



# Urinary matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 biomarkers for predicting renal scar in children with urinary tract infection

*Üriner sistem enfeksiyonu olan çocuklarda renal skarın biyobelirteçleri olarak üriner matriks metalloproteinaz -9 ve metalloproteinaz-1 doku inhibitörü*

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## ABSTRACT

**Objective:** Urinary tract infection occurs in 1.8–6.6% of children under 6 years old. The aim of this study was to assess the urinary concentrations of matrix metalloproteinase 9 (MMP9) and tissue inhibitor of metalloproteinase 1 (TIMP1), in children with acute pyelonephritis (APN) and the potential to develop renal scarring.

**Material and methods:** Children who had experienced an episode of APN were divided into 2 groups. Group 1 included children with APN who exhibited scarring and group 2 included children with APN who had a normal <sup>99m</sup>Tc dimercaptosuccinic acid scan. Urinary levels of MMP9 and TIMP1 were measured in the acute phase of infection. A receiver operating characteristic curve was generated to allow calculation of cut-off values.

**Results:** Sixty-one children were enrolled across the 2 groups: group 1 contained 16 patients (all female); group 2, 38 children (36 female and 2 male). Urinary levels of MMP9 and TIMP1 were significantly higher in group 1 than in group 2 (p=0.037 and 0.022 respectively). For comparison of groups 1 and 2, the cut-off values were measured as 75.5 ng/mL (sensitivity 62.5%, specificity 71.1%, positive predictive value, PPV, 48%, negative predictive value, NPV, 82%), 16.1 ng/mL (sensitivity 75%, specificity 55.3%, PPV 41%, NPV 84%), and 1310.7 ng/mL (sensitivity 75% specificity 60.5%, PPV 44%, NPV 85%) for MMP9, TIMP1, and MMP9×TIMP1 levels, respectively.

**Conclusion:** Evaluation of urinary MMP9 and TIMP1 levels may help to identify children with APN who are at risk of developing renal scarring.

**Keywords:** Children; matrix metalloproteinase; pyelonephritis; tissue inhibitor of metalloproteinase 1; urinary tract infection; vesico-ureteral reflux.

## ÖZ

**Amaç:** Altı yaş altı çocukların %1,8-6,6'sında üriner sistem enfeksiyonu oluşmaktadır. Bu çalışmanın amacı akut piyelonefritli (APN) renal skarlaşma geliştirme potansiyeli olan çocuklarda matriks metalloproteinaz -9 (MMP9) ve metalloproteinaz-1 doku inhibitörü (TIMP1) adlı iki biyobelirtecin idrardaki konsantrasyonlarını değerlendirmektir.

**Gereç ve yöntemler:** Bir APN atağı geçirmiş çocuklar iki gruba ayrılmıştır. Grup 1 skarlaşma gösteren APN'li, Grup 2 ise APN'li ancak normal DMSA bulguları olan çocukları içermiştir. Enfeksiyonun akut döneminde idrar MMP9 ve TIMP1 düzeyleri ölçülmüştür. Kestirim değerlerinin hesaplanmasına olanak tanımak için alıcı işletim karakteristik eğrisi oluşturulmuştur.

**Bulgular:** Çalışmaya Grup 1 (16 kız çocuk) ve Grup 2 (36'sı kız ve 2'si erkek çocuk) olmak üzere toplam 61 çocuk dahil edilmiştir. Grup 1'de MMP9 ve TIMP1 düzeyleri anlamlı derecede daha yüksekti (sırasıyla p=0,022 ve 0,037). İki grubun karşılaştırılmasında ölçülen kestirim değerleri (MMP9 için) 75,5 ng/mL %62,5 duyarlılık, %71,5 özgüllük, %48 PPV ve %82 NPV; (TIMP1 için) 16,1 ng/mL %75 duyarlılık, %55,3 özgüllük, %41 PPV ve %84 NPV ve (MMP9×TIMP1 için) 1310,7 ng/mL %75 duyarlılık, %60,5 özgüllük, %44 PPV ve %85 NPV'e sahipti.

**Sonuç:** Üriner MMP9 ve TIMP1 düzeylerinin değerlendirilmesi renal skar gelişme riski olan APN'li çocukların saptanmasına yardımcı olabilir.

**Anahtar Kelimeler:** Çocuk; matriks metalloproteinaz; piyelonefrit; metalloproteinaz-1'in doku inhibitörü, idrar yolu enfeksiyonu, vezikoüreteral reflü.

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## Introduction

Urinary tract infection (UTI) is the most common serious bacterial infection that occurs in infancy and early childhood.<sup>[1]</sup> The prevalence of UTI in male and female children under the age of 6 is 1.8% and 6.6%, respectively.<sup>[1]</sup> UTI is classified into 3 types: acute pyelonephritis (APN), lower UTI and asymptomatic bacteriuria. APN is the most severe form of the disease.<sup>[2]</sup> Permanent renal damage, characterized by scarring, has been observed after APN in 15–60% of affected children.<sup>[1]</sup>

The most important consideration in the evaluation and treatment of UTI is lowering the risk of scar formation.<sup>[3,4]</sup> Children with a higher tendency towards scarring must be carefully monitored. Renal scintigraphy using <sup>99m</sup>Tc-dimercaptosuccinic acid (<sup>99m</sup>Tc-DMSA) is the gold standard method for detecting renal parenchymal involvement.<sup>[5]</sup> DMSA renal scintigraphy can facilitate the diagnosis of APN, owing to its high sensitivity for detection of renal inflammation and scarring, though its ability to differentiate between the two is limited.<sup>[3]</sup> In the acute phase of APN, DMSA is unable to detect the potential for developing permanent renal damage.<sup>[2,6]</sup> For confirmation of scarring, it is necessary to repeat renal scintigraphy 4–6 months after the acute phase of infection. In the recent years several imaging methods have been tested as alternative predictors of renal scarring.<sup>[7]</sup> Urinary biomarkers, including metalloproteinases, represent new and promising candidates for predicting renal scarring in patients.<sup>[8,9]</sup>

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, known to play a role in tissue remodeling through the degradation of extracellular matrix components.<sup>[10–12]</sup> These biomarkers comprise a family of enzymes with 24 members, including collagenases, gelatinases, stromelysins, and membrane-type MMPs.<sup>[13]</sup> MMP expression and activity increase in injured tissue and under inflammatory conditions.<sup>[14]</sup> It has been suggested that the development of renal fibrosis is the result of excessive accumulation of extracellular matrix (ECM) components (type IV collagen, proteoglycan, laminin, etc.) due to increased production and concomitant decreased degradation of matrix.<sup>[15]</sup> These matrix components are metabolized by MMPs. Of these, the gelatinases (MMP2 and MMP9), in particular, have specific activity on type IV collagen, which is the main constituent of matrix.<sup>[15,16]</sup> The degradation of ECM is restrained by tissue inhibitors of metalloproteinases (TIMPs), which inhibit MMPs. Disruption of the balance between MMPs and their inhibitors may lead to fibrinogenesis and scar formation in the kidney.<sup>[16]</sup> Given the important role of MMPs and TIMPs in renal dysfunction, we measured the urinary levels of MMP9 and TIMP1 and evaluated their association with scar formation in children with APN.

## Material and methods

This prospective study was approved by the Ethics Committee of Mazandaran University of Medical Sciences, and was performed between April 2013 and Dec 2015. Children aged 2 months to 6 years old with clinically diagnosed APN were enrolled in the study. APN was clinically suggested by the occurrence of fever with or without urinary symptoms, and was confirmed by urinary examination and culture. Urine analysis was considered positive based on the presence of one or more of the following indices: pyuria (more than 5 leukocytes in each microscopic field), bacteriuria, positive leukocyte esterase, or positive nitrite. APN was confirmed by positive urine culture, based on the route of urine collection:  $>10^5$  colony counts for midstream collection,  $>10^3$  colony counts for catheter collection, and any colony count for the suprapubic method of examination. Urine was collected by catheter or suprapubic aspiration in all patients under 2 years old, and those unable to cooperate for urine excretion. Samples were obtained by the midstream method for cooperative children over 2 years old.

All patients with a clinical diagnosis of APN were admitted, and appropriate antibiotics were administered intravenously, either third generation cephalosporin or aminoglycoside for empirical therapy. Drugs were changed according to antimicrobial sensitivity test results. Kidney scintigraphic imaging by <sup>99m</sup>Tc-DMSA was used for assessment of parenchymal involvement. The scan was performed using a tomographic gamma camera (Siemens DH E-CAM) with a low-energy high-resolution collimator. Inflammation was defined as an attenuation in uptake in some or all portions of kidney with intact layout contour. Scarring was defined as any break in kidney contour, or any volume loss. Patients with any evidence of scarring in the initial DMSA scan were excluded from the study. Scanning was repeated 4–6 months later for children with inflammatory changes.

An ultrasonography study USG with a Siemens G-50 scanner and 2–5 MHz curved-array transducer was performed on all patients. Assessment of vesicoureteral reflux (VUR) was performed in children with any abnormal findings on DMSA or USG, and for those with repeated APN. For diagnosis of VUR, conventional voiding cystourethrography (VCUG) was performed for male children, and radionuclide cystography (RNC) for female children. The severity of VUR was classified as either mild (equal to grade 1 or 2 on VCUG), moderate (equal to grade 3 on VCUG) or severe (equal to grade 4 or 5 on VCUG).

Patients were divided into 2 groups. Patients with evidence of scarring on the second <sup>99m</sup>Tc-DMSA scan comprised group 1. Patients with a normal first <sup>99m</sup>Tc-DMSA scan performed in the acute phase of APN comprised group 2; patients with abnormal inflammatory <sup>99m</sup>Tc-DMSA findings on the first radioisotope

scan, but who had completely normal findings on the later scan were also enrolled in group 2. Patients with a previous history of APN, evidence of scarring on first  $^{99m}\text{Tc}$ -DMSA, or renal function impairment were excluded from the study.

### Measurement of biomarkers

All children were assessed for complete blood count, blood urea nitrogen, plasma creatinine (Cr), and urinary levels of MMP9, and TIMP-1. Fresh voided urine samples were obtained 72 hours after starting antibiotic therapy when urine culture became negative. The samples were collected in sterile polypropylene containers. Aliquots (1 ml) were centrifuged at 4000 g for 10 min and the supernatant fraction was stored at  $-80^{\circ}\text{C}$  until analysis. Urine levels of MMP-9 and TIMP-1 were determined by enzyme-linked immunosorbent assay (ELISA) kits (GenWay, San Diego, CA), according to the manufacturer's instructions. The absorbance values for the standards and samples were obtained at 450 nm, and standard curves constructed for each assay were compared and used to minimize inter-assay variation. Concentrations were extrapolated from standard curves and expressed in ng/ml. The lower limit of detection of MMP-9 and TIMP-1 was 0.05 ng/mL. To avoid any bias, all samples were analyzed in duplicate, in a blinded fashion, with the appropriate standards on 96-well microplates.

### Statistical analysis

Categorical variables are expressed as percentages, while continuous variables are expressed as mean  $\pm$  standard deviation (SD) or median (25-75<sup>th</sup> quartiles). The Kolmogorov-Smirnov test was used for assessment of normality of distribution. Statistical analysis of the difference between groups with normal distributions was performed using the Student's *t* test and Fisher's exact test for quantitative data, or chi-square test for qualitative data. Non-parametric tests, such as the Mann-Whitney test, were used for variables without normal distribution. A receiver operating characteristic (ROC) curve was constructed, and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the different cut-off points for MMP9, TIMP-1 and MMP9 $\times$ TIMP1 were determined. The cut-off point was chosen according to the ROC curve. Area under the curve was also calculated. Results are expressed with a 95% confidence interval (CI). A *p* value  $<0.05$  was considered statistically significant. All statistical analysis was performed using Statistical Package for the Social Sciences software (SPSS Inc., Chicago, IL, USA).

## Results

### Basic and demographic data

Sixty-one children with APN were enrolled in this study; of these, 7 did not complete their follow-up and were excluded from the study. The mean patient age was  $10.9\pm 2.5$  months, and all but

2 were female. The most common microorganism identified in urine was *E. coli*. After a mean time of 6.95 (5-11) months, patients were divided into 2 groups based on  $^{99m}\text{Tc}$ -DMSA results: group 1 comprised children with evidence of scarring ( $n=16$ , all female); group 2 comprised patients without scarring ( $n=38$ , 2 male and 36 female). Study demographics and other patient characteristics are summarized in Table 1. No significant differences in sex, gestational maturity or type of delivery were observed between groups ( $p=0.49$ ,  $p=0.79$ , and  $p=0.14$ , respectively). VCUG was performed for 41 children: 31 (76%) had VUR, and the frequency of VUR was 80% (12 of 15 VCUGs performed) for group 1, and 73% (19 of 26) for group 2. The severity of VUR in both groups is presented in Table 1. No significant difference in the presence or severity of VUR was observed between groups ( $p=0.46$  and  $p=0.29$ , respectively).

### Biomarker measurements

The median and 25<sup>th</sup> and 75<sup>th</sup> quartiles of urinary levels of MMP9, TIMP-1, TIMP/MMP are presented in Table 2. A comparison between the 2 groups revealed significant differences in the absolute levels of MMP9 and TIMP-1, but not in the ratios MMP9 to TIMP1 (Table 2). We additionally assessed the levels of biomarkers in 31 patients with VUR and 23 without VUR.

**Table 1. Demographic and clinical findings of patients with acute pyelonephritis with or without scar formation on late DMSA scan**

Parameter	Group 1 (with scar)	Group 2 (without scar)	<i>p</i>
<b>Sex</b>			
Female (n)	16	36	0.35
Male (n)	0	2	
Age: mo. (Mean $\pm$ SD)	44.1 $\pm$ 25.1	34.8 $\pm$ 37.2	0.58
<b>VUR: No (%)</b>			
Grade 0	3 (20)	7 (27)	
Grade 1	0	4 (15)	
Grade 2	4 (27)	9 (35)	0.29
Grade 3	5 (33)	4 (15)	
Grade 4	1 (7)	0	
Grade 5	2 (13)	2 (8)	
BUN mg/dL (Mean $\pm$ SD)	24.2 $\pm$ 10.4	17 $\pm$ 5.3	0.1
Creatinine mg/dL (Mean $\pm$ SD)	0.55 $\pm$ 0.16	0.6 $\pm$ 0.11	0.89
<b>Germ of UTI (n)</b>			
<i>E. coli</i>	12	30	
<i>Klebsiela</i>	1	0	0.24
<i>Pseudomona</i>	0	1	

VUR: vesicoureteral reflux; Urinary tract infection (UTI); DMSA:  $^{99m}\text{Tc}$ Technetium dimercaptosuccinic acid

**Table 2. Urine MMP9, TIMP1, MMP9/TIMP1 and MMP9×TIMP1 levels in pyelonephritic patients with or without scar formation**

Parameter	Group 1 (with scar)	Group 2 (without scar)	p
MMP9 (ng/mL)	80 (71.15-88.50)	71 (61.58-80.70)	0.037
TIMP1 (ng/mL)	18.65 (15.90-26.83)	15.90 (14.5-19.15)	0.022
MMP9/TIMP1	0.22 (0.20-0.26)	0.22(0.20-0.26)	0.677
MMP9 × TIMP1	1697.7 (1289.55-2563)	1129 (914.85-1583.82)	0.008

All measures presented as median (25-75<sup>th</sup> quartiles). MMP9: matrix metalloproteinase 9; TIMP1: tissue inhibitor of metalloproteinase 1

**Table 3. Cut-off value, sensitivity, specificity, PPV and NPV value of urinary MMP9, TIMP1 and MMP9×TIMP1 for comparing children with pyelonephritis with and without scar formation on late DMSA scan**

Parameter	Cut off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MMP9	75.5 (ng/mL)	62.5	71.1	48	82
TIMP1	16.1 (ng/mL)	75	55.3	41	84
MMP9 × TIMP1	1310.7	75	60.5	44	85

MMP9: matrix metalloproteinase 9; TIMP1: tissue inhibitor of metalloproteinase 1; DMSA: <sup>99m</sup>Techetium dimercaptosuccinic; PPV: positive predictive value; NPV: negative predictive value

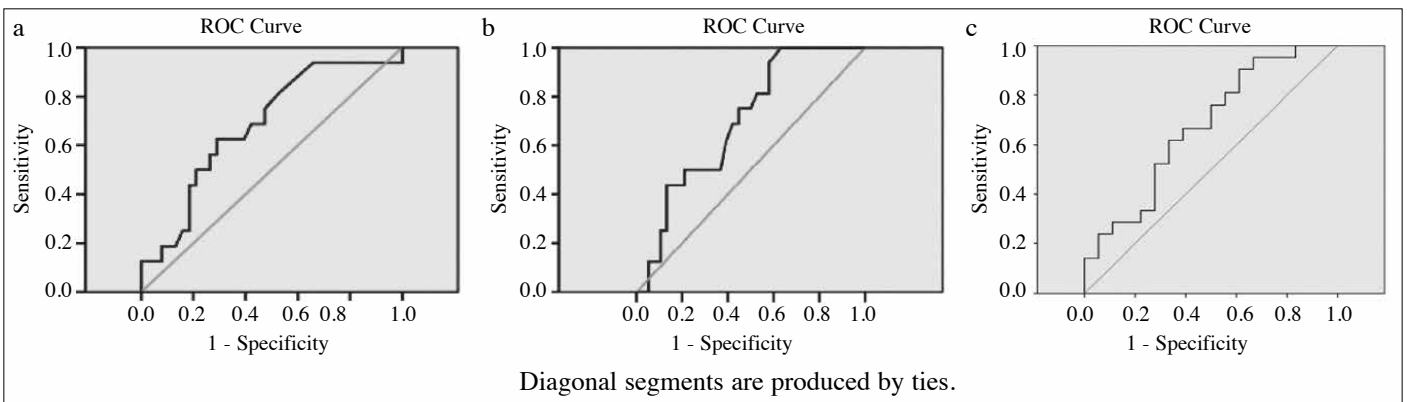


Figure 1. Receiver operating curve to detect scar formation (detect group 1 from 2). (a) for MMP9\* [area under curve (AUC) =0.681 (SE: 0.079, 95 % CI: 0.525 to 0.836)]. (b) for TIMP1\*\* [area under curve (AUC)=0.699 (SE: 0.072,95% CI: 0.558 to 0.840)]. (c) for MMP9×TIMP1 [area under curve (AUC)=0.730 (SE: 0.069, 95% CI: 0.596 to 0.865)].

\* Matrix metalloproteinase 9 \*\*Tissue inhibitor of metalloproteinase 1

The mean level of MMP9 was not different between children with VUR and those with normal VCUG (82.4±23.8 ng/mL vs. 74.6±10.4 ng/mL, p=0.137), but the mean concentration of TIMP1 was significantly lower in children with VUR (18.7±7.9 ng/mL vs. 36.4±28 ng/mL, p<0.05).

**ROC analysis**

The sensitivity and specificity of MMP9 and TIMP-1 were calculated from ROC curves (Figure 1), for comparisons of group 1 and group 2, and to identify cut-off values for distinguishing patients with scarring from those without.

The cut-off values for MMP9 and TIMP-1 were 75.5 ng/mL (sensitivity 63%, specificity 71%, PPV 48%, NPV 82%) and

16.1 ng/mL (sensitivity 75%, specificity 55%, PPV 41%, NPV 84%), respectively (Table 3). In order to maximize specificity, both biomarkers were considered simultaneously. As shown in Table 4, levels of both markers were lower than the cut-off value in 21 patients, 19 of them in group 1 (specificity 90%).

**Discussion**

Urinary tract infection is an important childhood disorder in terms of morbidity and permanent renal damage. Of particular concern in UTI management is detection of the potential for scar formation (4, 6). Early diagnosis of children with such potential facilitates more effective treatment, and allows more rigorous follow-up (1).

**Table 4. Combined results of urinary MMP9 and TIMP1 levels in anticipating scar formation on late DMSA scan in children with acute pyelonephritis**

	No Scar	Scar
High MMP+ High TIMP	9	8
High MMP+ Low TIMP	2	2
Low MMP+ High TIMP	8	4
Low MMP+ Low TIMP	19	2

High MMP and High TIMP = concentrations higher than cut off values presented on Table 3. Low MMP and low TIMP = concentrations lower than cut off values presented on Table 3. MMP9: matrix metalloproteinase 9; TIMP1: tissue inhibitor of metalloproteinase 1; DMSA: <sup>99m</sup>Tc-Technetium dimercaptosuccinic acid

The role of urinary biomarkers in assessment of febrile UTI and its' main complication, scar was studied widely. The three main biomarkers: Kidney injury molecule - 1(KIM-1), Cystatin-c and Lipocalin (NGAL) have been presented.<sup>[17-20]</sup> The validity of NGAL was assessed in two recent study; Ghassemi reported that with the cutoff point of 5 mg/L the NGAL get the NPV of 76.3%, the specificity of 97.83%, the PPV of 96.7%, and the sensitivity of 67.4% in diagnosis of APN.<sup>[21]</sup> Nickavar found that using a cutoff of 0.20 ng/mL, sensitivity and specificity of urine NGAL were 76% and 77% for prediction of APN, respectively.<sup>[22]</sup> The same results were obtained for Cystatin-c and KIM-1.<sup>[23,24]</sup>

In our study, we assessed the accuracy of a non-invasive modality for replacing <sup>99m</sup>Tc-DMSA as a means for predicting scar formation in patients in the acute phase of APN. We found that elevated levels of MMP9 had relatively good specificity (71%), and elevated levels of TIMP1 had good sensitivity (75%) for predicting scar formation. Combined analysis of both markers conferred a specificity of 90%.

Collagen is thought to be the most important molecule in scar formation.<sup>[15,25]</sup> Many factors and markers can affect collagen remodeling in the glomerular basement membrane, including gelatinases and their inhibitory protein TIMP.<sup>[12-14,16,26]</sup> Elevated levels of biomarkers associated with matrix regulation in UTI were reported recently. Hatipoglu et al.<sup>[27]</sup> measured the ratio of MMP9 to NGAL to Cr in children with symptomatic UTI, and compared them with asymptomatic patients with a contaminated urine exam, as well as with healthy children. The authors found that the mean ratio was higher in UTI patients than controls, and using a cut-off value of 0.08 ng/mg, sensitivity and specificity for diagnosing UTI were 98.6% and 97.3%, respectively.

In another study, Tenderenda et al.<sup>[28]</sup> assessed urinary levels of MMPs and TIMPs in 42 patients with pyelonephritis before and after treatment, and compared them with 30 healthy children. The authors found that MMP9 and TIMP1 levels were higher in APN. Since diagnosis of APN was an inclusion criterion in

our study, comparison with healthy children was not possible. In our study, levels of MMP9 were similar in children with and without VUR, but refluxing children had lower levels of TIMP1. The latter observation was expected based on basic information. Inhibitors of gelatinases are expected to be reduced in conditions of fibrosis; although we observed a slight increase in MMP9 levels, it was not statistically significant. Only 10 patients in our study had normal VCUG, and these few cases may not be sufficient for such comparison. Two other studies have reported divergent results: Yilmaz et al.<sup>[8]</sup> found that urinary levels of both TIMP1 and MMP9 are higher in patients with VUR, while Taranta-Janusz et al.<sup>[29]</sup> found that levels of TIMP1 are lower, and MMP9 higher in those with VUR. The difference between the two studies and ours is not obvious. Our study focused on APN and scar and only 4 children in group 1 and 13 children in group 2 had VUR; 7 of them with great severity. Therefore the conclusion is not reliable and we have to trust to other focused studies.

The principal aim of this study was to elucidate the relationship between biomarker levels and scar formation. We found that urinary MMP9 and TIMP1 levels are higher in children with acute pyelonephritis who had evidence of scar formation on a late DMSA scan, relative to those without scarring. We calculated a sensitivity of 62.5-75%, and a specificity of 55-90% for cutoff values of each of the 2 biomarkers or both together. Yilmaz et al.<sup>[8]</sup> also analyzed the levels of the 2 biomarkers in patients with VUR that had scar formation, and found that levels of MMP9 and TIMP1 were significantly higher in patients with scarring. These findings are similar to ours, though in their study urine was collected at different time points after UTI, because the researchers assessed children with VUR, either with or without UTI. Consequently, it was not possible to differentiate acute changes in biomarker levels from chronic ones. Chromek studied 40 patients with APN and 15 children with non-renal fever. He found that urinary MMP-9/Cr and TIMP-1/Cr ratios were significantly higher in children with APN compared with ratios from the same children at 6-week follow-up, and with children with non-renal fever. Out of 40 children with APN, 23 had urinary TIMP-1 levels higher than MMP-9 levels, with a difference of more than 0.1 ng/mmol. These children had significantly more severe changes in both the acute and follow-up DMSA scans, indicating a higher degree of acute tissue damage and renal scarring.<sup>[9]</sup>

In this study, we focused on children with documented APN and followed them for evaluation of scar formation. We found that urinary levels of TIMP1 and MMP9 in the acute phase are higher in children who went on to develop scar formation by the second DMSA scan. Our data suggest that future scar formation can be predicted with reasonable sensitivity (75%) and good specificity (90%), and that biomarker levels are important



clinical indicators in the acute phase of APN. This information may facilitate diagnosis and identification of patients with the potential to develop renal damage in the acute phase of APN, allowing early more intensive treatment.

In conclusion, children with APN who have elevated urinary levels of MMP9 and TIMP1 are significantly susceptible to scar formation. The elevated levels of MMP9 had relatively good specificity (71%), and elevated levels of TIMP1 had good sensitivity (75%) for predicting scar formation. Combined analysis of both markers conferred a specificity of 90%. The DMSA has its main role in diagnosis of scar formation yet.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the ethics committee of Mazandaran University.

**Informed Consent:** Written informed consent was obtained from the parents of the patients who participated in this study.

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