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ORIGINAL ARTICLE

Matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 in vesicoureteral reflux

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Abstract The aim of this study was to investigate whether urine levels of matrix metalloproteinase 9 (uMMP9) and tissue inhibitor of metalloproteinase 1 (uTIMP1) are novel biomarkers of vesicoureteral reflux (VUR) and to determine the optimal cut-off levels of these enzymes to predict VUR in children. The study group consisted of 67 children with VUR and 20 healthy children. Urine MMP9 and TIMP1 levels were measured by an enzyme-linked immunosorbent assay. Children with VUR had significantly higher uMMP9 (1,539.8 vs. 256.4 pg/mL; p=0.0001) and uTIMP1 (182 vs. 32.6 pg/mL; p=0.0001) levels than healthy children. For the prediction of VUR, the sensitivity of uMMP9 was 67%, with a specificity of 85% [cut-off value 1,054 pg/mL; area under the curve (AUC) 0.77], and the sensitivity of uTIMP1 was 74%, with a specificity of 65% (cut-off value 18.7 pg/mL; AUC 0.73). Both uMMP9 and uTIMP1 levels were significantly higher in patients with renal scar (uMMP9: 3,117.3 vs. 1,234.15 pg/mL; p=0.0001; uTIMP1: 551.05 vs. 128.64 pg/mL; p=0.0001). Urine MMP9 levels had a sensitivity of 81.2%, with a specificity of 85% to predict renal scar in the VUR group (cut-off 1,054 pg/mL; AUC 0.88). The sensitivity of uTIMP1 was 75%, with a specificity of 90% to predict renal scar (cut-off 243.7 pg/mL; AUC 0.82). Based on these results, we suggest that uTIMP1 may be a useful marker to predict renal scarring with a different cut-off value from VUR and a high specificity at this cut-off point. Although uMMP9 seemingly cannot distinguish renal scar from VUR, the simultaneous increase in the level of both markers may indicate ongoing renal injury due to VUR.

Keywords Matrix metalloproteinase 9 · MMP 9 · Renal scar · TIMP · Tissue inhibitor of metalloproteinase 1 · Vesicoureteral reflux · Children · Biomarker

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Introduction

Vesicoureteral reflux (VUR) is a major urological problems in children which may lead to recurrent urinary tract infection (UTI) and reflux nephropathy [1]. It was also shown to be the most common cause of chronic kidney disease (CKD) in Turkish children in 1995 [2]. According to a more recent assessment of the etiology of CKD, VUR is still the most common cause of CKD in Turkey [3, 4].

Reflux nephropathy is characterized histologically mainly by the presence of periglomerular and interstitial fibrosis [5]. It has been suggested that the development of renal fibrosis is a result of an excessive accumulation of extracellular matrix (ECM) components (type IV collagen, proteoglycan, laminin, etc.,) due to an increased production and concomitant decreased degradation of matrix [6–8]. These matrix components are metabolized by matrix metalloproteinases (MMPs), which form a family of enzymes containing 24 members. Of these, the gelatinases (MMP2 and MMP9) in particular

degrade type IV collagen, which is a main constituent of the matrix, being expressed in mesangial, epithelial, and tubular cells of glomeruli. The degradation of ECM is restrained by tissue inhibitors of metalloproteinases (TIMPs) via inhibition of MMPs [6–10]. Disruption of the balance between MMPs and their inhibitors may lead to fibrogenesis in kidneys [6–8].

A voiding cystourethrography (VCUG) is an essential imaging study in children with UTI or antenatal dilation of the urinary tract for the differential diagnosis and investigation of VUR. However, VUR exists in only 8–50% of children evaluated with UTI and 10% of newborns evaluated for antenatal hydronephrosis [1, 11]. Since the procedure is invasive and associated with exposure to radiation, other diagnostic procedures, such as the use of sensitive and specific markers for the prediction of VUR in children, are being sought to avoid unnecessary imaging studies. Although there have been several experimental studies investigating the role of MMPs and TIMPs in renal fibrogenesis, to the best of our knowledge, there is only one clinical study that has evaluated the urine levels of MMP in acute pyelonephritis and associated renal scarring. In that study, Chromek et al. [8] showed that the urinary MMP9/creatinine (uMMP9/Cr) and uTIMP1/Cr ratios were high in acute pyelonephritis and that patients with higher levels of uTIMP1 than uMMP9 had more severe renal scarring. Therefore, the aims of this study were to assess whether uMMP9 and uTIMP1 are novel biomarkers for the prediction of VUR and to determine the optimal cut-off level for these biomarkers to predict VUR in children. We chose spot urine as the test material instead of serum so that the test was easy, painless, and more applicable.

Patients and methods

This multicenter study was conducted over a 1-year period between 2009 and 2010. The patient group consisted of 67 children with VUR diagnosed by VCUG within 6 months prior to recruitment. The last UTI episode prior to sampling was 1–8 months in the patient group. Twenty-nine patients had one or two culture-proven UTI episodes, and the remaining 38 patients had three or more episodes; all of these children had a history of at least one episode of pyelonephritis. The data of these 67 children with VUR were compared to those of 20 healthy, age-matched children with no history of UTI and/or antenatal hydronephrosis. Children with abnormalities based on the urinalysis and ultrasound examination results were not enrolled in the control group.

Medical histories of the patients were obtained from the hospital files and the following parameters studied: blood pressure, proteinuria, blood test results, including serum urea and Cr, additional systemic disease, UTI episodes, and data from radiological imaging tests, such as ultrasound and dimercaptosuccinic acid (DMSA) radionuclide scans. UTI was defined as significant bacteriuria (≥100,000 cfu/mL) in the previous urine culture obtained by collecting bag or midstream urine. Pyelonephritis was defined as high fever (>39°C), leukocytosis (leukocyte count greater than the normal value according to age [12], and positive C-reactive protein (>5 mg/L) associated with a positive previous culture result in the patient's medical history. Impaired renal function was defined as an estimated glomerular filtration rate (eGFR; calculated using the Schwartz formula [13]) of ≤90 mL/min/1.73 m^2. Patients older than 18 years or those with impaired renal function, hypertension, proteinuria, additional systemic disease, and a UTI at the time of sampling were excluded from the study.

DMSA assays were performed in all of our patients. A renal scar was defined as a marked localized deformity of the kidney outline with no DMSA uptake on at least two DMSA scans in the absence of UTI, and functional lateralization was defined as one of the kidneys contributing <44% of total renal function as evidenced on the DMSA scan [14].

Biochemical methods

Random urine samples were collected for the measurement of uMMP9, uTIMP1, microalbumin, and Cr levels in both groups at the same time as the urinalysis and urine culture were performed. Urinary parameters were measured according to their measurement features in the pre-analytic and analytic process [15]. Physical and chemical features of the urine samples (color, pH, density) were analyzed by the dipstick test, and there were no features which could lead to measurement interference. Urinalysis, including the leukocyte esterase reaction, nitrite test, and microscopic analysis of urine, was performed on an Iris IQ 200 fully automated urine analyzer (Iris Diagnostics, Chatsworth, CA). Concentration or dilution was not performed prior to analysis of the urine samples. The urine samples were collected and immediately centrifuged at 4°C for 15 min at 4,000 *g*. Aliquots of the supernatant were stored at−80°C until assay. Centrifugation and storage occurred under the same conditions and assaying was performed at the same time for the urine of patients and controls.

MMP-9 and TIMP-1 levels in the urine were determined using the Human MMP-9 ELISA kit (cat no: ELH-MMP-9-001) and Human TIMP-1 ELISA kit (cat no: ELH-TIMP1-001), purchased from RayBiotech (Norcross, GA), in 100-μL urine samples following the manufacturer's instructions. These ELISA kits are in vitro enzyme-linked immunosorbent assays for the quantitative measurement of the proform and active form of human MMP-9 and of human

TIMP1 in urine. In these assays, uMMP9 and uTIMP1 levels are expressed as picograms per milliliter.

Urine microalbumin concentration was measured in 100-μL urine samples by an immunoturbidimetry test using the end-point method. This analysis was carried out using an Abbott Architect C16000 auto analyzer (Multigent microalbumin assay; Architect c16000 system; Abbott Diagnostics Europe, Wiesbaden, Germany). In this assay, microalbumin levels are expressed as micrograms per milliliter.

Statistical analysis

All data were stored and analyzed using the NCSS ver. 2007 (NCSS, Kaysville, UT) software program for Windows. In addition to standard descriptive statistical calculations [mean, standard deviation (SD), median, and geometric mean], one-way analysis of variance (ANOVA) was used to compare groups, and the unpaired *t* test was used to compare two groups. The chi-square test was applied for the comparison of qualitative data between groups, and the Pearson correlation test was used to analyze relationship between variables. The results were evaluated within a 95% confidence interval (CI). Significance was set at $p<0.05$.

Urine levels of MMP9 and TIMP1, the MMP9/Cr, TIMP1/Cr, and MMP9/TIMP1 ratios, and microalbumin were tested for their normal distribution. Logarithmic transformations were applied as needed to achieve a normal distribution. Geometric means were used for uMMP9, uTIMP1, MMP9/Cr, TIMP1/Cr, and MMP9/TIMP1. Urine levels of the biomarkers were expressed as the geometric mean and interquartile range (IQR). To calculate the sensitivity, specificity, positive and negative predictive values, and positive and negative possibility ratios at varying cut-off values, we generated a conventional receiver operating characteristic curve (ROC) and calculated the area under the curve (AUC) for the candidate biomarkers.

Ethics statement

This study was approved by the local ethical committee. Informed consent was obtained from the parents of both the patients and the controls. Authors have no conflict of interest.

Results

Characteristics of the patients

Sixty-seven patients with VUR (38 female, 29 male) and 20 healthy children (7 female, 13 male) were enrolled in the study. The mean age of the children with VUR and control group children was 4.6±4.0 years and 6.04±3.85 years, respectively. Age and gender distribution were not different between the groups ($p < 0.05$).

VUR was unilateral in 43 and bilateral in 24 children. Of the 91 renal units with VUR, 11 of them had high-grade VUR (grade IV–V). Urine microalbumin levels were not different between the children with and without VUR and also not between those with and without scar ($p > 0.05$) (Table 1).

VUR and urine MMP9 and TIMP1 levels

Mean uMMP9 was significantly higher in the VUR group than in healthy children ($p=0.0001$) (Table 1). According to the ROC analysis, the optimal cut-off level was 1,054 pg/mL for the prediction of VUR. Statistical values of this cut-off level are given in Table 2. When the uMMP9 level was higher than this cut-off value, the possibility of having VUR increased by 4.5-fold (Table 2).

Mean uTIMP1 was also significantly higher in children in the VUR group than in healthy children ($p=0.0001$) (Table 1). According to the ROC analysis, the optimal cut-off level was 18.7 pg/mL for the prediction of VUR. When uTIMP1 was higher than this cut-off value, the possibility of having VUR increased by twofold (Table 2).

A comparison of all patients in the VUR group according to bilateral and unilateral VUR revealed that both uMMP9 and uTIMP1 levels were not significantly different between these two patient groups ($p>0.05$) (Table 4). A comparible study of patients classified with high-grade (grade IV–V) and low-grade (grade I–III) VUR, respectively, demonstrated that the uMMP9 level was lower in children with high-grade VUR ($p=0.012$), while the uTIMP1 level was higher in children with high-grade VUR ($p=0.012$) (Table 4).

Renal scarring and urine MMP9 and TIMP1 levels

There were 16 patients in the VUR group who had renal scar on their DMSA scintigraphy scan (13 unilateral, three bilateral). Both uMMP9 and uTIMP1 levels were significantly higher in children with renal scar.

The mean levels of uMMP9 in patients with renal scar were significantly higher than those of healthy controls (3,117.3 vs. 256.4 pg/mL; $p=0.0001$) and also higher than VUR patients without scar (3,117.3 vs. 1,234.1 pg/mL; $p=0.032$) (Table 2). The optimal cut-off value to predict renal scar was 1,054 pg/mL, the same as the cut-off value for predicting VUR. The statistical values for this cut-off level are seen in Table 3. When uMMP9 was higher than this cut-off value, the probability of renal scar increased by fivefold (Table 3).

Table 1 Comparison of urine levels of MMP9 and TIMP1

	VUR(−) (n = 20)	VUR (+) (n = 67)	VUR (+)		p_{VUR}[a]	p_{SCAR}[b]
			Scar (−) (n = 51)	Scar (+) (n = 16)		
Urine MMP9 (pg/mL)	256.4 (29.5–993.6)	1,539.8 (509.8–5,912)	1,234.1 (412–5,908)	3,117.4 (1,994.5–5,922.5)	0.0001	0.0001
Urine TIMP1 (pg/mL)	32.6 (12–58.4)	182.08 (17.2–1,440)	128.6 (17–1,037)	551.05 (107.8–3,817)	0.0001	0.0001
Urine MMP9/Cr (pg/mg)	779.6 (329–2,789.9)	6,672.3 (2,902–40,789)	5,716.6 (1,603–41,694)	10,920.9 (3,783.7–38,124)	0.0001	0.0001
Urine TIMP1/Cr (pg/mg)	99.1 (23.2–1,031)	789 (54.5–7,422.2)	595.9 (50–7,422.2)	1,930.5 (364–19,286.2)	0.004	0.002
Urine MMP9/TIMP1 (pg/pg)	7.9 (1.1–55.5)	8.4 (1.2–118.5)	9.5 (1.2–151)	5.66 (1.3–15.2)	0.920	0.698
Urine microalbumin (μg/mL)	10.97 (7.2–17)	15.02 (7–20)	14.39 (7–18)	17.22 (10.7–26.5)	0.205	0.554

MMP9, Matrix metalloproteinase 9; TIMP1 tissue inhibitor of metalloproteinase 1; Cr, creatinine; VUR, vesicoureteral reflux

The results are presented as the geometric mean, with the interquartile range (IQR) given in parentheses

[a] p_{VUR} Comparison of VUR (−) and VUR(+) patients

[b] p_{SCAR} Comparison of VUR (−) and scar(+) patients

The mean uTIMP1 level was also significantly higher in patients with renal scar than in both healthy controls and VUR patients without scar [551.05 vs. 32.6 pg/mL (p=0.0001) and 128.6 pg/mL (p=0.032), respectively] (Table 1). The optimal cut-off value to predict renal scar was 243.7 pg/mL, and the possibility of renal scar increased by approximately sevenfold when uTIMP1 levels were above this cut-off value (Table 3).

Functional lateralization was present in 13 patients with renal scarring. Both uMMP9 and uTIMP1 levels were significantly higher in the patients with functional lateralization than in those without functional lateralization [mean uMMP9: 2,169.5 (IQR 965.5–5,929.7) vs. 1,271.6 (IQR 414.8–5,899) pg/mL; mean uTIMP1 481.9 (IQR 82.6–3,742.7) vs. 105.7 (IQR 17–801.6) pg/mL, respectively; p=0.012].

Mean uMMP9/Cr, uTIMP1/Cr, and uMMP9/uTIMP1 ratios

The mean uMMP9/Cr and uTIMP/Cr ratios were significantly higher in both the VUR and renal scar groups than in the controls (Table 1). The optimal cut-off value of uMMP9/Cr to predict VUR was 1,830.8 pg/mgCr. The possibility of having VUR increased by threefold above this cut-off value (Table 2). The optimal cut-off point of uTIMP1/Cr was 100 pg/mgCr, with the possibility of having VUR increasing by twofold when the uTIMP1/Cr ratio was higher than this value (Table 2).

The uMMP9/Cr and uTIMP1/Cr values were not significantly different between VUR patients when these were compared according to unilateral or bilateral VUR (p>0.05) (Table 4). The comparison of VUR patients with high-grade and low-grade VUR, respectively, revealed that the uMMP9/Cr value was not significantly different between these groups (p=0.88), whereas the uTIMP1/Cr value was higher in patients with high-grade VUR (p=0.036) (Table 4).

For the prediction of scar, the optimal cut-off value of uMMP9/Cr was 1,830.8 pg/mgCr, and the possibility of scar increased approximately fourfold above this value (Table 3). Urine levels of MMP9/Cr were quite sensitive for the prediction of scar (93%). Similarly, the optimal cut-off point for the uTIMP1/Cr ratio to predict scar was 1,165.5 pg/mgCr, with the possibility of scar increasing by 4.5-fold above this cut-off value (Table 3).

The mean uMMP9/Cr and uTIMP1/Cr values were not significantly different between the patients with and without functional lateralization [mean uMMP9/Cr: 7,992.4 (IQR 3,900–24,762.7) vs. 6,032.7 (IQR 1,603.2–45,050.6) pg/mL; mean uTIMP1/Cr: 1,775.3

Table 2 The optimal cut-off value for predicting VUR

	Cut-off	Sensitivity (%)	Specificity (%)	PPV	NPV	PLR	NLR	AUC
Urine MMP9 (pg/mL)	>1,054	67	85	93.7	43.6	4.5	0.4	0.77
Urine TIMP1 (pg/mL)	>18.7	74	65	87.7	43.3	2	0.4	0.73
Urine MMP9/Cr (pg/mg)	>1,830.8	79	75	91.7	51.7	3.1	0.2	0.79
Urine TIMP1/Cr (pg/mg)	>100	71	70	88.9	42.4	2.3	0.4	0.70

PPV, Positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; AUC, area under the curve

Table 3 The optimal cut-off values for predicting renal scar

	Cut-off	Sensitivity (%)	Specificity (%)	PPV	NPV	PLR	NLR	AUC
Urine MMP9 (pg/mL)	>1,054	81	85	81.2	85	5.4	0.2	0.88
Urine TIMP1 (pg/mL)	>243.7	75	90	85.7	81.8	7.5	0.3	0.82
Urine MMP9/Cr (pg/mg)	>1,830.8	93	75	75	93.7	3.7	0.08	0.88
Urine TIMP1/Cr (pg/mg)	>1,165.5	68	85	78.6	77.3	4.5	0.3	0.75

(IQR 266.5–22,429.4) vs. 5,01.7 (IQR 46.5–4,722.8) pg/mL, respectively; $p>0.05$].

The urine MMP9/TIMP1 ratio was not different in children with and without VUR as well as between those with and without renal scars ($p>0.05$) (Table 1). On the other hand, the uMMP9/TIMP1 ratio was lower in children with high-grade VUR than in those with low-grade VUR ($p=0.011$) (Table 4). Urine MMP9/TIMP1 was not significantly different between the patients with and without functional lateralization [mean: 4.4 (IQR 0.6–17.8) vs. 12 (IQR 1.7–167.5) pg/pg, respectively; $p=0.13$].

Discussion

Hypothesizing that the mechanisms underlying reflux nephropathy are linked to some biomarkers of hyalinization and fibrosis, and considering the importance of prediction of children with high risk for having VUR before invasive radiological imaging studies can be performed, we evaluated the possible relationship between uMMP9 and uTIMP1 levels and VUR in this prospective analysis. The results of our study support the existence of a positive relationship between higher levels of these biomarkers and the presence of VUR and renal scars in children.

Reflux nephropathy is characterized by glomerular lesions, including focal segmental hyalinosis and sclerosis, periglomerular fibrosis, and tubulointerstititalal lesions, such as tubular atrophy, interstitial infiltration of mononuclear cells, fibroblasts, and collagen leading to cortical and medullary fibrosis [5, 6]. Renal fibrosis begins with an induction phase, in which increased interstitial mononuclear cell and fibroblast infiltration occurs due to the release of chemokines and growth factors by some renal cells [16]. This phase is followed by the matrix synthesis phase, which is characterized by the increased production and decreased degradation of matrix, resulting in excessive accumulation of ECM [16]. These two phases represent physiological wound healing, whereas the third phase, the post-inflammatory matrix synthesis phase, leads to renal scarring due to sustained fibroblast activation and excessive deposition of ECM components despite diminished cellular infiltration [16]. The main mediator of this fibrosis process is considered to be the increased number of fibroblasts [16]. Two mechanisms are considered to contribute to this increase in fibroblasts: the proliferation of resident interstitital fibroblasts and the transformation of tubular epithelial cells into fibroblasts, i.e., the epithelial mesenchymal transition (EMT) [6, 16].

The role of MMPs in this process is complicated. The current concept suggests that MMP9 contributes to the prevention of renal scarring via degradation of the glomerular mesangial matrix, especially type IV collagen, and that TIMP1 assists scar formation by inhibiting MMP9 [6, 10].

In the studies of Lelongt and Bengatta [17, 18], MMP9-deficient mice were found to have a higher degree of crescent formation and fibrin deposition in crescentic proliferative glomerulonephritis and also more severe renal lesions and increased apoptosis in experimental acute kidney injury than wild-type mice. Additionally, it has been demonstrated that the inhibition of MMP9 may participate in renal fibrogenesis in experimental models of obstructive uropathy: renal fibrosis was found to be more severe in homozygote human TIMP1 transgenic mice than in controls [7]. On the other hand, disruption of the tubular

Table 4 Comparison of urine levels of MMP9 and TIMP1 in a subgroup of VUR

	Unilateral VUR (n = 43)	Bilateral VUR (n = 24)	p^a	High-grade VUR (n = 11)	Low-grade VUR (n = 56)	p^b
Urine MMP9 (pg/mL)	1,738.2 (509.8–5,899)	1,239.3 (366.5–5,918.7)	0.84	1,314.6 (414.8–5,932)	1,588.3 (533–5,905.7)	0.012
Urine TIMP1 (pg/mL)	174.3 (15.2–1,440)	196.9 (21.7–1,552.7)	0.84	927.4 (323.7–14,575)	132.2 (17–846.7)	0.012
Urine MMP9/Cr (pg/mg)	6,702.2 (2,956.4–22,856.2)	6,619 (1,927.9–46,788.6)	0.98	6,088.5 (1,603.2–40,788)	6,793.4 (2,915.5–38,802.7)	0.88
Urine TIMP1/Cr (pg/mg)	672 (37–7,422.2)	1,051.5 (190–18,545.8)	0.55	4,295.3 (641–127,511.4)	565.6 (51–4,679.3)	0.036
Urine MMP9/TIMP1 (pg/pg)	9.8 (1.4–151)	6.3(1.1–94.5)	0.50	1.3 (0.2–7.7)	12 (1.7–163.3)	0.011

The results are presented as the geometric mean, with the interquartile range (IQR) given in parentheses

[a] Comparison of unilateral and bilateral VUR

[b] Comparison of high-grade and low-grade VUR

basement membrane and type IV collagen by increased MMP9 may trigger EMT and finally result in compensatory accumulation of ECM [6, 16]. Under physiological conditions, the synthesis and degradation of ECM components are in balance and continue at variable rates [10]. According to these studies, the function of MMP9 seems to be versatile for providing the balance in matrix turnover under variable conditions.

There are very little data on the clinical effect of MMP9 and TIMP1 in renal disease. Endo et al. [19] reported that patients with glomerulonephritis (immunoglobulin A nephritis, membranous nephropathy, membranoproliferative glomerulonephritis, minimal change nephrotic syndrome, or lupus nephritis) had significantly increased peripheral blood levels of TIMP1 compared to healthy controls, while their MMP9 levels were not different. Similarly, in a study with anti-neutrophil cytoplasm autoantibody-associated vasculitis patients, uMMP9 was not associated with kidney disease, but uTIMP1 was significantly higher in patients than in the control group [20]. Chromek et al. [8] showed that both uMMP9/Cr and uTIMP1/Cr values were high in children with acute pyelonephritis, similar to our results. In addition, they observed that patients having higher levels of uTIMP1 than uMMP9 had significantly more severe renal scarring, as evidenced on the DMSA scans. TIMP1 levels were found to be increased but MMP9 levels were not decreased in these clinical studies. Recently, Qin et al. [21] found that serum and urine levels of both MMP9 and TIMP1 and the MMP9/TIMP1 ratio were higher in patients with Henoch–Schönlein nephritis than in the patients without nephritis and the controls, but they concluded that the serum level of MMP9 was more sensitive for the diagnosis of Henoch–Schönlein nephritis.

In our study, we expected to find a decrease in the uMMP9/TIMP1 ratio due to an increase in the uTIMP1 level and a decrease in the uMMP9 level in children with renal scar due to a disrupted balance of matrix turnover in favor of ECM accumulation. However, our findings demonstrate that uMMP9 increased along with uTIMP1 in patients with renal scar and, consequently, the uMMP9/TIMP1 ratio was not different from that of the control group. This increase in uMMP9 may be either a consequence of the effort to prevent renal fibrogenesis or an indicator of excessive MMP9 production contributing to fibrogenesis. Although the results from some animal studies have demonstrated that MMP9 deficiency results in renal fibrosis, MMP levels have not been found to be decreased in any of the human studies that have evaluated renal diseases to date; furthermore, the MMP9/TIMP1 ratio has been found to be higher in Henoch–Schönlein nephritis. Renal injury continues as long as VUR continues. Therefore, the increased uMMP9 level may be a response against ongoing trauma due to VUR, which would explain why the uMMP9/TIMP1 ratio was not decreased in our patients. In addition, MMP production and expression are affected by many factors, such as interleukin-1 alpha, tumor necrosis factor-alpha, and transforming growth factor beta [7, 10]. These regulating factors may influence the MMP levels of patients with renal scars due to VUR. However, our study was designed to demonstrate whether uMMP9 and uTIMP1 increased in children with scars and to determine the optimal cut-off levels of these biomarkers to predict scarring, rather than to find the reason for this increase. According to our results, when uMMP9 and uTIMP1 are higher than the determined cut-off values, the possibility of renal scar increases by five- and sevenfold, respectively.

Another result of our study is that both uMMP9 and uTIMP1 levels increase in VUR. This finding is likely to be related to the presence of fibrosis in the refluxing ureteral ending. Supporting this opinion, Oswald et al. [22] determined fibrosis and increased expression of MMP1, another matrix degrading enzyme in the MMP family, in the refluxing ureteral ending. However, we consider that increased uMMP9 and uTIMP1 levels may be the result of the presence of a low degree of renal parenchymal damage which cannot yet be detected as a scar by DMSA. Supporting the latter hypothesis, both uMMP9 and uTIMP1 levels were higher in our VUR patients without scar than in the control group, and they were much higher in the renal scar group. This finding suggests that uMMP9 and uTIMP1 levels increase as renal parenchymal damage becomes progressively more severe. Based on this finding, we determined the optimal cut-off levels of uMMP9 and uTIMP1 that would allow us to use these enzymes as biomarkers to predict the presence of VUR in daily practice. When uMMP9 and uTIMP1 levels were higher than these cut-off values, the possibility of having VUR increased by 4.5- and twofold, respectively. Regarding VUR grade, the uTIMP1 level was significantly increased patients with high-grade VUR, as expected. On the other hand, uMMP9 was significantly decreased in children with high-grade VUR and, consequently, the uMMP9/TIMP1 ratio was found also decreased in these patients in our study. These conflicting findings may be explained as a versatile effect of MMP9, which may contribute to fibrosis triggering EMT, as we stated above. Our results suggest that uTIMP1 is the more reliable biomarker with more consistent results.

There are some limitations of our study. First, patients with impaired renal function, proteinuria, or hypertension were not enrolled in the study in order to recruit a homogenous patient group without additional risk factors apart from VUR and/or renal scarring for elevated MMP9 or TIMP1. Therefore, our results cannot be generalized to all patients with VUR or scarring. Secondly, both MMP9

and TIMP1 are non-specific markers that are overexpressed in the urine in other renal and urologic conditions.

Although the expressions of MMP9 and TIMP1 are not unique factors for scar formation, it is known that they play very important roles in this process. Our study revealed that uTIMP1 especially may be a useful marker to predict renal scar with a different cut-off value from VUR and a high specificity at this cut-off point. Although it would appear that uMMP9 cannot distinguish renal scar from VUR, the simultaneous increase of both markers may indicate an ongoing renal injury due to VUR. The use of these markers as diagnostic tools may avoid unnecessary imaging studies, including VCUG and DMSA. Since there are insufficient data on this issue in the literature, further studies with a larger number of subjects are required to verify the predictive role of uMMP9 and uTIMP1 levels in children with VUR.

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