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Proximal tubular injury in Chinese herbs nephropathy: Monitoring by neutral endopeptidase enzymuria

JOËLLE L. NORTIER, MONIQUE M. DESCHODT-LANCKMAN, SYLVIE SIMON, NATHALIE O. THIELEMANS, ERIC G. DE PREZ, MICHEL F. DEPIERREUX, CHRISTIAN L. TIELEMANS, CLAUDE RICHARD, ROBERT R. LAUWERYS, ALFRED M. BERNARD, and JEAN-LOUIS VANHERWEGHEM

Laboratoire Pluridisciplinaire de Recherche Expérimentale Biomédicale, Faculté de Médecine, Université Libre de Bruxelles; Unité de Toxicologie Industrielle et de Médecine du Travail, Faculté de Médecine, Université Catholique de Louvain; Department of Nephrology, Institut E. Cavell; and Departments of Pathology and Nephrology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium

Proximal tubular injury in Chinese herbs nephropathy: Monitoring by neutral endopeptidase enzymuria. Neutral endopeptidase (NEP) is a 94 kDa ectoenzyme of the proximal tubule brush border, physiologically released into the urine with apical membrane fragments. As proximal tubular atrophy was a histological hallmark of Chinese herbs nephropathy (CHN), this study firstly determined renal excretion of NEP in healthy control subjects ($N = 31$), in patients with CHN ($N = 26$) and in women having consumed Chinese herbs and whose renal function was normal but running the risk of developing CHN ($N = 27$). Another patient group consisted of female patients with glomerular diseases ($N = 12$). At the same time, measurements of urinary microproteins (Clara cell protein, retinol binding protein, β_2-microglobulin and α_1-microglobulin) were performed, as indicators of tubular dysfunction. Cell damage was estimated by the excretion of N-acetyl-β-D-glucosaminidase (NAG). In the control group, the physiological NEP enzymuria was 43.1 μg/24 hr (geometric mean). In CHN patients, levels of urinary NEP were significantly decreased in those with moderate renal failure (26.7 μg/24 hr; $N = 21$; $P < 0.05$) and almost abolished in end-stage renal failure patients (4.35 μg/24 hr; $N = 5$; $P < 0.05$). In patients at risk as well as in patients with glomerular diseases, urinary NEP levels were not statistically different from those observed in control subjects (40.68 μg/24 hr and 48.5 μg/24 hr, respectively). Several degrees of tubular dysfunction and injury were noted in patients groups, as attested by increased urinary microproteins and NAG excretions. Considering the data from control and CHN patients, NEP enzymuria positively correlated with individual creatinine clearance values ($r = 0.76$; $P = 0.0001$) and negatively correlated with urinary microproteins levels ($r = -0.55$; $P = 0.00001$). Finally, NEP was regularly quantitated in the urine of 6 CHN patients for a period ranging from six months to two years and in 19 patients at risk during two years, respectively. In the first group, renal function progressively deteriorated in 3 patients, leading them to renal replacement therapy after 38 to 115 weeks. Stable parameters were observed in the remaining 3 patients. A direct correlation between creatinine clearance and NEP excretion was found longitudinally in each case. In the second group, no significant change of urinary NEP levels was observed (45.9 μg/24 hr), in parallel with stable renal function. Taken together, these results indicate that, in CHN patients, NEP enzymuria provides a rapid and noninvasive determination of the degree of structural impairment affecting the proximal tubular population and further reflecting the severity of the renal disease. The interest of this urinary marker in monitoring the progression of other tubulointerstitial diseases remains to be assessed.

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Neutral endopeptidase 24.11 (NEP) is a zinc metallopeptidase anchored to the cell surface of many tissues [1] and involved in regulatory processes affecting target cell responses by cleavage of several peptidic hormones [2, 3]. In the kidney, this ectoenzyme is abundantly localized on the brush border membranes of the proximal tubular epithelium, processing filtered peptides present in the tubular fluid [4]. An excreted form has been identified in human urine and is probably kidney-derived [5]. Indeed, the large size of the soluble plasmatic enzyme (~94 kDa) and the low concentration of this circulating form (~13 ng/ml) [6] permit the exclusion of the possibility of a significant plasma contribution to the urinary excretion, suggesting that NEP enzymuria mainly reflects the amount of the renal enzyme. This was supported by the recent work of Aviv et al, reporting increased NEP content both in the kidney and in urine after a chronic salt loading in the rat [7]. In humans, no data on NEP enzymuria are available, except transiently increased urinary levels of NEP early after renal transplantation [8]. These *in vivo* results are in agreement with morphological studies performed in experimental models of ischemia, demonstrating that the proximal tubular epithelium was a preferential target of ischemic injury [9–12].

In addition to the complex mechanisms related to hemodynamic modifications, toxic agents may directly or not damage the proximal tubule. Numerous studies over the last decades have pointed out the differences in susceptibility to certain nephrotoxicants of several parts of the nephron, according to their distinct morphological, biochemical, and hence, functional properties [reviewed in 13]. Although physiopathological mechanisms remain to be elucidated, an interesting example was provided by the recently described Chinese herbs nephropathy (CHN) [14]. Cases of rapidly progressive interstitial renal fibrosis were observed in Belgium in young female patients and related to a slimming regimen including Chinese herbs called *Stephania tetrandra* and *Magnolia officinalis*. Phytochemical analyses of batches of the so-called *S tetrandra* resulted in the identification in most of the herbs of aristolochic acids [15], known for their nephrotoxic properties [16], instead of the expected tetrandrine. Careful examination of renal biopsy material from these patients revealed that the destruction process of the tubules started in the superficial cortex, mainly composed of proximal tubules, and progressed towards the deep cortex [17]. Along these lines, we tested the

hypothesis that NEP enzymuria could contribute to the evaluation of the severity of this tubulointerstitial nephritis at different stages of the disease.

The aims of the present study were first to measure NEP concentration in normal human urine and in urine from patients with several degrees of renal failure due to CHN. Data were compared with those obtained from patients having consumed Chinese herbs and whose renal function was normal, but running the risk of developing CHN, and from patients with glomerular diseases. Secondly, we wanted to relate these results with urinary measurements of well-known markers: N-acetyl-β-D-glucosaminidase (NAG) (EC 3.2.1.30), another enzyme of renal origin, abnormally released from lysosomes into the tubular fluid in case of intracellular damage [18, 19]. The severity of tubular dysfunction was investigated by measuring the capacity of proximal tubular cells to reabsorb filtered microproteins such as Clara cell protein (CC16; 15.8 kDa), retinol binding protein (RBP; 22.2 kDa), β_2-microglobulin (β_2m; 11.8 kDa) and α_1-microglobulin (α_1m; 26 to 31 kDa) [20]. Finally, we had the opportunity to perform a long-term follow-up of 6 CHN patients and 19 patients running the risk of developing CHN, in order to relate NEP enzymuria with renal outcome.

Methods

Normal and patient populations

Thirty-one healthy female volunteers from the laboratory staff, aged 22 to 63 years, previously confirmed to have normal renal function parameters, were tested for NEP and NAG enzymuria and for urinary excretion rate of CC16, RBP, β_2m and α_1m.

The group of CHN patients was composed of 26 female patients (ages ranging from 33 to 58 years) admitted in the Nephrology departments of either Erasme Hospital or Institut Médical E. Cavell, between November 1992 and July 1993. All had followed a slimming regimen including Chinese herbs (CH) from May 1990 to June 1992. The exposure period of the patients to these Chinese herbs varied from 4 to 27 months at a dosage of 300 to 900 mg of powder extract per day for the so-called *Stephania tetrandra*.

Five out of 26 patients were admitted in end-stage renal failure (ESRF) requiring immediate hemodialysis. The remaining 21 patients entered for diagnostic management of their renal insufficiency (including renal biopsy, which was accepted and successfully performed for 17 out of 20). None of these patients received corticoids during the study. Six out of them were regularly (every 6 week period, approximately) re-evaluated in terms of their tubular markers in addition to the usual parameters of renal function. This follow-up period ranged from six months to two years.

The group of patients running the risk of developing CHN was composed of 27 women who consumed CH during the same critical period and according to identical oral administration prescriptions (see above). They consulted us to check on their renal function. They were informed that serum creatinine level was normal (between 70 and 105 μmol/liter) and that a follow-up was advisable. Among them, 22 accepted a one-year follow-up and 19 were still present after two years.

The last group of patients contained 12 patients with different glomerular diseases, characterized by moderate to severe albuminuria (geometric mean 1.1 g/24 hr). Two were in ESRF and the remaining 10 had different degrees of renal failure. Histological diagnosis was obtained for 11 out of these patients, the last one having congenital renal hypoplasia. None of these patients received corticotherapy.

Collection of urine and serum samples

Twenty-four-hour urine samples and peripheral venous blood were collected from normal volunteers and each patient. Each patient was free of Chinese herbs and other drugs for at least two months at the moment of the first 24-hour urine collection. For NEP assay, fresh urine samples were kept at 4°C until the analysis which was performed in the 72 following hours, taking into account preliminary stability experiments [21]. For other markers, measurements were performed on urine samples after one freezing-thawing cycle. Because of the stability of the measured microproteins in acid urines (except β_2m susceptible to be degraded by several mechanisms and for which measurements may be underestimated), no precaution was taken to alkalinize urine samples during or after collection.

Markers assay methods

Urine and serum creatinine was determined by the Jaffé reaction and the creatinine clearance was calculated on the basis of the 24-hour urine collection.

Endopeptidase enzymatic activity was measured by a spectrofluorimetric assay [8] after 1/15, 1/30 and 1/60 dilution of the urine samples with 50 mM Tris HCl buffer, pH 7.6. In brief, the synthetic substrate succinyl-alanyl-alanyl-phenylalanine-7-amido-4-methyl coumarine (Suc-Ala-Ala-Phe-AMC; Bachem, Bubendorf, Switzerland) was cleaved by NEP to produce Phe-AMC. This compound, after incubation with aminopeptidase M (EC 3.4.11.2; Pierce, Rockford, IL, USA), generates AMC. By the use of a standard curve established with purified human renal NEP, it was possible to convert the rate of AMC production into enzyme amounts.

Urinary levels of CC16 were determined by a sensitive latex immunoassay relying on the agglutination of latex particles [22]. The same methodology was used for β_2m, RBP, α_1m and albumin measurements in urine samples, as previously reported [23]. The activity of NAG was measured by a fluorimetric assay according to the method described by Tucker et al [24].

Statistical analysis

Differences between values of tubular markers excretion in control and patient populations as well as in patient groups were assessed by one-way analysis of variance followed by the Dunett's multiple comparison test. Results were reported as statistically significant at $P < 0.05$. Data on urinary CC16 protein were log transformed before statistical analysis, and the normality of all variables distribution was checked using the Kolmogorov-Smirnof's one-sample test. Linear regression tests were performed by calculating the correlation coefficient.

Results

NEP enzymuria and other tubular markers in controls and in patients previously exposed to Chinese herbs

Experimental data obtained at the beginning of the study, from 24-hour urine samples of healthy women and patients having consumed CH and presenting or not presenting with renal failure,

Table 1. Experimental data obtained from 24-hr urine samples of healthy women, patients at risk and patients with Chinese herbs nephropathy

	Control population ($N = 31$)	Patients at risk ($N = 27$)	Patients with renal failure ($N = 21$)	End-stage renal failure patients ($N = 5$)
Creatinine clearance *ml/min*[a]	113 ± 27	98 ± 29	40 ± 14[c]	8 ± 2[c]
Albuminuria *mg/24 hr*[b]	6.33 (1.62–346)	7.8 (2.33–128)	63.9[c] (11.5–444)	54.3[c] (13–184)
NEP *μg/24 hr*[b]	43.1 (26.7–67.8)	40.68 (22.7–87.2)	26.7[c] (13.5–45.5)	4.35[c] (1.1–12.4)
NAG *UI/24 hr*[b]	0.68 (0.23–2.93)	1.35 (0.33–3.62)	2.15[c] (0.77–6.24)	1.42[c] (0.7–2.3)
CC16 *μg/24 hr*[b]	3.49 (0.49–65.4)	10.94[c] (0.99–359)	891[c] (96.4–4,180)	963[c] (599–2,420)
β_2m *μg/24 hr*[b]	50.9 (0.42–363)	82.1 (17.5–199)	13,970[c] (708–104,400)	38,200[c] (15,800–77,400)
RBP *μg/24 hr*[b]	45.2 (1.19–363)	100 (22.2–316)	7,760[c] (186–97,400)	47,900[c] (18,600–134,000)
α_1m *mg/24 hr*[b]	3.5 (0.26–10.7)	7.1 (2.27–33.1)	111[c] (9.8–717)	199[c] (124–443)

[a] Data are mean ± SD
[b] Data are geometric mean with the range between brackets
[c] $P < 0.05$, compared to control values

are represented in Table 1. Patients at risk, that is, without renal insufficiency, had urinary levels of markers in the physiological range, except for CC16 excretion rates which were significantly higher than the values obtained in the reference female population. In CHN patients, the urinary NEP excretion rate was significantly decreased. This reduction was more pronounced in ESRF patients than in those with moderate to severe renal insufficiency, suggesting a progressive atrophy process affecting the proximal tubular population. As opposed to NEP, other tubular markers (NAG, CC16, β_2m, RBP and α_1m) were markedly increased in urine. This increased excretion of microproteins was particularly drastic, reaching values more than 1,000 times above normal, which testifies to a severe proximal tubule function failure. On the other hand, the increment of albuminuria in the patient population was not so dramatic, confirming the tubular rather than glomerular character of proteinuria.

Considering individual results of urinary NEP excretion and creatinine clearance measured in all patients and in healthy subjects, a positive correlation between NEP enzymuria and creatinine clearance was observed ($r = 0.76$, $P = 0.0001$; Fig. 1A). Significant correlations were also found for urinary microproteins (r values ranging from -0.58 to -0.64; $P = 0.0001$). According to this and knowing that plasma CC16 protein followed the filtration-proximal tubular uptake route, it was assumed that various degrees of proximal tubular dysfunction might be related to the relative severity of structural impairment reflected by the urinary release of NEP from the remaining intact brush borders. The examination of NEP and microproteins measurements (CC16 as well as β_2m, α_1m and RBP) performed in the total patient population and in the control group, indeed revealed that these urinary markers were negatively correlated ($r = -0.55$, $P = 0.0001$; Fig. 1B). By contrast, no significant correlation was found between NEP and NAG enzymurias (data not shown).

NEP enzymuria and other tubular markers in patients with glomerular diseases

Table 2 summarizes the results obtained in the patient group with glomerular diseases. Several types of glomerulonephritis were investigated, at different stage of renal failure (mean creatine clearance ± SD, 43.8 ± 24.7 ml/min). Proteinuria of glomerular type was present, as attested by significative albuminuria (geometric mean 1.1 g/24 hr). A marked discrepancy was observed in NEP values, ranging from 27.9 to 177.8 μg/24 hours (geometric mean 48.5 μg/24 hr, not statistically significant from the control population). High levels of urinary microproteins and NAG compatible with tubular impairment versus damage were noted. No significant correlation was found between NEP enzymuria and creatinine clearance or albuminuria (data not shown).

Anatomopathological analysis in patients with CHN

As indicated in the **Methods** section, renal tissue was successfully obtained in 17 out of 20 women admitted for renal failure. Renal biopsies of 15 out of these 17 patients are described in detail in Reference 4. For the purpose of the present study, the percentage of tubules remaining intact was estimated from the cortex surface area occupied by apparently unatrophied and uninjured tubules. Four patients had more than 95% of the tubules remaining intact (group A) and 3 patients had less than 5% of the tubules remaining intact (group B). Comparing group B to group A, creatinine clearance was significantly lower (mean ± SD, 30 ± 3 vs. 53 ± 15 ml/min; $P < 0.05$) as well as NEP urinary excretion (mean ± SD, 18.0 ± 5.3 vs. 30.5 ± 12.5 μg/24 hr; $P < 0.05$). For the 10 patients with a percentage of the tubules remaining intact estimated around 20 to 50%, the imprecise nature of this estimation led to a poor correlation with NEP excretion (creatinine clearance 37 ± 10 ml/min and NEP urinary excretion 29.8 ± 8.0 μg/24 hr).

Prospective studies on patients exposed to Chinese herbs

In 6 CHN patients, NEP and NAG enzymurias as well as urinary microproteins were assayed for a follow-up period ranging from six months to two years. Individual data for creatinine clearance and urinary NEP excretion are represented in Figure 2. In 3 patients, renal function progressively deteriorated, reaching the end-stage renal failure after 38 to 115 weeks, respectively (Fig. 2A). Urinary NEP levels fell in parallel with the decrement of

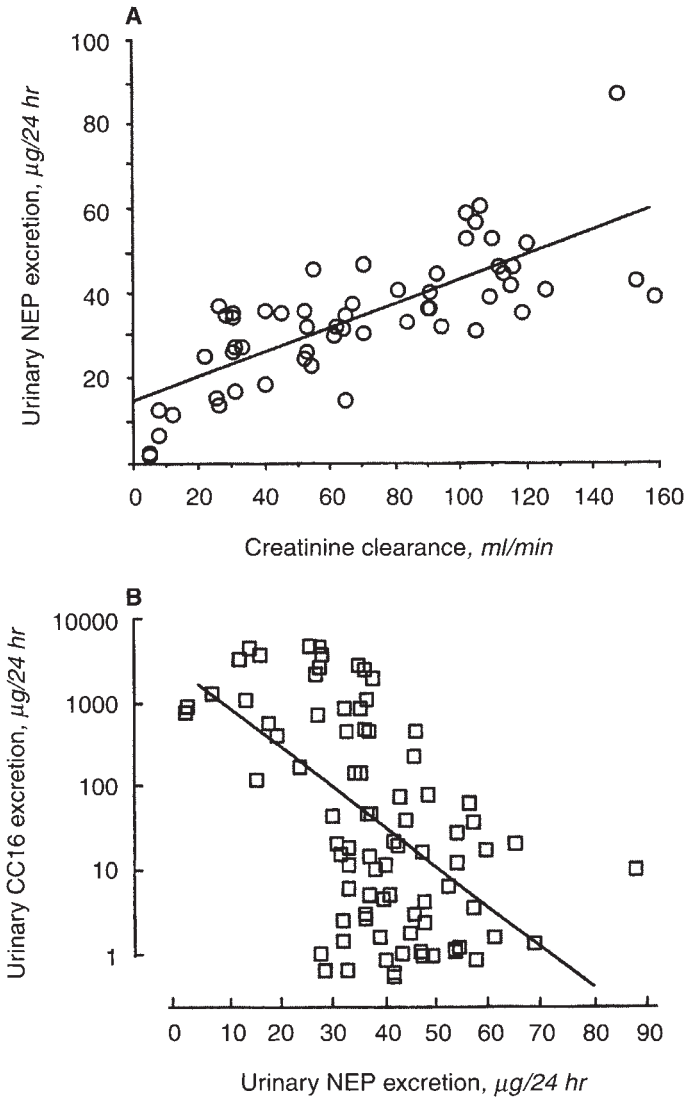

Fig. 1. *Cross-sectional correlations between* (**A**) *urinary NEP excretion and creatinine clearance* ($y = 0.29x + 14.35$; $P = 0.0001$; $r = 0.76$) *or* (**B**) *urinary CC16 excretion* ($y = -0.05x + 3.14$; $P = 0.0001$; $r = -0.55$) *in 26 patients with CHN, 27 patients at risk, and in 13 healthy subjects.*

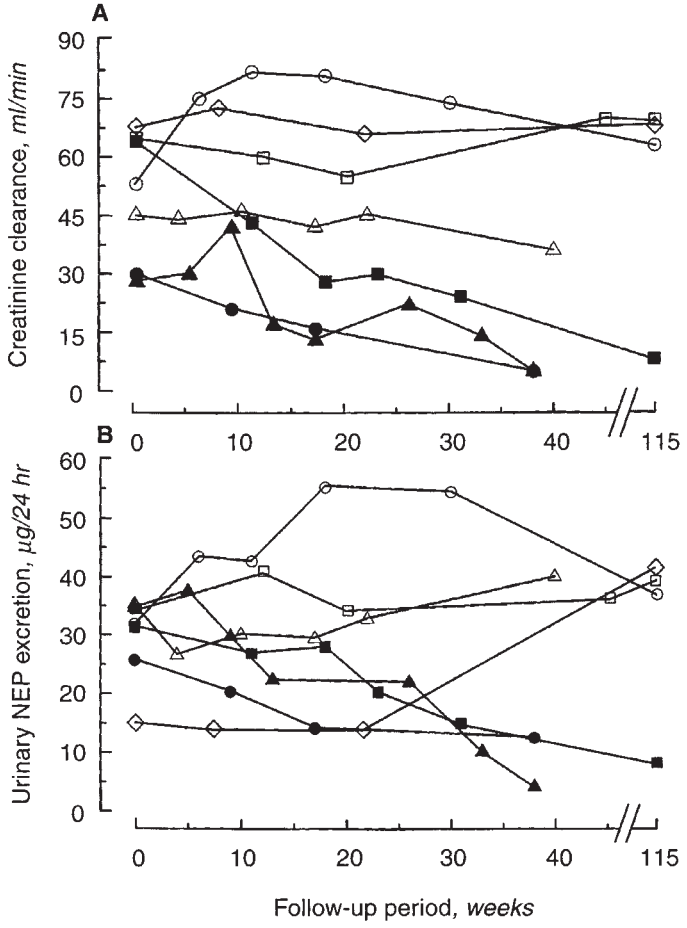

Fig. 2. *Time-courses of creatinine clearance* (**A**) *and urinary NEP excretion* (**B**) *in 6 patients with CHN.* Three subjects (■, ●, ▲) were submitted to hemodialysis treatment at the end of the follow-up, while the remaining 3 patients (□, ○, △) kept stable parameters.

creatinine clearance (Fig. 2B). In the remaining 3 patients, both renal function parameters and urinary NEP remained stable. Such a longitudinal correlation between creatinine clearance and NEP enzymuria could not be found for any other marker tested (data not shown).

In the group of at risk patients, urinary NEP and CC16 levels were regularly quantitated during one year ($N = 22$) or two years ($N = 19$), in parallel with usual parameters of renal function. No significant change of urinary NEP levels was observed after one year (geometric mean 37.1 μg/24 hr, range 22.3 to 66.6) or two years of follow-up (45.9, 33.8 to 97.9; Fig. 3A). Urinary CC16 levels, slightly elevated at time 0 of the follow-up and after one year (geometric mean 9.8 μg/24 hr, 0.6 to 112.5), significantly decreased after two years, reaching the normal range (2.9, 0.58 to 71.3; Fig. 3B). Serum creatinine remained stable, whereas decreased individual values of creatinine clearance were noted. Mean (± SD) creatinine clearance was 87 ± 23 ml/min after one year and 85 ± 23 ml/min after two years of follow-up (Fig. 3C), which was not statistically different from values obtained at the beginning of the follow-up (98 ± 29 ml/min).

Discussion

Our results provide evidence that, in CHN, the histologically-proven atrophy process affecting the proximal tubular population is quantitatively reflected by a progressive decrease in urinary NEP levels. The correlation of NEP with creatinine clearance is not reproduced in glomerular diseases. The usefulness of NEP enzymuria in monitoring the proximal tubular injury in other tubulointerstitial diseases remains to be assessed.

This is the first study to report a long-term follow-up of the structural and functional defects of the proximal tubular epithelium in patients having consumed CH. According to previous studies about ultrastructural localization of NEP in the kidney and its identification in human urine [4, 5], the amount of urinary NEP actually reflected the proportion of brush borders remaining intact at the apical side of the proximal tubules. In CHN patients, significantly decreased urinary excretion rates of NEP were found, showing quantitative evidence of the structural impairment of the proximal tubular epithelium, as seen by the pathologist on renal

Table 2. Histological findings and experimental data obtained from 12 women with glomerular diseases

Patient number	Renal histology	C_{Cr} *ml/min*	Albuminuria *g/24 hr*	NEP *μg/24 hr*	NAG *UI/24 hr*	CC16 *μg/24 hr*	RBP *μg/24 hr*
1	Diabetic GS	29	0.02	98	3.52	80.3	5034
2	Diabetic GS	10	1.14	19.2	3.97	1290	68200
3	Diabetic GS	11	2.18	37.7	5.63	1590	152000
4	Membranous GN	42	0.3	36.9	1.42	22.4	47.7
5	Membranous GN	25	4.0	41.5	4.52	145	6690
6	SLE	21	1.1	27.9	2.63	250	3590
7	Membranoproliferative GN (II)	74	9.6	178	17.7	290	1680
8	Focal segmental GN	22	1.21	45	6.51	697	31970
9	Focal segmental GN	65	3.5	50.8	3.31	33.3	2709
10	Focal segmental GN	95	0.96	33.5	2.17	45.4	133
11	IgA GN	89	0.5	70.2	1.50	83.3	152
12	—	42	1.48	58.7	3.44	173	740

Abbreviations are: C_{Cr}, creatinine clearance; GS, glomerulosclerosis; GN, glomerulonephritis; SLE, systemic lupus erythematosus; IgA GN, Berger's disease.

biopsies. However, to firmly establish that NEP was a marker of the proximal tubular mass, careful histomorphometric measurements of this proximal tubular mass should have been done on renal biopsy specimens and correlated with respective urinary NEP levels. Such histological experiments (that is, specific staining with antibodies against brush border antigens) could not be performed for technical and ethical reasons. Indeed, prospective correlations would also be necessary, requiring repetitive renal biopsies in these patients.

In parallel with the loss of integrity of the apical membrane domain reflected by decreased NEP enzymuria, functional repercussions of brush border alterations were documented by abnormally increased urinary levels of microproteins (CC16, RBP, β_2m and α_1m), revealing severe reabsorptive defects at the luminal side of the tubule. Such a typical pattern of tubular proteinuria was previously detailed by Kabanda et al in CHN patients, showing that this proteinuria was independent of glomerular albuminuria or overflow proteinuria [25]. We also found a higher ratio of urinary CC16 or RBP over albuminuria in our CHN patients than in patients with glomerular diseases (data not shown). In this latter group, whereas signs of tubular dysfunction were attested by increased urinary microproteins levels, NEP enzymuria remained in the normal range or increased. Although the size and the heterogeneity of this group make a clear-cut interpretation of the data difficult, several underlying mechanisms might explain these results. First, no extensive lesion of tubular atrophy was seen on histological preparations of renal tissue specimen. Even if they were present in other parts of the cortex, cellular adaptation processes might take place in remnant nephrons, where synthesis of tubular and glomerular markers might occur. This tubular hypermetabolism process was reported by Scherberich et al on the basis of histochemical evaluation of kidney sections of hemodialyzed patients and of increased urinary excretion of angiotensinase A [26]. The authors hypothesized that kidney tissue proteinuria derived not only from tubulointerstitial involvement of the disease, but also from synthesis of structural proteins by remnant nephrons. The progressive loss of this balance could lead to the worsening of the renal disease. Our data from ESRF patients (patients 2 and 3) and from patients with severe renal failure (creatinine clearance ranging from 20 to 40 ml/min) excreting significant amounts of NEP support this hypothesis.

Taking into account that urinary NEP was decreased in CHN patients, we investigated the importance of this reduction in terms of renal function. Despite the lack of predictive value of urinary NEP levels in terms of renal outcome, on the basis of the six cases

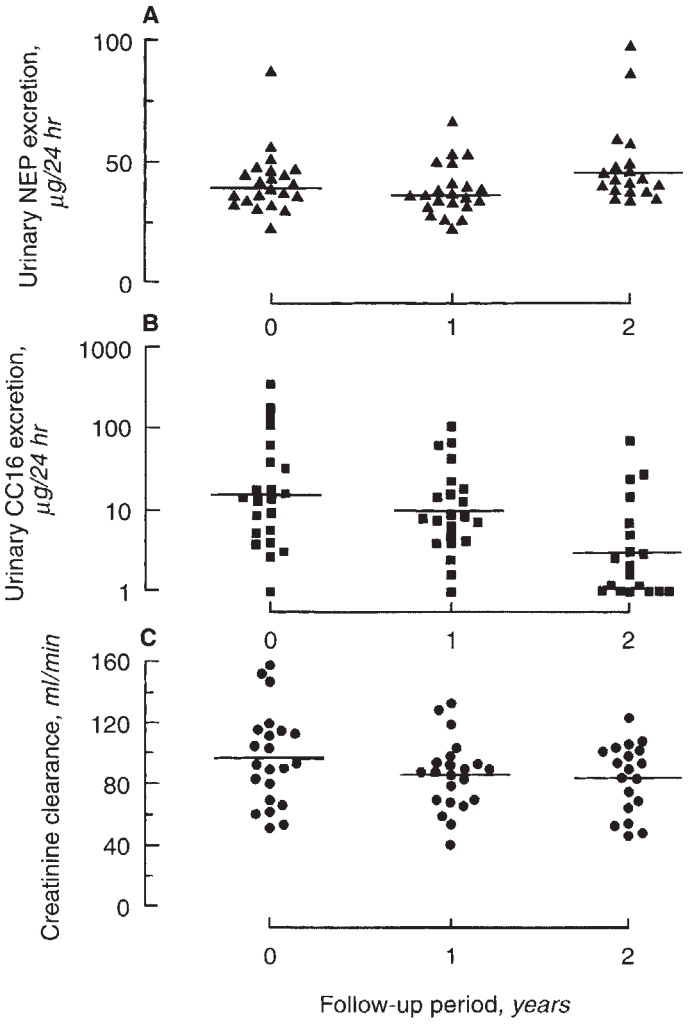

Fig. 3. *Urinary excretion of NEP* (**A**) *and CC16* (**B**) *and corresponding creatinine clearance values* (**C**) *recorded in patients at risk to develop CHN, at admission time and after one and two years of follow-up.* Horizontal bars indicate the geometric (A, B) and arithmetic means (C), respectively.

longitudinally studied (Fig. 2), NEP levels paralleled the creatinine clearance values. At this point it may be important to refer to the histological study of Depierreux et al on renal biopsy specimens from CHN patients [17]. In their work, the authors were not able to find a significant correlation between the renal function and the severity of the histologic changes at the time of the renal biopsy (based on the chronic interstitial score calculated as the sum of the score for tubular atrophy and the score for interstitial fibrosis). However, the patients with more severe lesions found in the biopsy had a rapid and poor evolution of the renal disease. From these observations and our data demonstrating a clear cut correlation between NEP enzymuria and the decline of creatinine clearance, we can reasonably conclude that a progressive time-dependent atrophy process affecting the proximal tubular compartment represents the major factor of the decline of renal function in CHN. This is actually in agreement with Fine et al's pathophysiological hypothesis suggesting that the final common pathway for progressive loss of renal function could be a chronic injury of the tubular epithelium, whatever the prior events (whether from vascular, tubular or glomerular origin) [27].

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Reprint requests to Dr. Joëlle Nortier, Laboratoire de Recherche Expérimentale Biomédicale (LPREB), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, CP 603, route de Lennik, 808, B-1070 Brussels, Belgium.

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