Urinary metalloproteinases and tissue inhibitors of metalloproteinases as potential early biomarkers for renal fibrosis in children with nephrotic syndrome

Beata Bienias, MD, PhD°, Przemyslaw Sikora, MD, PhD

Abstract

In chronic glomerulopathies, renal fibrosis (RF) results from extracellular matrix remodeling processes regulated by matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP). We assessed urinary (u-) and serum (s-) MMP-1, -2, -9, TIMP-1, -2 concentrations and MMP-1, -2, -9/TIMP-1, -2 ratios in children with nephrotic syndrome. Steroid-dependent and steroid-resistant nephrotic patients (SDNS-Ps and SRNS-Ps, respectively) were compared with respect to measured parameters. The correlations of measured parameters with magnitude of proteinuria and histopathological diagnosis were determined.

The study comprised of 39 children with nephrotic syndrome and 20 healthy controls. Twenty-three patients had SDNS and 16 ones—SRNS. The concentrations MMPs and TIMPs were measured using enzyme-linked immunosorbent assay.

In nephrotic patients, higher u-MMP-1, -2, -9/creatinine ratios and u-TIMP-1, -2/creatinine ratios were observed as compared with controls. Nephrotic children were also characterized by lower MMP-1, -2, -9/TIMP-1 ratios. In SRNS-Ps, u-MMP-2/creatinine ratio and u-TIMP-1/creatinine ratio were higher as compared with SDNS-Ps. Magnitude of proteinuria correlated positively with u-MMP-2/creatinine ratio and negatively with u-MMP-2/TIMP-1. In minimal change disease (MCD) patients as compared with those with other glomerulopathies, there was higher u-MMP-2/TIMP-1 ratio. No significant differences in s-MMPs, s-TIMPs, and s-MMPs/TIMPs ratios between nephrotic patients and controls were observed.

Children with nephrotic syndrome are characterized by increased u-fibrotic biomarkers excretions. U-MMP-1, -2, -9 excretions and u-MMP-2/TIMP-1 ratio may become potential early biomarkers for RF. SRNS-Ps, those with heavier proteinuria and other than MCD glomerulopathies, seem to be more susceptible to early RF.

Abbreviations: ACE = angiotensin-converting enzyme, CKD = chronic kidney disease, Cr = creatinine, ECM = extracellular matrix, FSGS = focal segmental glomerulosclerosis, HSP = Henoch–Schönlein purpura, MCD = minimal change disease, MMP = matrix metalloproteinase, MPGN = membranoproliferative glomerulonephritis, NS = nephrotic syndrome, RF = renal fibrosis, s = serum, SDNS = steroid-dependent nephrotic syndrome, SRNS = steroid-resistant nephrotic syndrome, TIMP = tissue inhibitors of metalloproteinase, u = urinary.

Keywords: children, metalloproteinases, nephrotic syndrome, renal fibrosis, tissue inhibitors of metalloproteinases

1. Introduction

Progressive renal fibrosis (RF) associated with chronic glomerulopathies results from imbalance between extracellular matrix (ECM) formation and degradation. Identification of early biomarkers for kidney fibrosis is of great importance to patients with chronic glomerulopathies because early institution of nephroprotective treatment may protect them from chronic kidney disease (CKD) occurrence and its progression.

Matrix metalloproteinases (MMPs) are a large family of zinc-containing enzymes that are involved in ECM remodeling processes.[1–3] However, recent studies revealed that MMPs may be implicated in initiation and progression of kidney fibrosis and CKD development.[2,4–6] Tissue inhibitors of metalloproteinases (TIMP) are endogenous, specific inhibitors of MMPs.

As yet the mode of action of only a few renal MMPs and TIMPs was unraveled. The most important role in ECM remodeling processes seems to play MMP-1, -2, -9 and their inhibitors TIMP-1 and -2.[7,8] MMP-1 is interstitial collagenase, which degrades native collagen and is hypothesized to be antifibrotic enzyme.[9,10] MMP-2 and -9 are gelatinases, which cleave denatured collagen, type IV collagen, and laminin.[11] MMP-2 is expressed in glomeruli, proximal tubules,[11,12,13] and collecting ducts.[14] The expression of MMP-9 was detected mainly in glomeruli,[11,12,13] although there are reports of its expression in proximal and distal tubules[16] and in collecting ducts.[14] MMP-2 and -9 were also showed to be pivotal to recruitment and chemotaxis of inflammatory cells.[17] MMP-2 has also the potential to activate MMP-1 and -9 by cleaving their prodomains.[18] Tan et al[19]
disclosed that MMP-9 contributes to the pathogenesis of RF via macrophage recruitment through osteopontin cleavage. It was proved that MMP-2 and -9 induced tubular cell epithelial–mesenchymal transition. Experimental data and human studies demonstrated that increased MMPs glomerular expression correlates positively with severity of glomerular damage and progression of kidney disease. Recent studies in patients with chronic kidney disease (CKD), diabetic nephropathy, and after kidney transplantation demonstrated that higher serum levels and/or urinary excretions of MMPs and TIMPs might be biomarkers for an early stage of RF or its progression. Therefore, the assessment of utility of selected MMPs and MMPs/TIMPs ratios as potential early biomarkers for kidney fibrosis in children with chronic glomerulopathies is justified and may be clinically relevant.

The purpose of the study was to assess urinary and serum MMP-1, -2, -9 and TIMP-1, -2 concentrations in children with nephrotic syndrome (NS). Steroid-dependent and steroid-resistant patients were compared with respect to measured parameters. The correlations of measured parameters with magnitude of proteinuria, histopathological diagnosis, duration of the disease, and number of relapses were determined. Similar comparison was made between children treated and those nontreated with cyclosporine A.

2. Patients, materials and methods

2.1. Patients

Baseline characteristics of patients and controls are presented in Table 1. The study comprised of 39 children (33 boys and 6 girls) aged 4 to 18 years (median 13.7 years) with NS treated in the Department of Pediatric Nephrology, Children’s University Hospital in Lublin, Poland. According to KIDIGO guidelines, 23 patients were affected with steroid-dependent nephrotic syndrome (2 or more relapses of proteinuria during corticosteroid therapy, or within 14 days of ceasing therapy, SDNS) and 16 ones— with steroid-resistant nephrotic syndrome (failure to achieve complete remission of proteinuria after 8 weeks of corticosteroid therapy, SRNS). In the majority of patients, a histopathological examination revealed minimal change disease (MCD)—25 (64%). Focal segmental glomerulosclerosis (FSGS), mesangio proliferative glomerulonephritis, membranoproliferative glomerulonephritis (MPGN), and membranous glomerulonephritis were diagnosed in 4 (10.3%), 6 (15.5%), 2 (5.1%), and 2 (5.1%) children, respectively. Before enrolment into the study, all patients were treated with steroids according to KIDIGO 2012 guidelines. Treatment of onset of NS was started with prednisone in dose of 60 mg/m² per d or 2 mg/kg per d (maximum, 60 mg/d) for 4 to 6 weeks, then switched to 40 mg/m² or 1.5 mg/kg (maximum, 40 mg) on alternate days for 4 weeks and tapered for 2 to 5 months. In relapse of NS, the initial dose of prednisone was used until urinary protein was negative in 3 consecutive days. Steroid-free time interval before enrolment to the study was from 1 day to 6 months.

Eighteen patients were treated with cyclosporine A in dose of 2 to 3 mg/kg per d (C0 cyclosporine plasma level of 85–162 ng/mL) for 2 to 4 years. The median duration of NS and the median number of relapses were 6 years (0.25–14 years) and 6 (2–13), respectively. All children had normal estimated glomerular filtration rate calculated by the Schwartz formula: 0.55 × body height (cm)/serum creatinine (Cr) level (mg/dL). None of the patients showed clinical evidence of infection, had diabetes, malignancies or peripheral vascular disease, smoked. In 20 patients, hypertension was diagnosed. In all, blood pressure was well controlled with the use of angiotensin-converting enzyme (ACE) inhibitors (14 patients) and calcium channel blockers (6 patients). Nineteen patients had nephroprotective treatment with ACE inhibitors or angiotensin II receptor antagonists.

The age- and sex-matched 20 apparently healthy volunteers (median age 12.3 years) were controls.

2.2. Methods

The mid-stream first-morning urine specimen was collected from each study participant on the same day.

Routine laboratory techniques were used to measure proteinuria. Concentrations of serum and urinary Cr were determined by Jaffe test.

Urinary MMP-1, -2, -9 and TIMP-1, -2 concentrations were measured using commercially available enzyme-linked immunosorbent assay test kits (R&D Systems) after prior preparation of urine samples following the manufacturer’s instructions. Monoclonal antibody specific for MMP-1, -2, -9 and TIMP-1, -2 was used to detect MMPs and TIMPs in the urine samples. Horseradish peroxidase-conjugated avidin was added, followed by a color-forming peroxidase substrate containing tetramethylbenzidine. The color was then measured at 450 nm by a microtiter plate reader. The results were calculated by reference to standard curves.

The urinary enzyme excretions and urinary inhibitor excretions were expressed as enzyme/Cr and inhibitor/Cr ratios in nanograms per milligram of Cr (ng/mg). Similarly, the urinary protein excretion was expressed as protein/Cr ratio in milligrams per milligram of Cr (mg/mg).

The statistical analysis was performed using STATISTICA 7.1. Nonparametric statistic methods were used as the data were not normally distributed in Shapiro–Wilk test and the patients groups were relatively small.
Differences between groups were assessed using nonparametric Mann–Whitney U test and correlation coefficients were calculated using Spearman test. $P < 0.05$ was considered significant.

### 3. Results

In children with NS significantly higher median values of urinary MMP-1, -2, -9/Cr ratios and TIMP-1, -2/Cr ratios were observed as compared with controls ($P < 0.05$). In comparison with controls, patients with NS were also characterized by significantly lower median values of MMP-1, -2, -9/TIMP-1 ratios (Table 2). In children with SRNS, the median values of urinary MMP-2/Cr ratio ($P = .01$) and urinary TIMP-1/Cr ratio ($P = .02$) were significantly higher as compared with children with SDNS (Table 3).

We disclosed significant positive correlation between the median value of urinary MMP-2/Cr ratio and magnitude of proteinuria ($r = 2.37$, $P = .03$) whereas correlation between the median value of urinary MMP-2/TIMP-1 ratio ($r = -1.42$, $P = .05$) and magnitude of proteinuria was significantly negative. In children with MCD as compared to those with other glomerulopathies, there was significantly higher median value of urinary MMP-2/TIMP-1 ratio ($P < .05$). The correlations between measured biomarkers and duration of the disease/number of relapses did not show any significant differences (Table 4).

No significant differences in median values of serum MMPs, TIMPs, and MMPs/TIMPs ratios between nephrotic patients and controls were observed (Tables 5 and 6).

There were no significant correlations between measured biomarkers (urinary MMPs and TIMPs) and dose of prednisone. Similarly, no correlations between measured biomarkers and

### Table 2

The results of urinary excretion of MMPs and TIMPs in study and control groups.

| Group | N | Median (range) | Statistical analysis |
|-------|---|----------------|---------------------|
| MMP-1/Cr, pg/mg | NS 39 | 272.2 (101.2–1646.6) | .02 |
| MMP-2/Cr, pg/mg | Controls 20 | 164.4 | .04 |
| MMP-9/Cr, pg/mg | NS 39 | 585.3 (159.5–85134.8) | .04 |
| TIMP-1/Cr, pg/mg | NS 39 | 7138.7 (551.9–15164) | .0003 |
| MMP-1/TIMP-1 | NS 39 | 114.0 (2823.8) | .04 |
| MMP-9/TIMP-2 | NS 39 | 5417.9 (20504.3–136530.9) | .001 |
| MMP-2/TIMP-1 | NS 39 | 0.1 (0.08) | .03 |
| MMP-9/TIMP-1 | NS 39 | 0.1 (0.04) | .03 |
| TIMP-2/Cr, pg/mg | NS 39 | 4377.1 (2713–9070.1) | .02 |
| MMP-2/TIMP-2 | NS 39 | 490.1 | .02 |
| MMP-1/TIMP-2 | NS 39 | 503.9 (250–1057) | .02 |
| TIMP-1/Cr, pg/mg | NS 39 | 7138.7 (551.9–15164) | .04 |

$\text{Cr} = $ creatinine, MMP = matrix metalloproteinase, NS = nephrotic syndrome, TIMP = tissue inhibitors of metalloproteinase.

The bold values in Tables are statistically significant.

### Table 3

The results of concentrations of urinary MMPs and TIMPs in SDNS, SRNS, and controls.

| SDNS median (range) | SRNS median (range) | Control group median (range) | Statistical analysis (P) |
|---------------------|---------------------|-----------------------------|-------------------------|
| MMP-1/krea, pg/mg | 14.649 (7.6–25.2) | 23.61 (9.1–23.2) | 14.6 (7.4–26.9) | .85 .06 .06 |
| MMP-2/krea, pg/mg | 0.17 (0.03–1.6) | 0.66 (0.04–2.23) | 0.12 (0.01–1.07) | .45 <.01 .02 |
| MMP-9/krea, pg/mg | 13.36 (3.7–16.2) | 13.70 (3.69–23.03) | 4.77 (2.1–10.4) | .15 .01 .6 |
| TIMP-1/krea, pg/mg | 0.87 (0.06–4.06) | 1.77 (0.2–9.8) | 0.28 (0.06–1.06) | .02 <.01 .02 |
| TIMP-2/krea, pg/mg | 7138.7 (551.9–15639) | 8612.7 (861.9–15639.1) | 5417.9 (20504.3–136530.9) | .61 .02 .18 |
| MMP-1/TIMP-1 | 0.1 (0.03–0.3) | 0.2 (0.04–0.62) | 0.44 (0.109–0.66) | <.01 .05 .5 |
| MMP-2/TIMP-1 | 1.3 (0.02–2.2) | 0.4 (0.03–3.1) | 0.9 (0.2–4.2) | .02 .02 .06 |
| MMP-3/TIMP-1 | 0.3 (0.03–0.9) | 0.3 (0.04–1.51) | 1.2 (0.35–16.2) | <.01 .04 .4 |
| MMP-1/TIMP-2 | 0.1 (0.04–0.21) | 0.1 (0.06–3.3) | 0.1 (0.02–3.2) | .9 .7 .8 |
| MMP-2/TIMP-2 | 0.2 (0.01–0.34) | 0.1 (0.02–4.2) | 0.1 (0.02–3.2) | .09 .06 .7 |
| MMP-9/TIMP-2 | 0.1 (0.08–0.26) | 0.1 (0.06–28) | 0.1 (0.04–3.2) | .8 .09 .7 |

$\text{Cr} = $ creatinine, MMP = matrix metalloproteinase, NS = nephrotic syndrome, SDNS = steroid-dependent nephrotic syndrome, SRNS = steroid-resistant nephrotic syndrome, TIMP = tissue inhibitors of metalloproteinase.

The bold values in Tables are statistically significant.
steroid-free time interval before enrolment to the study were observed. There were no significant differences in median values of urinary MMPs and TIMPs excretions between children treated and those nontreated with cyclosporine A.

4. Ethical aspects

The study was approved by Ethics Committee of the Medical University of Lublin. Informed consent was obtained from all individual participants included in the study, either the patients or the parents or legal guardians.

5. Discussion

In chronic glomerulopathies, persistent or recurrent proteinuria may lead to progressive kidney fibrosis and CKD occurrence. There is a small number of reports on serum levels and/or urinary excretions of MMPs and TIMPs in patients with kidney diseases and none of them regard children with glomerulopathies. In vitro and animal studies showed higher activity of MMPs and TIMPs in kidney during fibrosis process. Experimental model of kidney fibrosis showed elevated MMP-1 activity in glomeruli and suggested its significant role in initiation of glomerular remodeling processes. Hirt-Minkowski et al revealed significant positive correlation between serum level and/or urinary excretion of MMP-1 and TIMP-1 and existing or early developing interstitial fibrosis in renal allograft. Elevated serum MMP-1 level was also observed in lupus nephritis. Morillas et al demonstrated higher serum MMP-1 and TIMP-1 levels in hypertensive patients with target organ damage.

In our patients with SDNS and SRNS, significant increase in urinary MMP-1 and TIMP-1 excretions was observed. Those patients were also characterized by significant decrease in urinary...

| Table 4 | The correlations between concentrations of urinary MMPs and TIMPs and magnitude of proteinuria, duration of the disease, and number of relapses in children with NS. |
|-----------------|-----------------|-----------------|-----------------|
| Protein/Cr, mg/mg | Duration of the disease (NS) | Number of relapses |
| r | P | r | P | r | P |
| MMP-1/Cr, pg/mg | 1.74 | .15 | −0.23 | .34 | 0.22 | .53 |
| MMP-2/Cr, pg/mg | 2.37 | .03 | −0.19 | .25 | −0.03 | .88 |
| MMP-9/Cr, pg/mg | 0.18 | .7 | −0.24 | .3 | 0.28 | .42 |
| TIMP-1/Cr, pg/mg | 0.1 | .87 | −0.26 | .34 | 0.42 | .3 |
| TIMP-2/Cr, pg/mg | 0.62 | .01 | −0.27 | .09 | −0.04 | .98 |
| MMP-1/TIMP-1 | −0.35 | .74 | −0.14 | .61 | −0.41 | .31 |
| MMP-2/TIMP-1 | −1.42 | .05 | −0.07 | .77 | 0.051 | .89 |
| MMP-9/TIMP-1 | −0.5 | .39 | −0.17 | .53 | 0.23 | .55 |
| MMP-1/TIMP-2 | −0.6 | .14 | 0.03 | .9 | −0.12 | .76 |
| MMP-2/TIMP-2 | 0.05 | .84 | 0.09 | .56 | −0.12 | .63 |
| MMP-9/TIMP-2 | −0.53 | .42 | 0.01 | .9 | −0.24 | .52 |

Cr = creatinine, MMP = matrix metalloproteinase, NS = nephrotic syndrome, TIMP = tissue inhibitors of metalloproteinase. The bold values in Tables are statistically significant.

| Table 5 | The results of serum concentrations of MMPs and TIMPs in study and control groups. |
|-----------------|-----------------|-----------------|-----------------|
| Group | N | Median | Range |
|-----------------|-----------------|-----------------|-----------------|
| MMP-1, pg/mL | NS | 39 | 314.6 | 213.7–896.6 | .76 |
| Controls | 20 | | 326.1 | 209.1–607.1 |
| MMP-2, pg/mL | NS | 39 | 360.877 | 163.847–695.384 | .68 |
| Controls | 20 | | 374.646 | 307.208–726.193 |
| MMP-9, pg/mL | NS | 39 | 1443.4 | 1083.0–2030.0 | .80 |
| Controls | 20 | | 1506.7 | 903.3–2185.4 |
| TIMP-1, pg/mL | NS | 39 | 12,479.6 | 5671.9–28,371.7 | .52 |
| Controls | 20 | | 13,303.5 | 6522.2–18,303.6 |
| TIMP-2, pg/mL | NS | 39 | 80,057.7 | 23,593.3–160,991.0 | .08 |
| Controls | 20 | | 90,671.2 | 24,504.3–173,531.2 |
| MMP-1/TIMP-1 | NS | 39 | 0.11 | 0.017–0.42 | .37 |
| Controls | 20 | | 0.1 | 0.03–0.35 |
| MMP-2/TIMP-1 | NS | 39 | 3.1 | 0.8–8.8 | .42 |
| Controls | 20 | | 2.9 | 2.4–3.4 |
| MMP-9/TIMP-1 | NS | 39 | 0.07 | 0.02–0.09 | .09 |
| Controls | 20 | | 0.1 | 0.1–0.2 |
| MMP-1/TIMP-2 | NS | 39 | 4.4 | 2.7–7.9 | .06 |
| Controls | 20 | | 3.3 | 2.0–5.0 |
| MMP-9/TIMP-2 | NS | 39 | 0.1 | 0.04–0.3 | .08 |

MMP = matrix metalloproteinase, NS = nephrotic syndrome, TIMP = tissue inhibitors of metalloproteinase.
MMP-1/TIMP-1 ratio. These findings may suggest activation of ECM remodeling processes in children with more severe forms of NS.

The profibrotic role of MMP-2 was reported in experimental studies. In a few studies, increased urinary MMP-2 excretion was observed at an early stage of diabetic nephropathy. In addition, positive correlation between urinary MMP-2 excretion and risk factors for diabetic nephropathy was demonstrated. Higher serum MMP-2 level was observed also in patients with interstitial fibrosis/tubular atrophy of transplanted kidney. Other authors showed increased serum MMP-2 level and increased urinary MMP-2 excretion in patients with CKD associated with progressive kidney fibrosis.

Higher serum MMP-2 and MMP-9 levels were found in patients with CKD, diabetic nephropathy, membranous nephropathy, FSGS, MPGN, lupus nephritis, and acute kidney injury. Our studies showed increased urinary MMP-2 excretion and decreased urinary MMP-2/TIMP-1 ratio. In children with SRNS, we observed higher urinary MMP-2/Cr ratio as compared with children with SDNS. These findings may suggest activation of ECM remodeling processes both in children with SDNS and in those with SRNS but steroid-resistant nephrotic children seem to be at higher risk for RF and CKD.

In our patients, positive correlation between magnitude of proteinuria and urinary MMP-2 excretion was observed whereas correlation between magnitude of proteinuria and urinary MMP-2/TIMP-1 ratio was negative. These results may suggest that patients with proteinuria are at higher risk for RF due to predominance of ECM degradation inhibition processes. In addition, our results may indicate an important role of urinary MMP-2 and MMP-2/TIMP-1 ratio as an early biomarkers for RF.

Taking into account histopathological diagnosis, children with MCD showed higher urinary MMP-2/TIMP-1 ratio in comparison with those with other glomerulopathies. These results may indicate that nephrotic children with other than MCD glomerulopathy are more susceptible to RF due to more intense activation of ECM remodeling processes.

Previously published studies with use of experimental models of FSGS, lupus nephritis, MPGN showed increased glomerular expression and/or activity of MMP-9. Similarly, increased glomerular MMP-9 activity was found in patients with lupus nephritis, IgA nephropathy, Henoch–Schönlein purpura (HSP), and postinfectious acute glomerulonephritis. Other authors reported higher urinary MMP-9 activity in patients with CKD and HSP with renal involvement. Our study showed increased urinary MMP-9 excretions and decreased urinary MMP-9/TIMP-1 ratio. These results may suggest activation of ECM remodeling processes in children with SDNS and SRNS.

Experimental studies conducted in glomerulosclerosis and interstitial fibrosis found higher expression and activity of TIMPs, particularly TIMP-1, in glomeruli. Urinary TIMP-1, -2 excretions were reported to be increased in patients with CKD. In our study, all patients showed elevated urinary TIMP-1, -2 excretions. In children with SRNS, urinary TIMP-1/Cr ratio was significantly higher than that in children with SDNS. This may suggest an increase in activity of ECM remodeling inhibition processes in SRNS. Furthermore, in our patients decreased urinary MMP-1, -2,-9/TIMP-1 ratios were disclosed. This may suggest predominance of ECM degradation inhibition processes and fibrosis development in SDNS and SRNS. No significant differences in serum MMPs and TIMPs between nephrotic patients and controls observed in our study may support the hypothesis that their local production and tubular excretion into urine is the most relevant to tubulo-interstitial injury and development of kidney fibrosis.

The main limitation of this study is relatively small group of patients. Further studies are required to confirm our findings.

6. Conclusions

Children with SDNS and SRNS are characterized by increased urinary excretion of fibrotic biomarkers. Urinary MMP-1, -2, -9 excretions and urinary MMP-1, -2,-9/TIMP-1 ratios, particularly urinary MMP-2 excretion and urinary MMP-2/TIMP-1 ratio, may become useful early biomarkers for kidney fibrosis. Children with SRNS, those with proteinuria and other than MCD glomerulopathies seem to be more susceptible to an early kidney fibrosis.

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