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A. El Shenoufy
Department of Urology,
Cairo University,
Cairo, Egypt

Urine Level of Interleukin-8 as a Non-Invasive Marker for Diagnosis of Vesicoureteral Reflux in Children

[1]A. El Shenoufy, [2]M. Issa, [3]M. Abd Elfatah and [4]N. Elabd

The objective of this study is to assess the urinary levels of IL-8 as a noninvasive marker of VUR in children after resolution of acute UTI. The study was conducted over a 6 month period on 45 children, aged 1-5 years. They were suffering from symptoms of UTI and attending the Pediatric Outpatient and Urology Clinic at Pediatric Specialized Hospital. These children underwent renal ultrasonography (RUS) and voiding cystourethrography (VCUG).The patients were subdivided into two groups: group A (n = 13), children with proven VUR and group B (n = 32), children with negative investigation for VUR. Thirty-nine healthy children with no history of UTI or a known underlying condition that might impair renal function were recruited as control group (group C). Urinary levels of IL-8 were evaluated in all cases using a sandwich enzyme-linked immunosorbant assay for the quantitative measurement of urinary IL-8. To avoid dilution effects, urinary levels of IL-8 were expressed as the ratio of cytokine-to-urinary creatinine. The mean urinary IL-8 levels standardized to the urinary creatinine levels were significantly higher in group A than in group B and C (p-value<0.001). No significant differences were observed between Group B and C (p-value>0.05). A positive correlation was noted between the urinary IL-8/creatinine concentrations and reflux grade (r = 0.338, p-value>0.05). While, no statistical significant difference was observed between the level of urinary IL-8/creatinine and age and sex of the patients and RUS abnormalities. Optimum limit of urinary IL-8/creatinine to establish presumptive diagnosis of VUR obtained by ROC analysis was found to be 10 pg μmol^{-1}, with sensitivity and specificity of 84.6 and 64.8%, respectively. This study demonstrates that urinary IL-8 levels are higher in children with VUR even in the absence of UTI and that it may be considered as an effective noninvasive marker for screening of VUR with high sensitivity and adequate specificity.

Key words: Interleukin-8, non-invasive marker, vesicoureteral reflux, children

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[1]Department of Urology,
[2]Department of Pediatrics,
[3]Department of Radiology,
[4]Department of Clinical and Chemical Pathology, Cairo University, Cairo, Egypt

INTRODUCTION

Vesicoureteral reflux (VUR) is estimated to occur in 1-2% of children, the incidence being less in black children (Chand *et al*., 2003). It is also a common finding in children presenting with Urinary Tract Infection (UTI) and prenatally diagnosed urinary tract dilatation and in relatives of index patients (Galanakis *et al*., 2006). Consequently, screening of all children at the time of the first recorded UTI has shown that about a third have VUR (Jacobson *et al*., 1999). It is common in females with the exception of infancy, when most studies show not only a male preponderance, but a more severe VUR. Primary VUR is usually familial and is inherited as a Mendelian dominant with partial expression (Esbejörner *et al*., 2004).

The cause of VUR is a developmental anomaly resulting in an inadequate length of intravesical submucosal ureter (Blumenthal, 2006). A substantial number of children also have dysfunctional voiding, which may initiate or perpetuate VUR. The VUR is often intermittent and varies in degree; being influenced by the state of hydration (Herndon, 2001). In 1981, an international grading system consisting of five grades was established (International Reflux Study Committee, 1981). Dilating reflux (grades 3-5) has been shown to be significantly associated with Reflux Nephropathy (RN). It has been noted that, VUR has a natural tendency to resolve as the intravesical part of ureter lengthens (Blumenthal, 2006).

Children with VUR are believed to be at risk of ongoing renal damage with subsequent infections resulting in hypertension and reduced renal function (American Academy of Pediatrics, 1999). The VUR provides access for both infection and transmission of bladder pressure to the kidneys; however, the progress from VUR and UTI to RN, renal parenchymal damage and renal scarring has not been thoroughly elucidated (Hellerstein, 2000).

Current American Academy of Pediatrics (American Academy of Pediatrics, 1999) guidelines recommend routine imaging (ultrasound and voiding cystourethrogram or radionuclide cystography) after the initial UTI in febrile infants and young children. The purpose of imaging studies is to detect anatomical abnormalities of the urinary tract system as well as VUR. Furthermore, radiologist often report of dilation of the collecting system of the kidney and urinary tract on renal ultrasound, suggesting that further investigation for VUR should be done (Mahant *et al*., 2002). Micturating cystourethrography (MCUG) is necessary to rule out VUR, but the procedure which is technically difficult, is not standardized and requires the cooperation of the child. Children and their parents find the experience very distressing. Other drawbacks are radiation exposure and the risk of iatrogenic UTI (Butler *et al*., 2005). Contrast enhanced ultrasound techniques, which are accurate and free from radiation will probably increase as an alternative to MCUG. These techniques are however expensive and time consuming (Darge, 2002).

Cytokines are soluble protein mediators of immune and inflammatory response, released primarily by monocytes and macrophages and to lesser extent by other cells (Krzemien *et al*., 2004). They are also well-known to modulate inflammatory response in UTI (Rao *et al*., 2001) and they are related to glomerular diseases (Haraoka *et al*., 1996).

IL-8 is a potent chemoattractant and activator of neutrophils. It is produced by epithelial cells of the renal tract in response to inflammatory stimuli and has been shown to increase during acute UTI (Jantausch *et al*., 2000). Many, experimental and clinical studies have shown high urinary excretion of interleukin-8 (IL-8) in patients with UTI (Hollowell, 2003), but only a few studied the secretions of this cytokine in urine after the acute infection has resolved.

This study aimed at determining the urine levels of IL-8 and evaluating its efficacy as a non-invasive marker of VUR in children in the absence of a recent UTI episode.

MATERIALS AND METHODS

The study was conducted over a 6 month period from January till June 2008 in Pediatric Urology Department Faculty of Medicine, Cairo University on 45 children, aged 1-5 years, suffering from symptoms of UTI, who were attending the Pediatric Outpatient and Urology clinic at Pediatric Specialized Hospital and patients were simply collected by taking the first 45 child coming to the clinic suffering from symptoms of UTI. The diagnosis of UTI was confirmed by the presence of both leukocyturia (defined as more than 5 leukocytes per field in boys and more than 10 leukocytes per field in girls) (Krzemien *et al*., 2004) and the presence of significant bacteriuria (defined as the presence of uniform growth of 10^5 colony forming units (CFU mL^{-1}) in 2 consecutive urine samples) (Hellerstein, 2000).

Urinary tract ultrasonography was done within a week from acute infection to determine kidney size and outline and to detect any dilatation or anomalies. Voiding cystourethrography (VCUG) was performed 4-6 weeks after onset of infection to determine the presence and grade of VUR. Reflux was graded according to the International Reflux Study Committee (International Reflux Study Committee, 1981).

Urine samples for determination of IL-8 levels were obtained from the subjects after resolution of acute UTI. Resolution of UTI was determined by clinical findings, normal White Blood Cell Count (WBCC), Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), urine analysis and culture. To compare the results from different children and avoid dilution effects, urinary levels of IL-8 was expressed as a ratio of IL-8 to urinary creatinine (pg μmol^{-1} creatinine).

The patients were then divided into two groups, group A included 13 children with proven VUR and group B included 32 children with negative investigation for VUR.

Thirty-nine healthy children with no history of UTI or a known underlying condition that might impair renal function were recruited as control group (Group C).

Laboratory investigations

- **Laboratory investigations:** Urine analysis and culture (Christenson Tucker and Auen, 1985), Erythrocyte Sedimentation Rate (ESR) (Katz *et al*., 1989) and White Blood Cell Count (WBCC): by routine analytical methods
- **C-Reactive Protein (CRP):** By immuneturbidimetric method on the Hitachi 917 autoanalyzer (Roche Boehringer Mannheim Düsseldorf, Germany) (Roberts *et al*., 2001)
- **Interleukin-8 assay:** Urine IL-8 was done by ELISA: A sandwich enzyme-linked immunosorbant assay for the quantitative measurement of urinary IL-8(RandD systems Abingdon, UK) (Ninan *et al*., 1999)
- **Urinary creatinine:** It was done by routine analytical method on the Hitachi 917 Autoanalyzer (Roche Diagnostics Gmb, D-68298 Mannheim, Germany) (Ninan *et al*., 1999)

Radiological investigations

- **Urinary tract ultrasonography:** Using the abdominal prob
- **Voiding cystourethrography:** Using the transurethral catheter for contrast medium injection (Oswald *et al*., 2002)

Statistical analysis: Data collected was revised, coded, tabulated and introduced to PC for statistical analysis. All data manipulation and analysis were performed using the 11th version of SPSS (Statistical Package for Social Sciences). Qualitative data was presented in the form of frequency tables (number and percentage) Quantitative data was presented in the form of Mean±SD. Pearson chi-squared was used with correction to test association between two qualitative variables. Independent sample t-test was also used to compare between two groups with quantitative continuous variables. One-way analysis of variance followed by post-hoc comparisons procedures was used to compare between three or more independent means. The correlation coefficients were calculated according to Pearson correlation test. p-values was considered significant if <0.05. Receiver Operating Characteristic (ROC) analysis was constructed by plotting the sensitivity versus the specificity for different cutoff concentrations of IL-8/creatinine.

RESULTS

We studied 45 children (19 males and 26 females with a mean age of 2.6 years ±1.2) suffering from urinary tract infection and attending the Pediatric outpatient and Urology clinics at Pediatric Specialized Hospital. They were further subdivided into 2 groups on the basis of presence or absence of VUR. Group A included 13 patients with VUR and group B included 32 patients without VUR. Thirty-nine healthy children of matching age and sex were recruited as controls. The demographic characteristics of the three groups are presented in Table 1.

Renal ultrasonography revealed urinary tract abnormalities in 7 patients (15.5%; 7/45). Five out of these 7 patients were in Group A (71.5%; 5/7) and their ultrasound showed various degrees of dilation of the urinary tract which was in turn suggestive of VUR. While only 2 cases in group B (28.5%; 2/7) showed ultrasonographic abnormalities. Despite that the presence of renal ultrasound abnormalities in Group A cases was statistically significant when compared to Group B patients (p-value= 0.01), still more than 60% of cases with VUR in Group A (8/13) were missed in this imaging technique (Fig. 1). The sensitivity of the ultrasound for detection of VUR was 38%; specificity was 93%. The positive predictive value of the ultrasound for VUR was 71% and the negative predictive value was 78%.

Table 1: The demographic data and the urinary IL-8/creatinine concentrations of the study participants

	Group			
Parameters	A (n = 13)	B (n = 32)	C (n = 39)	p-value
*Mean age (years)	2.7±1.3	2.5±1.2	2.6±1.1	0.91
Sex (M/F)	8/5	11/21	20/19	0.18
*Mean urinary IL-8/creatinine (pg μmol^{-1})	957.5±1130.5	15±50.6	11.2±19.5	<0.001

*Data are expected as Mean±SD

Table 2: Statistical comparison among the different grades of VUR

Parameters	Grade				p-value
	I VUR (n = 3)	II VUR (n = 3)	III VUR (n = 5)	IV VUR (n = 2)	
*Mean age (years)	1.4±0.4	2.8±1.9	3.1±1.2	3.8±0.2	0.25
Sex (M/F)	2/1	1/2	3/2	2/0	0.51
*Mean urinary IL-8/creatinine (pg μmol^{-1})	444.1±762	963.3±1490	912±1021.2	1832.1±1796	0.66

*Data are expected as Mean±SD

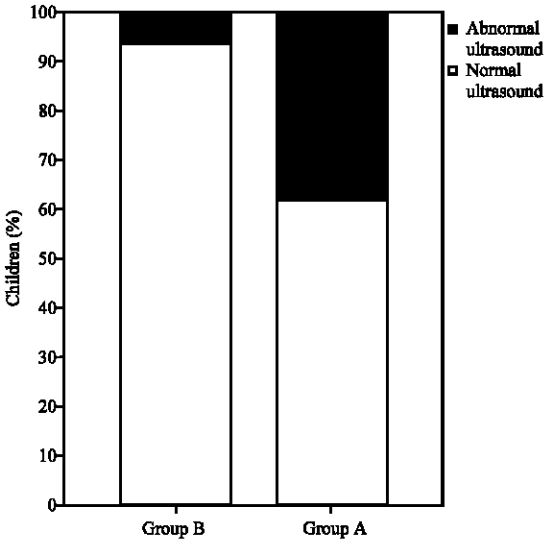

Fig. 1: Frequency of ultrasound abnormalities in Group A and B patients

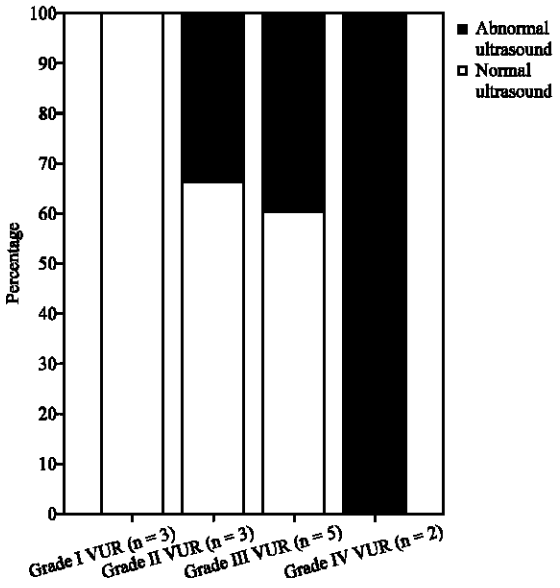

Fig. 2: Frequency of abnormalities of the urinary tract on ultrasonogram, according to the degree of VUR

Abnormal VCUG was found in 13 cases (group A). Three patients had VUR grade I, 3 patients had VUR grade II, 5 patients have VUR Grade III and 2 had VUR Grade IV; no cases of VUR grade V were noted in our study group. Reflux occurred on the right side in 6 cases and on the left side in 7 cases. The demographic characteristics of group A cases according to the grade of VUR is presented in Table 2. VUR of grade III or IV was more likely to occur among children with abnormal ultrasonographic findings than among those with normal findings (4 of 7 vs. 3 of 38, p-value = 0.003). The proportions of cases with ultrasonogram abnormalities of the urinary tract, according to the presence and degree of VUR are presented in Fig. 2.

The urine creatinine concentrations did not differ among the different study groups (2 μmol mL^{-1}±0.6 SD, 1.9 μmol mL^{-1} ±0.5 SD and 1.8 μmol mL^{-1}±0.4 SD in Group A, B and C, respectively with p-value>0.05). Mean urinary IL-8 levels standardized to the urinary creatinine level were significantly higher in group A than in both group B and group C (p-value<0.001). Statistical comparison between the different study groups regarding the urinary levels of IL-8/creatinine is presented in Table 1.

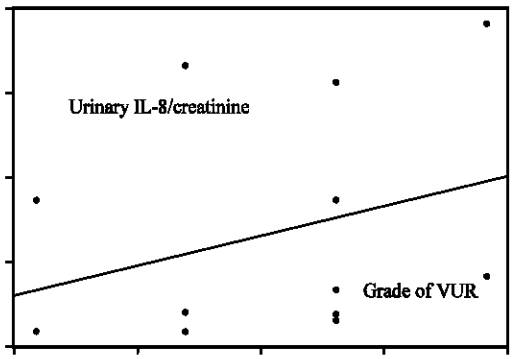

Fig. 3: Correlation between urinary IL-8/creatinine levels and Grade of VUR (r = 0.338, p-value=>0.05)

A positive correlation was noted between the urinary IL-8/creatinine concentrations and reflux grade (Fig. 3). While no statistical significant difference was noted between the level of urinary IL-8/creatinine and age and sex of the cases (Table 1), or the presence of ultrasonographic abnormalities.

The predictive values of urinary IL-8/creatinine concentration in children with VUR was done using

Table 3: The Predictive values for urinary IL-8/creatinine at various cut-off points

Cut-off value for urinary IL-8/creatinine (pg μmol^{-1})	Sensitivity (%)	Specificity (%)	Positive prognostic value (%)	Negative prognostic value (%)
5	92.3	46.4	24.0	97.0
10*	84.6	64.8	30.5	95.8
200	53.8	98.6	87.5	92.1

*Optimal cut-off point

several cut-off points (Table 3) and the optimal limit to predict the diagnosis of VUR in children (cut-off point) obtained by ROC analysis was found to be 10 pg μmol^{-1}. The sensitivity and specificity at this cut-off point was 84.6 and 64.8%, respectively. At the same cut-off point, the Positive Prognostic Value (PPV) was 30.5% and the Negative Prognostic Value (NPV) was 95.8%. At higher cutoff concentration (200 pg μmol^{-1}), specificity of the marker increased but sensitivity rapidly decreased.

DISCUSSION

VUR is a common finding in children presenting with UTI, in prenatally diagnosed urinary tract dilation and in relatives of index patients. Children with VUR are at risk of ongoing renal damage with subsequent infections. Therefore, national guidelines for the investigation and treatment of UTI's were devised (American Academy of Pediatrics, 1999). Those guidelines were aimed at determining the renal tract anomaly and to establish if VUR is present.

In this case-control study ultrasonography was able to predict the presence of VUR in 5 out of 13 cases (38.5%) while the remaining 8 cases were missed by this technique, with sensitivity and specificity of RUS for VUR of 38 and 93%, respectively. Therefore, in this study the ultrasound wasn't sensitive for VUR.

Several studies published have shown similar findings regarding the usefulness of renal ultrasound (RUS) as a screening tool for VUR. Mahant *et al.* (2002) studied the RUS findings in children under the age of 5 years hospitalized with UTI. Renal ulterasound (RUS) was suggestive of VUR in only 14 of 35 children with confirmed VUR and they found out that ultrasound was neither specific (76%) nor sensitive (40%) for VUR in these children. Similar findings were observed by Hoberman *et al.* (2003), where RUS was done for 309 children aged 1-24 months after a first febrile UTI. RUS was unreliable in identifying those with VUR with a sensitivity of 10%. Zamir *et al.* (2004), evaluated the value of RUS in young children hospitalized with UTI and they found abnormal RUS findings suggestive of VUR in 12.9% of their cases. The sensitivity and specificity of RUS for detecting VUR in their study was 17.7 and 87.6%.

Therefore, despite the fact that RUS is of a non-invasive nature, lacks radiation and is low in cost, which should make it an ideal tool for the initial screening investigation of children with UTI. However, it is not sensitive enough to detect the presence of VUR. The reason that RUS is unreliable for screening of VUR, can be explained by dynamic nature of VUR which cannot be consistently detected by ultrasonography and hence the need for VCUG for diagnosis of VUR (Hoberman *et al.*, 2003).

In this study 28.8% (13 out of 45) of the cases with UTI were diagnosed as having VUR by VCUG. The prevalence of VUR was 22% in research conducted by Mahant *et al.* (2002), which were nearly similar to present study. On the other hand, Hoberman *et al.* (2003) found that 39% (117 out of 309) of their cases had evidence of VUR, which was slightly higher then our study. While Zamir *et al.* (2004), diagnosed VUR in 18.4% (47 out of 255) of the children hospitalized with a first episode of uncomplicated UTI. Detection of VUR currently depends on VCUG (Galanakis *et al.*, 2006) and although it is the best technique for confirming VUR, it is technically difficult, requires the cooperation of the child and involves drawbacks of radiation exposure and risk of iatrogenic UTI (Blumenthal, 2006). Contrast enhanced ultrasound techniques, which are accurate and free from radiation will probably increase as an alternative to VCUG. These techniques are however expensive and time-consuming (Darge, 2002).

In the current study we present evidence that mean urinary IL-8 levels, a potent neutrophil and chemoattractant, standardized to urinary creatinine level, were significantly higher in children with than without VUR (p-value<0.001).

IL-8 is present only in trace amounts in urine of healthy subjects (Krzemien *et al.*, 2004). The elevation of urinary levels of IL-8 has been observed in patients with acute urinary tract infections (Rao *et al.*, 2001). It is produced by the urinary epithelium in response to bacterial infections (Krzemien *et al.*, 2004) and it is responsible for migration of neutrophils to the site of infection and for development of pyuria (Agace *et al.*, 1993). The mechanism by which IL-8 was secreted into the urine has not yet been completely defined. IL-8 is a protein induced by liposaccharide stimulation from peripheral monocytes or macrophages (Haraoka *et al.*, 1996). It has been reported that IL-8 is also secreted by histocytes and induced by many stimulants. To this

knowledge there have been few published papers in the literature investigating the secretion of IL-8 into the urine after acute inflammation has resolved.

Agace *et al.* (1993) reported that urinary IL-8 levels in patients with a UTI were elevated but remained undetectable in patients without UTI. However, in our study IL-8 levels in patients with VUR (group A) were elevated despite the clinical and laboratory evidence of resolution of attack of acute UTI. These findings suggest that in kidneys, with VUR, even after acute inflammation has resolved, IL-8 is secreted from an unknown origin.

Present findings are in concordance with Galanakis *et al.* (2006), who evaluated urine IL-8 levels in 59 infants. All infants selected for the study were free of UTI for a minimum of 3 weeks before IL-8 evaluation. The mean urine IL-8/creatinine concentrations were significantly higher in VUR group when compared to cases with history of UTI, but negative investigation for VUR (p-value = 0.0003) and in healthy controls (p-value = <0.001). Similar results were observed with Haraoka *et al.* (1996) who conducted a similar study, were they measured the IL-8 in urine of 32 children with VUR who were free from UTI. There were statistical significant differences between urinary IL-8-to-creatinine levels in children with then without VUR (p-value = 0.0246) in their study.

The similarities in results between us and the above mentioned studies, suggest that inflammatory process in VUR is ongoing even after UTI has resolved, pointing against the currently held belief that sterile reflux cannot harm kidneys (Smellie, 1999). The increase of IL-8 could not be explained by the residual inflammation caused by UTI, because this increase was not noted in children with UTI alone after the UTI has resolved (Galanakis *et al*., 2006).

A cutoff of 200 pg mL^{-1} for urine IL-8 has been proposed as a marker for diagnosing UTI (Rao *et al*., 2001). Urine IL-8 secretion in UTI is quickly reduced after clinical and laboratory resolution of the UTI, a finding confirmed in Group B patients of our study. Our findings suggest that a cut-off of 10 pg μmol^{-1} for urinary IL-8/creatinine, gave us the best sensitivity (84.6%) without markedly affecting specificity (64.8%) for diagnosing of VUR. Higher cutoffs were associated with higher specificity and but very low sensitivity. In another study the optimum cut-off point for the diagnosis of VUR was 5 pg μmol^{-1} and this gave a sensitivity of 88% and specificity of 69% (Galanakis, 2006).

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, detection of VUR by the current imaging modalities, particularly the VCUG, present with considerable limitations as screening tools because they are associated with radiation and invasiveness. Ultrasound is a useful noninvasive tool for the definition of gross urinary tract anatomy; however the modalities low sensitivity to detect reflux, poses certain limitations to its use as a screening method.

Present study clearly demonstrates that urinary Il-8 levels were significantly higher in children with VUR when compared to children without VUR and that urinary IL-8 would be an effective and promising non-invasive marker for VUR. The optimal cut-off point in determining the concentrations of urine IL-8/creatinine is 10 pg μmol^{-1}, for which the maximum sensitivity and specificity is needed to establish the diagnosis of VUR. However, this marker is not free from limitations, because IL-8 maybe elevated as a result of urinary tract manipulation, vaginitis, balanitis or as a result of undetected UTI. Nevertheless, routine determining of urine IL-8 levels after UTI has resolved may provide substantial help as a screening test in evaluating high-risk patients for VUR and siblings of patients diagnosed with VUR.

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