Urinary podocalyxin and nephrin levels as biomarkers in lupus nephritis patients: Relation to renal involvement and disease activity

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KEYWORDS
Systemic lupus erythematosus; Lupus nephritis; Urinary markers; Podocalyxin; Nephrin; BILAG

Abstract  Aim of the work: To evaluate the impact of systemic lupus erythematosus (SLE) on urinary levels of podocalyxin and nephrin and to determine their relationship to renal biopsy and disease activity in lupus nephritis (LN) patients.

Patients and methods: The study included 50 LN patients with their renal biopsy classified according to the international society of nephrology. Disease activity was determined using the British Isles Lupus Assessment Group (BILAG). All patients underwent clinical and laboratory evaluation. Urine samples were collected for the assessment of urinary podocalyxin (UPx) and nephrin (UN) by ELISA and for the estimation of protein (UP) and creatinine (Cr) concentrations. The UPx:Cr, UN:Cr and UP:Cr ratios were calculated.

Results: Urinary levels of podocalyxin (593.8 ± 282.2 ng/ml), nephrin (304.1 ± 236.8 ng/ml) and protein (2.36 ± 0.56 g/l) were significantly higher, while urinary creatinine levels (101.4 ± 28.7 mg/l) were lower in LN patients compared to control (38.1 ± 9 ng/ml, 19.2 ± 4.1 ng/ml, 0.34 ± 0.13 g/l and 155.4 ± 26.7 mg/l; p = 0.0008, p = 0.0003, p = 0.00002 and 0.0009, respectively). Consequently, UN:Cr, UPx:Cr and UP:Cr ratios were significantly higher in patients compared to control. There was a significant correlation of the estimated ratios with the LN class and with the BILAG scores being most significant with UPx:Cr ratio. ROC curve and regression analyses defined UPx:Cr ratio as the specific significant predictor of pathological LN grade.

Conclusion: SLE deleteriously affects fine glomerular structure as reflected by increased urinary levels of podocyte-related proteins; podocalyxin and nephrin. Urinary podocalyxin/creatinine ratio...
significantly predicts the pathological impact of SLE on the kidney and could be used as a non-invasive marker for such effect and its progression.

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1. Introduction

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease characterized by the production of antinuclear antibodies [1]. Lupus nephritis (LN) patients present with proteinuria that has generally been associated with immune complex deposition in the glomerular capillary wall and endo-capillary proliferation and inflammation [1]. Many studies focused on the importance of finding potential biomarkers, to estimate the degree of LN in SLE patients, such as serum cystatin C and urinary neutrophil gelatinase-associated lipocalin [2], cytokines [3], markers of oxidative stress [4], matrix metalloproteinase [5], adipokines [6,7] and growth arrest specific protein 6 [8]. The search for reliable markers is still necessary and ongoing.

Podocyte injury is an important feature of several renal diseases. Regulation of the podocyte actin cytoskeleton is of critical importance for sustained function of the glomerular filter and is mediated by several podocyte proteins such as nephrin and podocin [11,12]. Several markers of podocyte injury include nephrin, synaptopodin, podocalyxin and podocin [13,14]. The relationship between urinary podocyte protein and renal diseases is supported by their detection in patients with immunoglobulin A nephropathy, Henoch–Schönlein purpura nephritis, lupus nephritis, diabetic nephropathy and focal segmental glomerulosclerosis. The detection of urinary markers of podocyte injury would have broad implications for the evaluation of disease activity, the degree of dedifferentiation and the possibility of podocyte regeneration [15].

The aim of the current study was to evaluate the impact of SLE on fine glomerular architecture using podocyte injury related markers; podocalyxin and nephrin in urine and to determine their relationship to pathological classes of renal biopsy and disease activity.

2. Patients and methods

This study was conducted at Rheumatology, Internal Medicine and Clinical Pathology departments, Dallah and Ibn Sina College Hospitals, Kingdom of Saudi Arabia (KSA). The study included 50 lupus nephritis (LN) patients diagnosed by previous renal biopsy. The research protocol was approved by the Ethics Committee of our hospitals and informed written consents were obtained from all participants. Systemic lupus erythematosus (SLE) was diagnosed according to the systemic lupus international collaborating clinics (SLICC) classification criteria for SLE [16]. All patients underwent full clinical examination and laboratory tests were performed including complete blood count (CBC), C-reactive protein (CRP), complement (C3 and C4), anti-nuclear antibodies (ANA) and anti-double stranded deoxyribonucleic acid (anti-dsDNA). Anemia was defined by a hemoglobin (Hb) concentration of ≤12 g/dl for women and of ≤13.5 g/dl for men [17]. Proteinuria was measured by a dipstick method. The study also included 20 matched normal subjects free of renal disease as control group for estimated urinary biomarkers.

Disease activity was determined using the British Isles Lupus Assessment Group (BILAG) score which consisted of evaluation of 8 points of interest: general, mucocutaneous, neurologic, musculoskeletal, cardio-respiratory and renal manifestations, vasculitis and hematologic findings. To obtain a global score, BILAG component scores are assigned numerical values: A = 9 (most active disease), B = 3 (intermediate activity), C = 1 (mild, stable disease activity), D = 0 (inactive disease) and E = 0 (no activity), resulting in a potential summed range of 0–72 points with 72 = most active disease affecting the 8 organs [18].

Lupus nephritis was diagnosed depending on the presence of proteinuria and hematuria [19] and renal biopsy histopathology was classified according to the International Society of Nephrology/Renal Pathology Section (ISN/RPS) classification as minimal mesangial (class I), mesangial proliferative LN (class II), focal LN (class III), diffuse LN (class IV), membranous LN (class V) and advanced sclerosis (class VI) [20].

2.1. Urine sample collection

Urine (10 ml) was collected in plastic tubes, without preservative. Samples were clarified by centrifugation at 3,000 rpm for 5 min and supernatant collected in Eppendorf tubes and kept frozen at −80 °C till assayed for:

1. **Urine protein concentrations** were measured by the Bradford method [21].
2. **Estimation of urinary markers of podocyte injury**: urinary podocalyxin and urinary nephrin was estimated using commercially available ELISA kits (Exocell Inc., Philadelphia, PA). Urine samples were diluted with dilution buffer provided by the ELISA kits in a ratio of 1:2 for urinary podocalyxin [9] and 1:1 for Urinary nephrin [22]. Each sample was measured in duplicate. The values are expressed as ng/ml.
3. **Urine creatinine** was measured by the Jaffe reaction on the same aliquot of urine to [27] adjust the ratio of urinary podocalyxin to creatinine (UPx:Cr), urinary nephrin to creatinine (UN:Cr) and urine total protein-to-creatinine ratio (UP:Cr).

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2.2. Statistical analysis

Obtained data were presented as mean ± SD, ranges, numbers and ratios. Results were analyzed using Wilcoxon; ranked test for unrelated data (Z-test) and Chi-square test ($\chi^2$ test). Sensitivity and specificity of estimated parameters as predictors were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that $AUC = 0.05$. Regression analysis (Stepwise method) was used for stratification of studied parameters as specific predictors. Statistical analysis was conducted using the SPSS (Version 15) for Windows statistical package. $p$ value $< 0.05$ was considered statistically significant.

3. Results

The current study included 50 SLE patients; 39 females and 11 males (F:M 3.5:1) with a mean age of 45.7 ± 11.8 years (range: 22–69 years) and a mean disease duration of 5.1 ± 1.1 years (range: 3–8 years). In our clinics, there is high number of males for regular follow up so the number of males in our study was relatively high and there was no effect of gender on levels of the study parameters. All patients had LN diagnosed clinically and documented by renal biopsy that showed varied classes with focal LN being the most frequent. Details of patients’ distribution according to results of renal biopsy are shown in Table 1. Mean BILAG score was 17 ± 9.6 (range: 8–38). The BILAG score ranged from 8–10 in 24 patients; 11–17 in 13; 6; 20–29 in 13 and 32–38 in 7 patients. Systematic BILAG grading is shown in Table 2.

Hemoglobin detected 11 anemic patients (22%) with a mean Hb concentration of 10 ± 1.2 gm/dl, while it was 13.2 ± 0.5 gm/dl in the other 39 patients. The total leukocyte count (TLC) was 8.25 ± 2.7 $\times$ 10$^3$/dl, platelet count 165 ± 18.6 $\times$ 10$^3$/dl, erythrocyte sedimentation rate (ESR) 41 ± 15.6 mm/1st h, CRP 7.2 ± 4.6 mg/dl, Complement C3 94 ± 53.4 mg/dl, C4 60.4 ± 39.3 mg/dl, the ANA was positive in 100% of patients and the anti-ds DNA was 216.8 ± 99.2 U/l.

Urinary levels of nephrin, podocalyxin and protein were significantly higher in LN patients compared to controls, while urinary creatinine levels were significantly lower compared to control levels. Consequently, urinary nephrin/creatinine (UNCr), urinary podocalyxin/protein (UPxCr) and urinary protein/creatinine (UPCr) ratios are significantly higher in studied patients compared to control levels (Table 3).

### Table 1 Renal biopsy classification in lupus nephritis patients.

<table>
<thead>
<tr>
<th>Renal biopsy classification in LN patients</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISN/RPS Minimal mesangial LN</td>
<td>9 (18)</td>
</tr>
<tr>
<td>ISN/RPS Mesangial proliferative LN</td>
<td>16 (32)</td>
</tr>
<tr>
<td>ISN/RPS Focal LN</td>
<td>18 (36)</td>
</tr>
<tr>
<td>ISN/RPS Diffuse LN</td>
<td>3 (6)</td>
</tr>
<tr>
<td>ISN/RPS Membranous LN</td>
<td>3 (6)</td>
</tr>
<tr>
<td>ISN/RPS Advanced sclerosis</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

ISN/RPS: International Society of Nephrology/Renal Pathology Section, LN: lupus nephritis.

### Table 2 Disease activity as assessed by the British Isles Lupus Assessment Group (BILAG) grading in lupus nephritis patients.

<table>
<thead>
<tr>
<th>Manifestations</th>
<th>BILAG grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>General</td>
<td>5</td>
</tr>
<tr>
<td>Musocutaneous</td>
<td>6</td>
</tr>
<tr>
<td>Neurological</td>
<td>3</td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>3</td>
</tr>
<tr>
<td>Cardiorespiratory</td>
<td>3</td>
</tr>
<tr>
<td>Renal</td>
<td>7</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>10</td>
</tr>
<tr>
<td>Hematological</td>
<td>11</td>
</tr>
</tbody>
</table>

BILAG: British Isles Lupus Assessment Group. BILAG grades: A: severe = 9, B: intermediate = 3, C: mild = 1, D: inactive = 0, E: no activity = 0.

A stepwise increase of estimated ratios with increased pathological disease severity grade was found (Fig. 1). The UPxCr ratio significantly correlated with the UN:Cr ratio ($r = 0.43$, $p = 0.002$), UP:Cr ratio ($r = 0.37$, $p = 0.008$), BILAG score ($r = 0.31$, $p = 0.03$) and with the LN class ($r = 0.56$, $p = 0.008$). The UN:Cr ratio significantly correlated with the UP:Cr ratio ($r = 0.31$, $p = 0.037$), BILAG score ($r = 0.29$, $p = 0.04$) and with the LN renal biopsy class ($r = 0.38$, $p = 0.007$). The UP:Cr significantly correlated with the BILAG score ($r = 0.28$, $p = 0.046$) and with the renal biopsy class ($r = 0.36$, $p = 0.009$). The renal biopsy grading significantly correlated with the BILAG scores ($r = 0.29$, $p = 0.04$).

In a trial to determine a predictor for pathological LN disease severity, UPxCr ratio showed the highest AUC using ROC curve analysis that was the only significant area versus the null hypothesis (Table 4, Fig. 2). Regression analysis defined UPxCr ratio as the specific significant predictor of pathological grade, while UP:Cr ratio and BILAG scoring were non-significant predictors as shown in Table 4.

### Table 3 Urinary podocalyxin, nephrin and protein and their ratios relative to urinary creatinine level in lupus nephritis patients and control.

<table>
<thead>
<tr>
<th>Urinary parameter</th>
<th>LN patients ($n = 50$)</th>
<th>Control ($n = 20$)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocalyxin (ng/ml)</td>
<td>593.8 ± 282.2</td>
<td>38.1 ± 9</td>
<td>0.0008</td>
</tr>
<tr>
<td>Nephrin (ng/ml)</td>
<td>304.1 ± 236.8</td>
<td>19.2 ± 4.1</td>
<td>0.0003</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>2.36 ± 0.56</td>
<td>0.34 ± 0.13</td>
<td>0.0002</td>
</tr>
<tr>
<td>Creatinine (mg/l)</td>
<td>101.4 ± 28.7</td>
<td>155.4 ± 26.7</td>
<td>0.0009</td>
</tr>
<tr>
<td>Nephrin:creatinine</td>
<td>287.4 ± 163.3</td>
<td>12.7 ± 3.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Podocalyxin:creatinine</td>
<td>602 ± 284.5</td>
<td>25 ± 6.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein:creatinine</td>
<td>2.46 ± 0.72</td>
<td>0.23 ± 0.09</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

LN: lupus nephritis.
of SLE on fine glomerular architecture. Both UPx:Cr and UN:Cr ratios showed significant correlations with pathological changes in lupus nephritis more than other evaluated parameters like those related to podocytes alone [23].

The obtained results showed a significant correlation between both UPx:Cr ratio and UN:Cr ratios with the BILAG disease activity scores and UP:Cr ratio but no significant correlation between disease duration and the BILAG score. More interestingly, both UPx:Cr ratio and UN:Cr ratios showed a significant correlation with the ISN renal biopsy grade. The UPx:Cr ratio was found to be a significant predictor for renal pathological class thus it could be considered as a potential non-invasive biomarker for the grade of LN. This was in agreement to the results presented by Persisnakí et al. [24] reporting that glomerular expression of nephrin and podocin significantly correlated with and became pronounced at advanced renal biopsy histopathological classes (focal and diffuse proliferative LN). The expression of nephrin and podocin was reduced in early stages of LN (mesangial) and nephrin was also particularly reduced in diffuse proliferative LN. Similarly, Bollain-Y-Goytia et al. [25] reported that in LN patients, reduction of glomerular podocytes significantly correlated with the cumulative excretion of urinary podocytes and proteinuria. In line with the obtained results, Sabino et al. [23] reported that podocyturia correlated with the protein/creatinine ratio and both showed a significant correlation with the degree of lupus disease activity. They concluded that podocyturia with anti-podocin could be useful in monitoring disease activity in LN patients. Wang et al. [26] reported that podocyte damage was common in LN; urinary podocytes met the histological criteria of lupus podocytopathy. Pure lupus podocytopathy might act as an extreme form of lupus podocyte lesion, and more patients might present with severe podocyte effacement concealed in different types of LN. Sir Elkhaim et al. [27] documented that urine podocyte-related protein markers have been used with varying degrees of success to study glomerular diseases and the determination of urinary podocyte loss may become an important noninvasive tool in the evaluation of glomerular diseases. Rezende et al. [28] reported that in proliferative forms of LN there seems to occur structural podocyte damage, whereas in the pure membranous forms the predominant preserved pattern suggests a dysfunctional podocyte lesion that may account for the better long-term prognosis of proteinuria outcome.

Table 4 Receiver operating characteristic (ROC) curve and regression analysis of the ratios of estimated urinary parameters relative to urinary creatinine level and British Isles Lupus Assessment Group (BILAG) clinical scoring as predictors for International Society of Nephrology (ISN) pathological grading in lupus nephritis patients.

<table>
<thead>
<tr>
<th>Predictors for ISN grading in LN patients (n = 50)</th>
<th>AUC</th>
<th>CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROC curve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BILAG score</td>
<td>0.28</td>
<td>0.14–432</td>
<td>0.21</td>
</tr>
<tr>
<td>UPx:Cr</td>
<td>0.88</td>
<td>0.79–0.97</td>
<td>0.03</td>
</tr>
<tr>
<td>UN:Cr</td>
<td>0.68</td>
<td>0.3–1.06</td>
<td>0.31</td>
</tr>
<tr>
<td>UP:Cr</td>
<td>0.63</td>
<td>0.49–0.78</td>
<td>0.45</td>
</tr>
<tr>
<td>Regression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UN:Cr</td>
<td>0.35</td>
<td>3.15</td>
<td>0.003</td>
</tr>
<tr>
<td>UPx:Cr</td>
<td>0.54</td>
<td>4.86</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

ISN: International Society of Nephrology, LN: lupus nephritis, AUC: Area under curve; CI: confidence interval, β: Standardized coefficient. UPx:Cr: urinary podocalyxin:creatinine, UN:Cr: urinary nephrin:creatinine, UP:Cr: urinary protein:creatinine. Bold values are significant at p < 0.05.
The present study relied on the detection of podocyte-related markers using ELISA estimation of their urinary levels. Review of the literature detected that previous studies used ELISA estimation of urinary podocalyxin and nephrin in other kidney diseases, wherein Hara et al. [29] quantified urinary podocalyxin by ELISA in patients with glomerular diseases and patients with type 2 diabetes and found that levels of urinary podocalyxin were elevated in patients with various glomerular diseases and patients with diabetes. Also, Palacios de Franco et al. [30] reported significantly higher levels of urinary podocalyxin as measured using by ELISA in preeclampsia/eclampsia. Recently, in 2015, Wang et al. [31] using indirect immunofluorescence, ELISA and Western blotting of urinary sediment found that urinary podocyte number and nephrin level were significantly higher in patients with LN compared to those patients with other glomerular diseases and were higher in patients with severe proteinuria and urinary nephrin expression was related to podocyte and urinary albumin/creatinine ratio. Moreover, they found that urinary podocyte number and nephrin level dramatically increased in the focal segmental glomerulosclerosis group as compared to those of the mesangial proliferative glomerulonephritis and minimal change disease groups and concluded that the detection of the urinary podocytes and nephrin could be taken as non-invasive markers for glomerular disease severity.

It could be concluded that SLE deleteriously affects the fine glomerular structure as reflected by increased urinary levels of podocyte-related proteins; podocalyxin and nephrin. Urinary podocalyxin/creatinine ratio significantly predicts the pathological impact of SLE on the kidney and could be used as a non-invasive marker for such effect and its progression. A longitudinal larger scale study is recommended to further assess the value of these markers in response to therapy.

Conflict of interest

None.

References


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Biomarkers in lupus nephritis patients


