Detection of podocyturia in patients with lupus nephritis

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Abstract

Introduction: The podocyturia has been detected in glomerular diseases, such as lupus nephritis (LN), in which proteinuria is an important manifestation, and its occurrence seems to be limited to the active phase of the disease. **Objective:** To evaluate podocyturia in LN patients, and the possible association with clinical disease activity. **Methods:** We evaluated 56 patients with LN, that were classified in three groups according to the degree of clinical activity: Group B, no activity (n = 17), Group C with mild (n = 29) and Group D, moderate to severe activity (n = 10). The control group was composed by 29 healthy subjects (Group A). The podocyturia was studied by indirect immunofluorescence using primary antibodies to podocyte: anti-podocin, nephrin and synaptopodin, and a secondary antibody conjugated with FITC. We also evaluated serum creatinine levels, urinary protein/creatinine (P/C) ratio, hematuria and leucocituria. **Results:** The podocyturia with anti-podocin and anti-sinaptopodin correlated statistically with the P/C ratio ($p = 0.001$ and $p = 0.013$, respectively). The podocyturia with anti-podocin, as well as the P/C ratio showed significant correlation ($p < 0.001$) with the degree of lupus disease activity, unlike the other two antibodies, anti-nephrin and anti-synaptopodin. **Conclusion:** Our findings show that podocyturia with anti-podocin could be useful in monitoring disease activity in LN patients.

Keywords: glomerulonephritis, lupus nephritis, podocytes, proteinuria.

Introduction

Podocytes or visceral epithelial cells are highly specialized cells which line the urinary surface of the glomerular capillary tuft and, together with endothelial cells and the basement membrane, form the glomerular filtration barrier and provide for its selective permeability. After injury, podocytes may detach from the glomerular basement membrane and be excreted in urine; in this situation, they may still be viable, or have undergone apoptosis or necrosis.[1]

Animal experiments and clinical studies with glomerulopathy patients have shown that injury to podocytes plays a key role in the development of proteinuria.[2] The presence of podocytes in urine has been described in many glomerular diseases, such as IgA nephropathy, diabetic nephropathy, membranous nephropathy, and lupus nephritis (LN),[3,4] among others, reflecting the occurrence of glomerular injury.

One of the assays used to assess podocyturia is indirect immunofluorescence with specific antibodies directed against podocyte antigens in urinary sediments. The quantification of podocytes in the different stages of glomerular disease may contribute to the understanding of the condition's pathogenesis. Podocyturia tests may also be a practical means of monitoring patients with glomerulopathy. The noninvasive nature of the tests and the real-time data they provides on podocyte injury in the glomerulus yield potential applications in the assessment of glomerular disease clinical activity.

LN is one of the most severe clinical manifestations of systemic lupus erythematosus (SLE). Studies have shown that podocyte injury occurs in the early stages of glomerular damage in LN,[5] and that quantification of podocyturia could be used as a marker for active disease.[6,7]

This study aimed to analyze the use of immunofluorescence assays for podocyturia in patients with LN. This simple low cost technique requires commercially available podocyte-specific antibodies.

Methods

The series

The study included patients followed up in the Glomerulopathy Clinic of the Federal University of São Paulo (UNIFESP) diagnosed with LN and SLE as per the criteria of the American College of Rheumatology.[8]

Patients with LN were divided into three groups according to the degree of disease activity: 17 (20.0%) had no active disease (Group B, 14 females and three males, mean age 41.4 years); 29 (34.1%) had mild disease (Group C, 22 females and seven males, mean age of 37.8 years); and 10 (11.8%) had moderate to severe disease (Group D, all females, mean age of 29.6 years). Twenty-nine (34.1%) healthy individuals without urinary disorders were enrolled in the the control group (Group A, 21 females and eight males, mean age of 40.7 years). They were selected based on negative urine test strip results.

Clinical and workup criteria were applied by an experienced physician in the area of lupus nephritis monitoring to grade disease activity (no active disease, mild disease, moderate to severe disease). Clinical criteria included signs and symptoms manifested in the patients and treatment with immunosuppressants; workup criteria included the results of tests routinely used to monitor patients with SLE in our service as described by Solorzano *et al.*[9]

Podocyturia testing

Samples of midstream urine were collected in sterile vials and kept under refrigeration until the time of processing. Approximately 30 ml of urine were transferred to a tube and centrifuged at 2000 rpm for five minutes. After centrifugation, the supernatant was discarded and the sediment resuspended in 5 ml of 50% ethanol and washed with HDF (solution of ultra-pure water containing 137 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM $NaHCO_3$, and 0.2% EDTA). Then, the material was submitted to cytocentrifugation on microscope slides with adhesive filter paper.

The prepared slides were fixed in 2% formaldehyde and 4% sucrose PBS at room temperature for 10 minutes. The slides were then washed in PBS for 5 minutes and treated with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 10 minutes to increase the permeability of the material. Another washing cycle with PBS was performed for 5 minutes, and then the material was sent for incubation for one hour with blocking buffer solution (PBS with 0.2% BSA, 50 mM NH_4Cl, and 1% goat serum). After another washing cycle, the slides were incubated for 16 hours at 4°C with primary podocyte-specific antibodies: rabbit anti-podocin antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-nephrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-synaptopodin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the slides were incubated with secondary antibody fluorescein-conjugated goat anti-rabbit IgG (FITC, Sigma-Aldrich, St. Louis, MO) for 45 minutes at room temperature. DAPI was used to stain nucleic acid. The slides were examined and photographed under 400x magnification using a DM1000 epifluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany); positive and negative reaction controls were used in each procedure. The number of podocytes in 30 randomly chosen fields of the slides were counted and the results were corrected based on the levels of urine creatinine found in each sample.

Lab workup

Creatinine and proteinuria levels were measured using a commercial kit and an Olympus AU 400 Analyzer (Olympus Mishima Co. Ltd., Shizuoka, Japan).

Hematuria and leukocyturia in urinary sediments were assessed based on the mean number of red and white blood cells in 10 fields.

Measurements of serum creatinine, 24-hour proteinuria, hematuria, and leukocyturia were made only for subjects in groups B, C, and D. Controls were not tested for these parameters.

Statistical analysis

The following inferential statistics analyses were used: Spearman's rank correlation coefficient, the Kruskal-Wallis test, and the Mann-Whitney U test. A level of significance of 5% was established for all tests.

Results

The specificity of primary antibodies (rabbit anti-podocin antibody, rabbit anti-nephrin antibody, and rabbit anti-synaptopodin antibody) was confirmed by indirect immunofluorescence, using histological sections of normal human kidney tissue. When tested on slides with podocytes, the best results were obtained with the anti-podocin antibody, as it produced higher reactivity and lower nonspecific staining (Figure 1).

Podocyturia measurements by rabbit anti-podocin, anti-nephrin, and anti-synaptopodin antibodies are shown in Table 1.

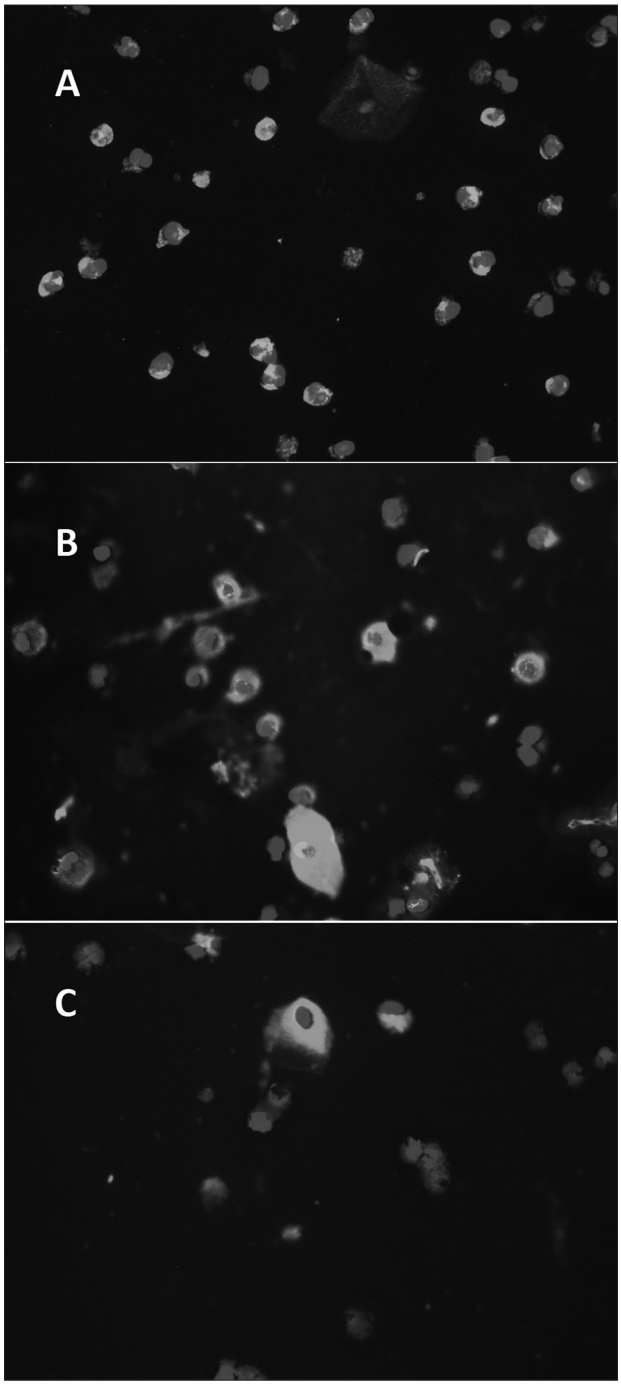
Lab test results are shown in Table 2. In the control group, only protein/creatinine ratios and podocyturia were measured (Table 2), since these subjects were included in the study for having negative results in the urine test strip.

The diagnosis of podocyturia based on the anti-podocin antibody was correlated with active disease according to clinical and workup parameters, contrary to what was observed with the other two antibodies, anti-nephrin, and anti-synaptopodin, whose results did not correlate with disease severity.

Inferential results revealed that scores of podocyturia with anti-podocin ($p < 0.001$) and anti-nephrin ($p = 0.047$) antibodies were different in groups A, B, C, and D. The same was not observed for levels of podocyturia with the anti-synaptopodin ($p = 0.107$) antibody.

Protein/creatinine ratios ($p < 0.001$) and 24-hour proteinuria levels ($p < 0.001$) were not similar between groups. The results of multiple comparisons between groups are shown in Figures 2 and 3.

Figure 1. Podocyturia testing through indirect immunofluorescence staining. A: Positive leveling for anti-podocin antibody; B: Anti-nephrin antibody; C: Anti-synaptopodin antibody; superimposition of images related to staining with DAPI (blue) and specific antibodies (green). The colors described refer to the original image stained by immunofluorescence. 400x magnification.



The possible correlations between variables were assessed using Spearman's rank correlation coefficient. Diagnoses of podocyturia with the anti-podocin and anti-synaptopodin antibodies

Table 1 Summary of levels of podocyturia based on anti-podocin, anti-nephrin, and anti-synaptopodin (podocytes/mg of creatinine) of individuals in groups A, B, C, and D

Antibody		Group A	Group B	Group C	Group D	Total
Podocin	N	29	17	29	10	85
	25th percentile	0.00	9.23	5.73	47.71	2.99
	50th percentile	3.78	17.47	22.59	109.13	16.28
	75th percentile	17.11	50.37	55.74	173.63	49.60
Nephrin	N	29	17	27	8	81
	25th percentile	0.00	0.00	0.00	0.00	0.00
	50th percentile	0.00	9.22	0.00	0.00	0.00
	75th percentile	2.98	17.47	8.02	9.63	8.96
Synaptopodin	N	29	17	28	9	83
	25th percentile	0.00	0.00	0.00	0.00	0.00
	50th percentile	0.00	0.00	0.00	2.82	0.00
	75th percentile	0.00	4.31	3.80	6.57	2.99

Table 2 Summary of levels of 24-hour proteinuria, serum creatinine, hematuria, leukocyturia, protein/creatinine ratio, podocyturia by anti-podocin, anti-nephrin, and anti-synaptopodin of individuals in groups A, B, C, and D

		Group A	Group B	Group C	Group D	Total
24-hour proteinuria (g/24h)	N	-	16	26	9	51
	25th percentile	-	< 0.05	0.58	3.09	0.25
	50th percentile	-	< 0.05	1.12	4.30	0.91
	75th percentile	-	0.26	1.89	5.73	2.56
Protein/creatinine ratio	N	29	17	29	10	85
	25th percentile	< 0.05	< 0.05	0.55	2.66	< 0.05
	50th percentile	< 0.05	< 0.05	1.03	3.78	0.17
	75th percentile	< 0.05	0.33	1.80	7.07	1.21
Serum creatinine (mg/dL)	N	-	17	29	10	56
	25th percentile	-	0.65	0.80	0.73	0.71
	50th percentile	-	0.71	1.07	0.95	0.93
	75th percentile	-	0.99	2.19	1.39	1.36
Hematuria (phpf)[a]	N	-	17	29	10	56
	25th percentile	-	5.00	5.00	26.25	5.00
	50th percentile	-	8.00	10.00	32.50	12.50
	75th percentile	-	20.00	35.00	100.00	35.00
Leukocyturia (phpf)[a]	N	-	17	29	10	56
	25th percentile	-	5.00	7.00	14.00	5.00
	50th percentile	-	5.00	12.00	20.00	11.00
	75th percentile	-	12.00	15.00	68.75	20.00

[a] Results expressed in cells per high-power field (phpf).

were positively and statistically correlated with the protein/creatinine ratio (r_s = 0.367, p = 0.001; and r_s = 0.272, p = 0.013, respectively); diagnoses using the anti-nephrin and anti-synaptopodin antibodies were correlated with each other (r_s = 0.317, p = 0.001); diagnosis of podocyturia using the anti-nephrin antibody was negatively and statistically correlated with 24-hour proteinuria (r_s = -0.334, p = 0.022). The other possible correlations were not statistically significant.

Figure 2. Levels of podocyturia (podocytes/mg of creatinine) of individuals in groups A, B, C, and D. A: Anti-podocin; B: Anti-nephrin.

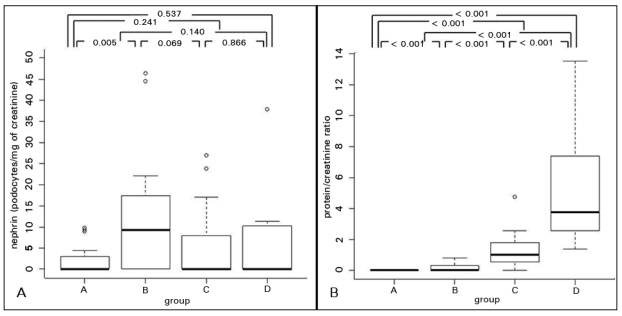
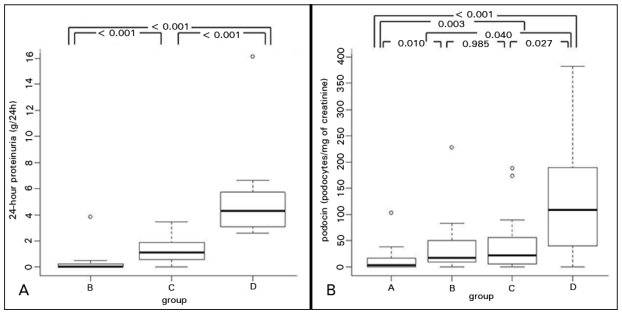


Figure 3. Levels of proteinuria. A: Urine protein/creatinine ratios of individuals in groups A, B, C, and D; B: 24-hour proteinuria levels (g/24h) of individuals in groups B, C, and D.



Discussion

Studies have described the presence of viable podocytes in the urine of patients with various glomerular diseases and proteinuria. Unlike proteinuria - present in active disease and in the chronic stages of glomerular injury - podocyturia seems to be present only in cases of active disease.[6,10] Podocyturia is not an indicator for proteinuria,[10] and could thus serve as a sensitive early marker of active glomerular injury and an indicator of great value in the definition of therapy.[1] Indeed, the levels of podocyturia in healthy individuals and patients with inactive disease has been shown to be significantly lower than the levels seen in patients with active glomerular disease.[11]

In our study, the levels of leukocyturia, hematuria, serum creatinine, protein/creatinine ratio, and 24-hour proteinuria grew according to the degree of clinical activity, as also described by Solorzano *et al.*[9] It was evident that the counts of podocin-positive and synaptopodin-positive cells in urine were significantly correlated with protein/creatinine ratios. In fact, a previous study carried out at our center[9] found the protein/creatinine ratios of isolated urine samples were a good indicator of renal activity. This finding is of great importance, since this is a fast, easy-to-perform, low cost and well-established test in clinical practice. Solorzano *et al.*[12] observed that the protein/creatinine ratio can also be used to monitor the degree of renal involvement in LN, as also found in our study.

The correlation of podocyturia with disease activity in patients with lupus nephritis suggested the anti-podocin antibody was the most suitable biomarker when compared with anti-nephrin and anti-synaptopodin. Garovic *et al.*[13] reported similar results in the assessment of a group of pregnant women with preeclampsia. The authors analyzed the renal tissue of this group of patients and found reduced glomerular expression of nephrin and synaptopodin, while both the control group and the group with preeclampsia had strong labeling for podocin. According to these authors, the podocytes excreted in urine could have lower expression of nephrin and synaptopodin than podocin, thus making the latter a more sensitive marker for the presence of podocytes in urine.[14]

A similar finding was observed in a study on diabetic nephropathy, wherein the counts of podocyte-related molecules was correlated with severity of albuminuria.[15]

Gene expression of podocyte proteins (nephrin, podocin, synaptopodin, podocalyxin, among others) in renal tissue is reduced in different glomerulopathies including lupus nephritis. Increased urinary excretion of these markers has been observed due to detachment of podocytes from the glomerular basement membrane and/or apoptosis.[16] Experimental studies have also described correlations between expression of nephrin and podocin and histological grade of lupus nephritis.[17]

Nakamura *et al.*[6] reported podocyturia in a group of LN patients through immunofluorescence with the anti-podocalyxin antibody. Podocytes were not found in the urine of normal control subjects and patients without signs of systemic or

renal disease. However, all patients with clinically active LN had podocytes in their urine samples. The authors concluded that podocyturia diagnosed with the anti-podocalyxin antibody could serve as an indicator of active LN.

Vogelmann *et al.*[3] studied patients with focal segmental glomerulosclerosis and LN and found that less than 1% of the nucleated cells from urine sediments stained with DAPI were podocalyxin-positive and that labeling for anti-WT1 was negative in all cases. Approximately 30% to 40% of the podocalyxin-positive samples were labeled with synaptopodin, GLEPP1, or podocin. These reports and our study elicit the difficulties inherent to defining the most adequate markers for podocyturia using immunofluorescence staining.

Podocyturia labeled by the anti-podocin antibody was increased in patients with active LN. Podocin-positive cells were significantly correlated with disease severity, and cell counts were higher in patients with severe disease when compared to subjects with mild disease.

Patients without active disease had levels of podocyturia similar to the group with mild disease, and higher levels of podocyturia than controls, indicating that podocyturia may occur secondarily to early glomerular injury.

Podocytes were also seen in the urine of healthy individuals in the control group in all three markers used, but counts were lower than in patients with LN. Facca *et al.*[18] studied women with preeclampsia and found podocytes in the urine of pregnant women without preeclampsia in the control group.

To sum up with, this study showed that podocyturia testing by indirect immunofluorescence may be useful in monitoring patients with LN. The anti-podocin antibody was the most suitable biomarker when compared to the anti-nephrin and anti-synaptopodin antibodies. The preliminary nature of our findings calls for more comprehensive investigations and clearer definitions for the clinical significance of podocyturia in various glomerulopathies. Methods with higher sensitivity to detect podocyturia are still needed.

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