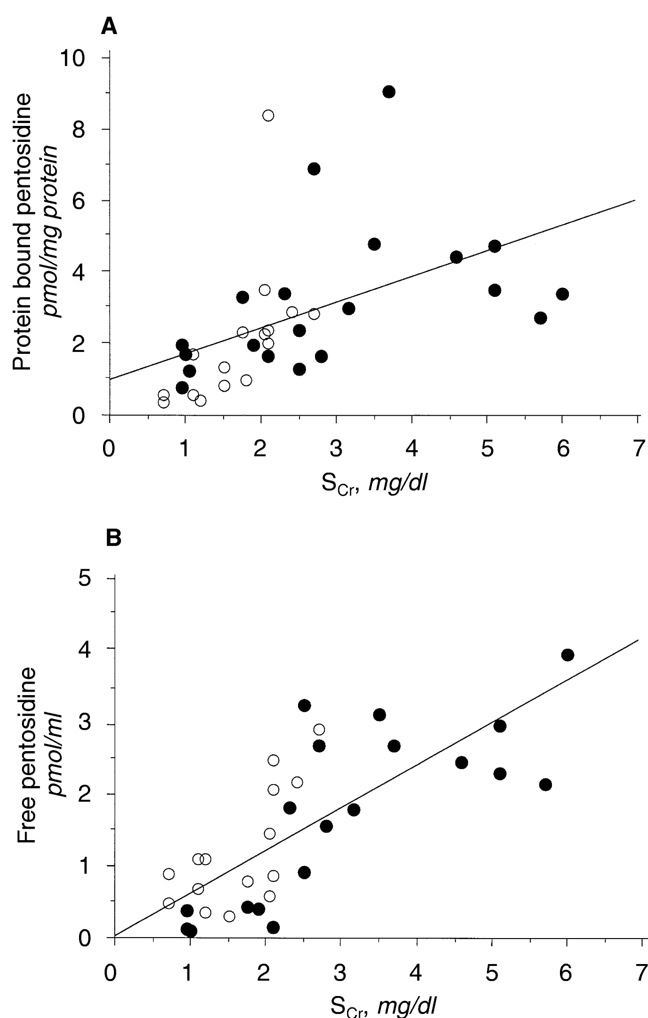

Fig. 2. Regression plots of protein bound and free pentosidine versus serum creatinine in "doublers" and paired "non-doublers" at baseline (○) and at time of doubling (●). (*A*) Protein bound pentosidine ($\rho = 0.50$, $P < 0.002$). (*B*) Free pentosidine ($\rho = 0.76$, $P < 0.0001$). See Table 2.

Table 4. Baseline immune activation markers by outcome and treatment group

Assigned to treatment group	"Doublers"		"Non-doublers"	
	ACE-inhibitor	Placebo	ACE-inhibitor	Placebo
Number	25	42	25	42
Interleukin-6 *pg/ml*	2.2 ± 0.2	2.4 ± 0.3	2.0 ± 0.2	2.1 ± 0.3
C-reactive protein *μg/ml*	3.5 ± 0.7	4.1 ± 0.9	3.3 ± 0.9	3.2 ± 0.9
Neopterin *ng/ml*	3.5 ± 0.5	3.9 ± 0.3	2.8 ± 0.4[a]	2.6 ± 0.2[b]

Plus/minus values are means ± SE. Comparisons are by 2 way ANOVA.
[a] $P < 0.005$, [b] $P < 0.001$ comparing "doublers" with "non-doublers"

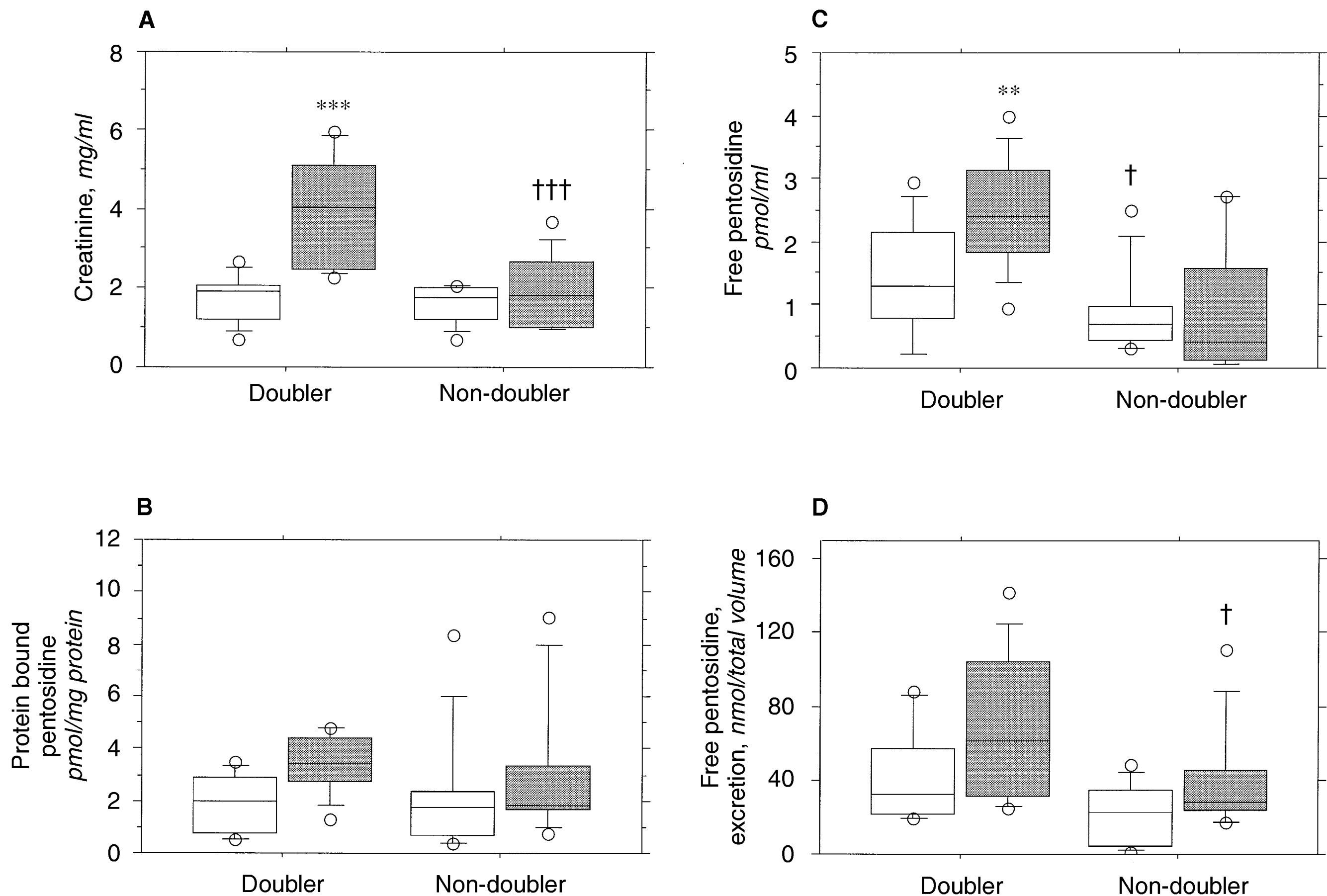

Fig. 3. Whisker plots showing the 10th, 25th, 50th, 75th and 90th percentile distribution of data obtained at baseline (□) and follow-up (▩) in 10 pairs of "doublers" and "non-doublers" (see Table 2). (*A*) Changes in serum creatinine (***$P < 0.0001$ baseline vs. follow-up, †††$P < 0.0001$ follow-up in "non-doubler" compared to follow-up in "doubler"). (*C*) Changes in serum free pentosidine (**$P < 0.0004$ baseline vs. follow-up, †$P < 0.02$ baseline in "non-doubler" compared to baseline in "doubler"). (*D*) Changes in urinary free pentosidine excretion (†$P < 0.05$, follow-up in "non-double" vs. follow-up in "doubler").

seen in the larger cohort of 67 pairs of subjects; Table 3). In addition, at follow-up, the total urinary excretion of free pentosidine remained lower in the "non-doublers" than in the "doublers" (Fig. 3D).

Because the simple grouping of patients by their "doubling" or "non-doubling" status did not take into account differences in times of entry into the study and lengths of observation, additional statistical analyses were performed. Based on cut-points determined from the ROC curves, patients with free pentosidine levels greater than 1.0 pmol/ml and those with neopterin levels above 3.0 ng/ml reached "doubling" of creatinine at a significantly more rapid rate, Figures 4 and 5. Serum free pentosidine and neopterin, and the excretion of free pentosidine were significant predictors for time to "doubling", in the proportional hazards model with a single covariate (Table 5). The multivariate proportional hazards model showed no effect of adjustment for age, baseline serum creatinine, baseline proteinuria or eventual treatment regimen (captopril or placebo; Table 6).

Optimal endpoints for serum free pentosidine (>1 pmol/ml) and neopterin (>3.0 ng/ml) demonstrated modest sensitivity and specificity for predicting doubling of serum creatinine (Table 7). Despite differences in Cumulative Survival as demonstrated by the Kaplan-Meier plots (Figs. 4 and 5), the predictive value of these parameters was not strong enough for either parameter to be taken as an isolated predictor for worsening of diabetic nephropathy, based on this data set.

DISCUSSION

In this subgroup of patients from the Collaborative Study Group Trial, serum free pentosidine, 24-hour free pentosidine excretion, and serum levels of the monocyte activation marker neopterin differed significantly at baseline in those whose serum creatinine levels ultimately doubled compared with those in a matched group who experienced a better outcome. At baseline, patients in the two groups had similar levels of serum creatinine, urinary protein excretion, glycohemoglobin and protein-bound pentosidine

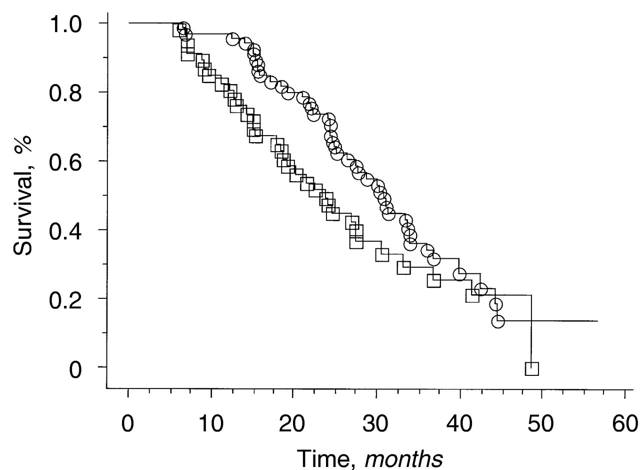

Fig. 4. Cumulative survival plots of time to doubling of serum creatinine in study subjects for serum free pentosidine cut points above (□) and below (○) 1.0 pg/ml. Difference between groups significant at $P < 0.01$.

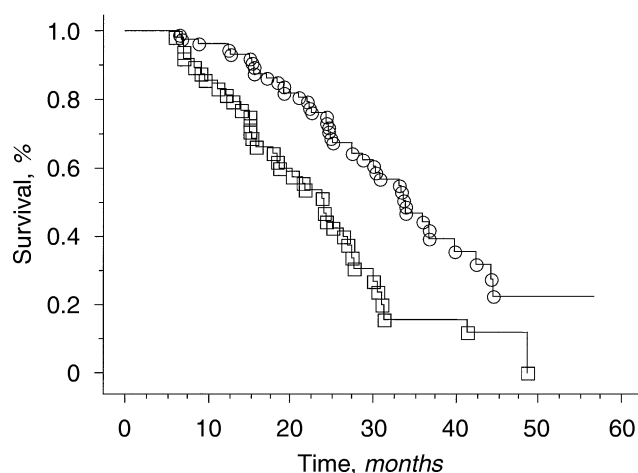

Fig. 5. Cumulative survival plots of time to doubling of serum creatinine in study subjects for serum neopterin cut points above (□) and below (○) 3.0 ng/ml. Difference between groups significant at $P < 0.002$.

(in serum and excreted). These results confirm the validity of the study design by demonstrating that subjects were well matched at baseline for the degree of diabetic severity and of renal impairment. Comparing baseline values between "doublers" or "non-doublers" without adjusting for observation intervals can introduce bias because of differences in length of observation throughout the study (the effects of continuing enrollment and variable lengths of follow-up). Therefore, the effects of these parameters were examined in a proportional hazards model. Serum free pentosidine and neopterin remained highly significant, whereas the effect of 24-hour free pentosidine excretion was not quite statistically significant ($P = 0.055$; Table 4). Grouping patients by serum neopterin and free pentosidine "cut-points" resulted in highly significant differences in survival curves (Figs. 4 and 5). These results suggest that higher levels of serum free pentosidine and neopterin may serve as markers of progression in patients with early nephropathy. The mechanism underlying this association remains speculative.

Table 5. Effect of markers on time to doubling of serum creatinine

Parameter	*N*	"Doublers"	Hazard ratio	Confidence interval	*P*
Serum free pentosidine	119	61	1.35	1.11–1.64	0.003
Bound pentosidine excretion	119	60	1.02	0.98–1.04	0.311
Free pentosidine excretion	114	54	1.01	0.99–1.02	0.055
Neopterin	128	63	1.15	1.03–1.28	0.012

Proportional hazards used to compare times to doubling taking into account censoring.

Table 6. Effect of neopterin and free pentosidine on time to doubling of creatinine in multivariate proportional hazards model

Parameter	Hazard ratio	Confidence interval	*P*
Age	0.99	0.96–1.04	0.99
Treatment[c]	0.82	0.47–1.43	0.49
Baseline serum creatinine	1.02	0.48–2.16	0.96
Baseline protein excretion	1.06	0.99–1.14	0.08
Serum free pentosidine[b]	1.49	1.11–1.98	0.007
Neopterin[a]	1.17	0.99–1.37	0.05

[a] Without free pentosidine in model, neopterin $P = 0.0001$, other parameters NS
[b] Without neopterin in model, free pentosidine $P = 0.0002$, other parameters NS
[c] Treatment (captopril vs. placebo)

Pentosidine is a very stable structure. Its resistance to acid hydrolysis forms the basis for the quantitative methods used in this paper. In the laboratory, acid hydrolysis breaks amino acid bonds, but leaves pentosidine intact for measurement by HPLC. A similar process which could result in the formation of free pentosidine from protein-bound pentosidine has not been identified in intact animals. Thus, the origin of free pentosidine in the circulation, or excreted into the urine remains unclear. Previous reports have shown that levels of protein-associated pentosidine and free pentosidine correlate well across a range of concentrations of both forms of the AGE [17, 18]. Nonetheless, greater than 95% of the pentosidine present in the circulation is tightly bound to protein, with less than 5% existing as free pentosidine [17, 18]. Pentosidine bound to protein undoubtedly forms through the Maillard reaction directly involving that protein. Consistent with previously reported levels, the range of protein-bound pentosidine found in these patients with diabetic nephropathy and mild renal insufficiency is two to four times normal [17, 19, 21]. Although plasma-free pentosidine levels were undetectable in a group of patients with diabetes and normal renal

Table 7. Predictive value of chosen cut-points for neopterin and free pentosidine

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Serum free pentosidine >1.0 pmol/ml	52.2%	59.4%	55.6%	56.2%
Neopterin >3.0 ng/ml	48.4%	71.2%	62.0%	58.8%

Cutpoints for neopterin and for free pentosidine were chosen using Receiver Operating Characteristic curves and clinical judgment. (See text.)

function [17], the patients in the Collaborative Study had mild to moderate renal insufficiency.

Animal studies demonstrate that the kidney plays a key role in removing circulating AGEs, both clearing AGE peptides and metabolizing AGE proteins by the endolysosomal apparatus of the proximal tubule [23]. The kidney has high levels of the enzyme 3-deoxyglucosone reductase, which is thought to prevent protein glycation in the presence of the Amadori-derived reactive intermediate, 3-deoxyglucosone [24]. Thus, loss of renal mass might result in loss of expression of this enzyme leading to accelerated rates of formation of AGEs. In addition, AGEs themselves can catalyze further AGE formation as demonstrated *in vitro* [25].

Free pentosidine in the circulation may have its origin in the intestinal absorption of food-associated AGEs. Free pentosidine can be detected in the plasma of both healthy and uremic rats fed pentosidine, and there is a consistently prolonged half-life in rats with renal impairment [17]. Recently, radiolabeled free pentosidine has been shown to accumulate in the proximal tubule when injected into rats [26]. Tubular processing of pentosidine, via an endolysosomal pathway may explain this finding, because a greater percentage of unbound radioactivity appeared in the urine than was immunoprecipitatable. In contrast, in rats with tubular dysfunction, the urine contained intact radiolabeled pentosidine [26]. Thus, impaired renal excretion of free pentosidine may explain accumulation of this metabolite in the serum of patients with renal failure. However, impaired excretion of free pentosidine does not account for the increased quantity of free pentosidine excreted in the urine in the presence of renal insufficiency. Therefore, increased total excretion of free pentosidine in the presence of diabetes and renal insufficiency suggests increased total formation of this analyte or of a precursor such as protein-bound pentosidine [17].

The receptor for AGEs (RAGE) has been isolated and cloned [27, 28]. It has been identified on an array of cells including endothelial and smooth muscle cells of the vasculature, and on macrophages and lymphocytes [29, 30]. Some workers have suggested that uptake and processing of protein-bound AGEs by monocytes and macrophages may provide a pathway for degradation with release of amino acids and free AGEs [31]. In addition, the increased cellular oxidant stress induced in cells [32, 33] by the uptake of AGEs, might lead to a vicious cycle in which accumulation of AGEs is accelerated. One explanation for the increased total excretion of free pentosidine seen in the "doublers" is that a vicious cycle involving monocytes and macrophages resulted in the accelerated formation of AGEs on proteins, with a further increase in the excretion of free pentosidine.

To obtain evidence of systemic inflammation, an inflammatory cytokine that modulates acute phase reaction proteins (IL-6), a marker of the acute phase reaction (C-reactive protein), and a monocyte/macrophage activation marker (neopterin) were measured in the baseline serum samples. IL-6 is produced by activated monocytes and macrophages, but also by activated T and B cells and endothelial cells. Renal insufficiency has been associated with increases in levels of IL-6 [34, 35], and it has been shown that IL-6 induces the release of acute phase reactants from the liver [36]. IL-6 may play an important role in nutritional abnormalities experienced by patients with renal failure [37]. C-reactive protein is one of the major acute-phase reactants produced in the liver in response to inflammatory cytokines (IL-6, IL-1β and TNF-α). C-reactive protein is a component of normal serum. Increased levels function in host defense in a manner similar to immunoglobulins [38]. Neopterin is a monocyte/macrophage product that derives from 7,8-dihydroneopterin triphosphate, the first intermediate in the biosynthesis of tetrahydrobiopterin from GTP. Because human monocytes and macrophages contain low levels of 6-pyruvoyl tetrahydrobiopterin synthase, neopterin derivatives accumulate and, after dephosphorylation, can leak from cells [39]. In chronic inflammatory and infectious diseases, neopterin has been found to be a valuable predictor of morbidity and mortality [40].

The monocyte activation marker neopterin and serum levels of protein-bound pentosidine have been previously found to have a strong positive correlation in a group of patients with renal insufficiency unrelated to diabetes across a wide range of renal function [21]. In the present study the baseline level of renal function was controlled, and there were no baseline differences in serum levels of protein-bound pentosidine. Therefore, the differences in baseline neopterin levels demonstrated in this study directly reflect inter-subject variability with respect to monocyte activation. There was a strong correlation between neopterin levels and free pentosidine levels in the serum of these patients with diabetic nephropathy, supporting our hypothesis that a relationship exists between monocyte activation and the possible release of free pentosidine from protein-bound pentosidine by these cells.

In summary, elevated levels of serum free pentosidine, the total urinary excretion of free pentosidine, and the

monocyte activation marker neopterin, are correlated with the rate of progression of diabetic nephropathy. Neopterin levels are highly correlated with serum-free and protein-bound pentosidine. In conclusion, differences exist in individual responsiveness to the handling and processing of AGEs that form during the course of diabetes. A more complete understanding of the nature of such differences has the potential to open new therapeutic approaches to the prevention of diabetic nephropathy and other complications of diabetes mellitus.

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