Original Article

A Novel Method for the Estimation of Podocyte Injury: Podocalyxin-Positive Elements in Urine

(glomerulus / glomerulonephritis / podocyte / podocalyxin / FACS)

P. HABARA[1,2,3], H. MAREČKOVÁ[2,3], Z. SOPKOVÁ[1], K. MALÍČKOVÁ[3], D. ŽIVOROVÁ[3], T. ZIMA[3], V. TESAŘ[1]

[1]Charles University in Prague, First Faculty of Medicine, Department of Nephrology, Prague, Czech Republic
[2]Charles University in Prague, First Faculty of Medicine, Institute of Immunology and Microbiology, Prague, Czech Republic
[3]Charles University in Prague, First Faculty of Medicine, Institute of Clinical Biochemistry and Laboratory Medicine, Prague, Czech Republic

Abstract. Podocytes form an outer aspect of the glomerular capillary wall and play a decisive role in its permeability for macromolecules. The main podocyte surface antigen podocalyxin, a highly electronegative sialoglycoprotein, prevents the podocyte foot processes from collapsing. Podocyte damage in glomerular disease is supposed to be accompanied by podocyte detachment, and shed podocytes and their fragments (marked by podocalyxin) may be identified in the urine. Using anti-podocalyxin monoclonal antibody, PCX+EL were counted by FACS in 38 patients with various types of active glomerulonephritis, 15 patients with chronic glomerulonephritis in long-term remission and 44 healthy controls. Urinary levels of PCX+EL were significantly higher in patients with active glomerulonephritis compared to patients with chronic glomerulonephritis in long-term remission (93 ± 100 vs. 6.3 ± 3.2/µl of urine, P < 0.000001) and healthy controls (4.4 ± 2.6/µl of urine, P < 0.000001 compared to active glomerulonephritis, n.s. compared to chronic glomerulonephritis in long-term remission). These preliminary data suggest the potential of this simple method to monitor the activity of glomerular disease. Further prospective studies of larger cohorts of patients with individual glomerular diseases are clearly warranted.

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Corresponding author: Peter Habara, Charles University in Prague, First Faculty of Medicine, Institute of Clinical Biochemistry and Laboratory Medicine, Laboratory of Clinical Immunology and Allergology (KIA), U nemocnice 2, 128 00 Prague 2, Czech Republic. Phone: + 420 224 962 574; e-mail: peter.habara@gmail.com; peter.habara@lf1.cuni.cz

Abbreviations: AAV – ANCA-associated vasculitis, ANCA – anti-neutrophil cytoplasmic antibody, anti-GBM – anti-glomerular basement membrane, CL – clearence, EryU – erythrocyturia, FACS – fluorescence-activated cell sorter, flow cytometer, FSGS – focal segmental glomerulosclerosis, GN – glomerulonephritis, HSPN – Henoch-Schoenlein purpura nephritis, IgA – immunoglobulin A, IgAN – IgA nephropathy, MCD – mimimal change disease, MPGN – membranoproliferative glomerulonephritis, LN – lupus nephritis, n.s. – not significant, PBS – phosphate-buffered saline, PCX+EL – podocalyxin-positive element, PRU – proteinuria, S-Krea – creatinaemia, SLE – systemic lupus erythematosus.

Introduction

Glomerular capillary wall has a unique structure consisting of the fenestrated capillary endothelium, glomerular basement membrane and slit diaphragm between the podocyte foot processes. Podocytes, epithelial cells connected to the glomerular basement membrane through their foot processes, express highly electronegative sialoglycoproteins (particularly podocalyxin) on their apical surface. Podocalyxin expression is relatively specific for the podocyte; parietal cells of Bowman capsule do not express podocalyxin on their surface.

Podocytes are believed to be the primary target of glomerular damage in so-called podocytopathies (minimimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy), but they may also be damaged in the glomerular disease affecting primarily the mesangial (e.g. IgA nephropathy (IgAN)), or glomerular endothelial (lupus nephritis (LN)) cells (Barrisoni et al., 2007).

Podocyte damage in glomerular disease is supposed to be accompanied by podocyte detachment, and shed podocytes and their fragments (marked by podocalyxin) may be identified in the urine. Urinary excretion of podocytes and/or their fragments could reflect the severity of podocyte damage and could be a useful tool in monitoring the activity of glomerular disease.

In this pilot cross-sectional study we evaluated urinary levels of podocalyxin-positive elements (PCX+EL)

in various chronic glomerulonephritides (GN) (both active and in long-term remission) and compared them to healthy controls.

Material and Methods

We examined 53 patients with biopsy-proved chronic glomerulonephritis and 44 healthy controls (mean age 47 years). Patients with chronic glomerulonephritis were subdivided into three groups:

1) 20 patients with proliferative forms of glomerulonephritis (ANCA-associated vasculitis (AAV), anti-GBM and Henoch-Schoenlein purpura nephritis (HSPN), diffuse proliferative changes in LN and membranoproliferative glomerulonephritis (MPGN), mean age 46, AAV – 7, LN class IV – 8, HSPN – 2, MPGN – 1, anti-GBM nephritis – 1, IgAN – 1),
2) 18 patients with non-proliferative forms of glomerulonephritis (FSGS, LN class V, MCD), mean age 55, FSGS – 13, LN class V – 2, and MCD – 3),
3) 15 patients in long-term remission of various chronic glomerulonephritides (mean age 48, FSGS – 2, LN, all class V – 5, AAV – 5, IgAN – 3). Patients in long-term remission had proteinuria < 0.5 g/day, no erythrocyturia, stable serum creatinine level; all patients were treated by maintenance immunosuppressive therapy).

Diagnoses were established in all patients by renal biopsy before start of the immunosuppressive therapy. There was no significant difference in age between the different groups of patiens. Patients with different glomerulonephritides were treated by defined immunosuppressive protocols based on the available evidence.

Urine samples obtained from the first morning urine were immediately stabilized with preservation solution (ca 25 ml urine + 20 ml of 1 part PBS : 1 part CellFix, Becton Dickinson, Oxford, UK). Preservation solution permits reliable investigation in the laboratory within three days compared to only one hour in fresh urine (Kouri et al., 2002). In the laboratory, 10 ml of urine were centrifuged 5 min at 800 *g*, then washed three times with 6 ml of physiological solution and centrifuged another 5 min at 800 *g* once again. The sediment was then (after washing and resuspending) divided into two tubes. Monoclonal antibody against podocalyxin conjugated with phycoerythrin (R&D systems, Minneapolis, MN) was added to the first tube; the second served as a native control. After 45 min of incubation at 4°C, washing three times with 2 ml of physiological solution and centrifugation 5 min at 800 *g* followed. The whole volume from tubes was transferred into Becton Dickinson TruCount tubes for absolute counting afterwards. The formula for absolute couting was as follows: Absolute concentration in µl of urine = [Elements in PCX gate]/[TruCount Beads gate]x[TruCount beads in test tube]/2000 µl. The result was then corrected by input volume according to the formula: [Input volume(the volume in tube with preservative 30-50 ml)]ml-20 ml(Volume of preservation solution)/[Input volume]ml.

Measurement was performed in a flow cytometer in the program BD FACS Diva. We counted the absolute number of elements with phycoerythrin fluorescence. First, we counted the absolute number of elements in the native control tube, then in an experimental tube. The definitive result was the difference between the experimental and the native tube expressed as the number of

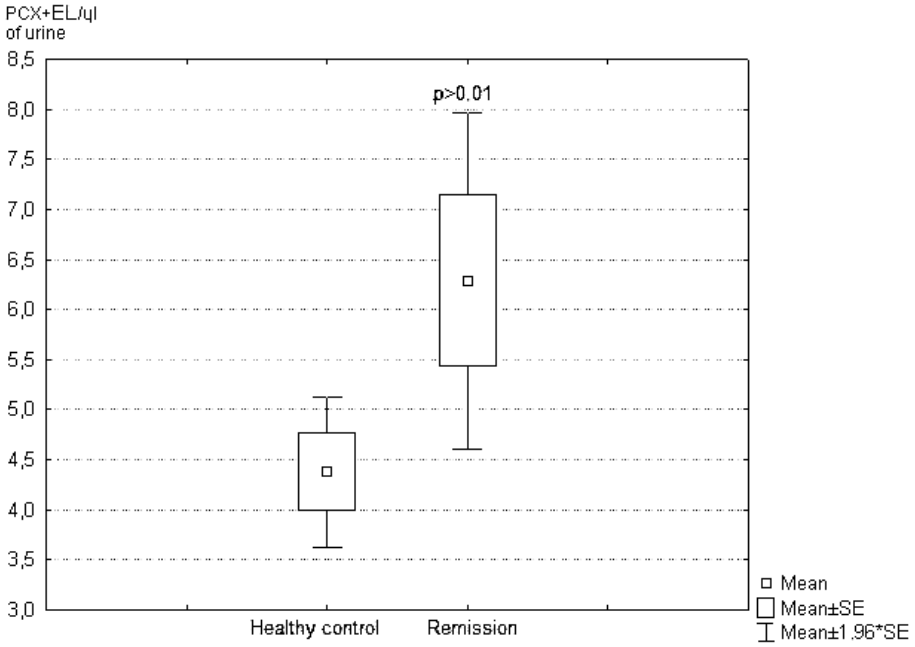

Fig. 1. Analysis of PCX+EL detected by FACS in urine of healthy controls and patients in long-term remission. Values are expressed as the number of PCX+EL in 1 µl of urine. The results show that although the number of PCX+EL in urine of patients in remission is slightly higher than the numbers observed in controls, the difference is not statistically significant ($P > 0.01$).

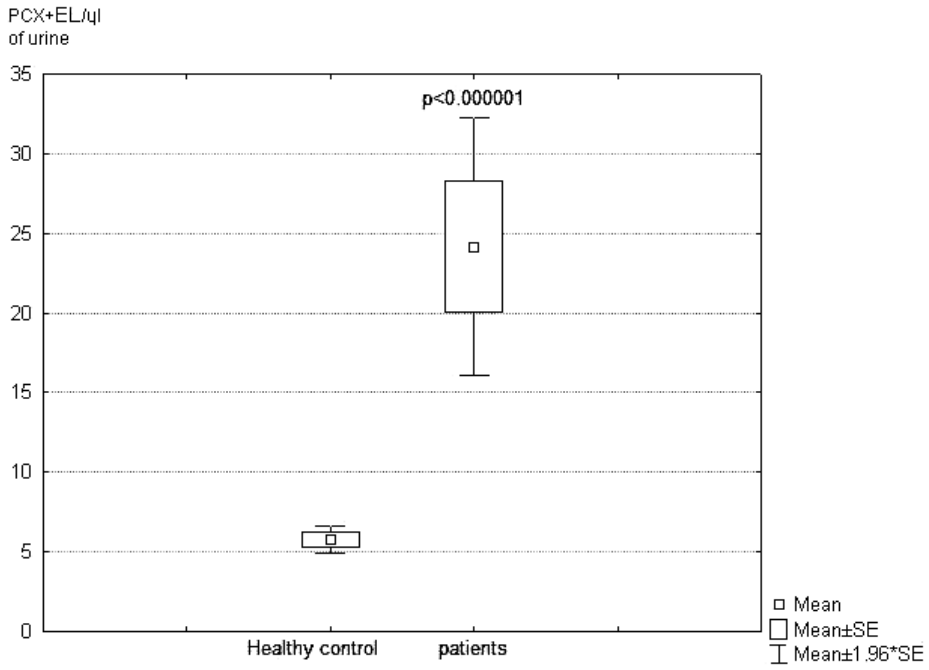

Fig. 2. Comparison of numbers of PCX+EL detected by FACS in urine of healthy controls and patients suffering the non-proliferative forms of GN. Results (expressed as the number of PCX+EL in 1 µl of urine) show elevated numbers of PCX+EL in urine of patients with active non-proliferative GN ($P < 0.00001$).

PCX+EL in a microlitre of urine. Counting in the native tube was necessary because urine displays very variable autofluorescence. Due to the non-specific binding of the monoclonal antibody (probably caused by the high level of albumin in patients' urine) we had to dilute the monoclonal antibody and the specimen by washing them more than once. As a cut-off point we established 10 PCX+EL/µl urine. The results were processed using the program Statitistica (Statsoft, Inc., ver. 7.1, Prague, Czech Republic). We tested the data by *t*-test independent of variables, determined the P and displayed them in Box-whisker plot. Our experiments were approved by the Ethical Committee and an informed consent was obtained from all patiens.

Results

The numbers of PCX+EL in urine of healthy controls and patients in remission were statistically unchanged (P = n.s. – Fig. 1).

We divided the active glomerulonephritides into two groups similarly as in Hara et al. (2005). In the first group with the non-proliferative forms, the glomerular damage was moderate, and in the second group with proliferative forms, the damage was heavier.

Patients with non-proliferative forms of glomerulonephritis had significantly higher counts of PCX+EL compared to healthy controls and patients in remission (Fig. 2); and patients with proliferative forms of glomerulonephritis had even higher urinary PCX+EL concentrations (Fig. 3). We didn't find any correlation between the number of PCX+EL and proteinuria, erythrocyturia and renal function (glomerular filtration rate (GFR) and serum creatinine). The biochemical data related to individual patients are listed in Tables Nos. 1–3. When the cut-off point was 10 PCX+EL/µl of urine, the sensitivity was 90 % and specificity 100 %. The difference between the group of patients in long-term remission (healthy controls and patients in remission) and both active non-proliferative forms of GN and active proliferative forms of GN was significant at $P < 0.001$.

The method thus seemed to discern the active and inactive glomerular lesions with a reliable sensitivity and specificity. The higher counts may reflect more severe damage to the glomerulus.

Discussion

Podocyte is a visceral epithelial cell of Bowman capsule (Mundel et al., 2002) of mesenchymal origin (Bariety et al., 2006). The main surface antigen of the podocyte is podocalyxin (Hara et al., 1995). Podocalyxin is a highly electronegative sialoglycoprotein, which maintains the stability of the podocyte foot processes and thus contributes to the preservation of normal permeability of the glomerular capillary wall (Mundel et al., 2002). Podocalyxin is also expressed on thrombocytes, high endothelial cells, and invasive carcinomas or malignant blood cells (Somasiri et al., 2004). Enhanced expression of podocalyxin on the podocyte surface is also accompanied by the damage to the basal surface of podocyte (Economou et al., 2004). Podocyte injury in various GN results in the shedding of podocytes and their fragments into the urine (Hara

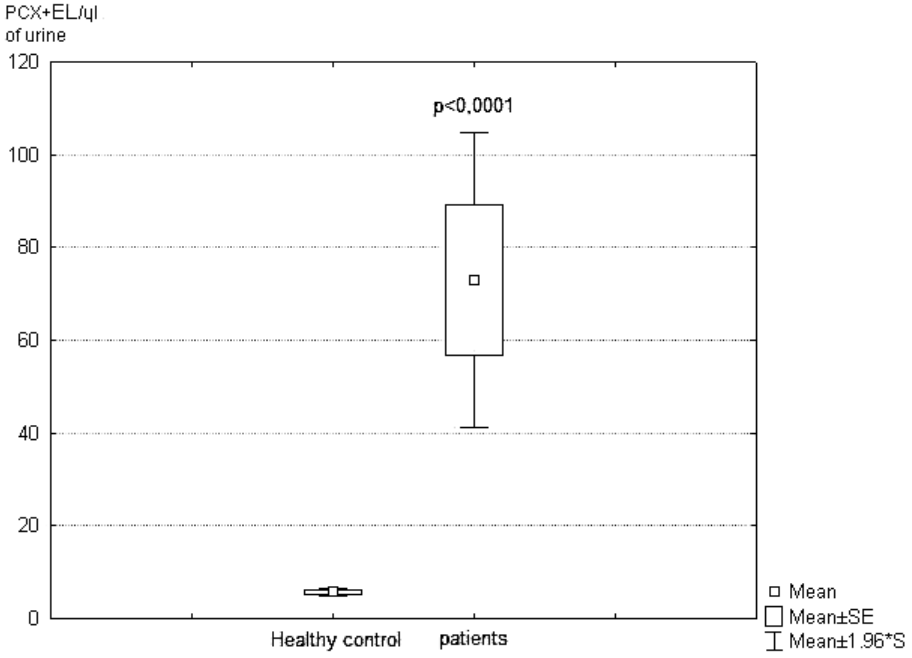

Fig.3. Comparison of numbers of PCX+EL detected by FACS in urine of healthy controls and patients with proliferative forms of GN. Results (expressed as the number of PCX+EL in 1 µl of urine) show elevated numbers of PCX+EL in urine of patients with active proliferative GN ($P < 0.0001$). The results document high numbers of PCX+EL with a relatively wide range of recorded values.

Table 1. Clinical and biochemical data of patients in long-term remission

Diagnosis	Age	Sex	PCX+EL/µl	S-Krea	CL(ml/s)	PRU(g/day)	EryU /ml
FSGS	56	F	4.95	60	2	0	0
AAV	70	F	10.78	180	0.5	0	0
AAV	51	M	8.32	600	0.3	0.39	0
SLE IV	42	M	5.15	100	1.6	0	0
AAV	42	F	0	60	2	0	0
AAV	70	F	6.85	60	2	0	0
MGN	66	M	9.38	80	1.22	3	0
FSGS	30	M	6.83	72	2	0.25	0
SLE IV	31	F	8.99	90	1.2	0.46	41
AAV	51	M	0.72	600	0.4	0.36	0
SLE IV	36	F	9.66	130	1	0.2	40
IgAN	29	M	7.32	330	0.4	0.2	12
IgAN	39	M	4.3	80	2	0.26	0
AAV	64	F	4.81	110	1.6	0.19	0
FSGS	47	M	3.74	109	1.4	0	0

et al., 1995, 2001, 2005; Kanno et al., 2003; Petermann et al., 2007).

Evaluation of podocytes in the urine was used for the first time as a marker of glomerular damage by Hara (Hara et al., 1995, 2001, 2005) and then followed in Germany by Petermann (Yu et al., 2005; Petermann et al., 2007). Podocytes were marked by anti-podocalyxin monoclonal antibody and counted using fluorescence microscope; total podocalyxin concentration was determined by ELISA and Western blot. Compared to controls, patients with nephrotic syndrome had only slightly increased levels of podocalyxin in the urinary sediment. On the other hand, patients with proliferative glomerulonephritis (IgAN, HSP, LN) had very high levels of podocalyxin in the urinary sediment and also increased urinary excretion of podocytes (Hara et al., 2005).

The disadvantages of the methods described in these reports were partly represented by a necessity of rather experienced laboratory assistants and particularly a considerably rare appearance of podocytes in urine (1–6/ml), even in patients with active glomerulonephritis. In case of determination of total podocalyxin in the urine, the major disadvantage was the rather complicated preparation of the sample. We have chosen the golden mean by evaluating all podocalyxin-binding particles using a fluorescence-activated cell sorter (FACS).

Table 2. Clinical and biochemical data of patients with non-proliferative forms of GN

Diagnosis	Age	Sex	PCX+EL/µl	S-Krea	CL(ml/s)	PRU(g/day)	EryU /ml
FSGS	63	M	20.01	200	0.52	6	0
SLE V	63	F	25.56	100	1.16	10	0
MCD	36	M	11.46	90	1.47	2	50
FSGS	49	F	11.88	130	0.90	4	1
FSGS	75	M	33.21	160	0.65	3.5	4
FSGS	63	F	21.09	200	0.52	6	200
FSGS	54	F	23.14	230	0.46	2.5	25
FSGS	44	F	53.29	190	0.60	8.3	30
FSGS	51	M	41.88	150	0.76	3.6	120
MCD	75	M	12.23	107	1.04	8	11
MCD	53	F	11.88	250	0.42	4.5	55
FSGS	49	F	21.24	115	1.04	5.3	12
FSGS	51	M	19.02	150	0.76	3.7	0
FSGS	39	M	22.33	99	1.29	4.5	3
FSGS	70	F	15.43	70	1.72	3.1	15
FSGS	63	F	31.24	220	0.47	10.3	17
FSGS	55	M	27.51	103	1.15	9.3	19
SLE V	29	F	20.17	80	1.76	15.3	0

Table 3. Clinical and biochemical data of patients with proliferative forms of GN

Diagnosis	Age	Sex	PCX+EL/µl	S-Krea	CL(ml/s)	PRU(g/day)	EryU /ml
SLE IV	26	F	44.72	230	0.53	10	12
SLE IV	26	F	92.34	260	0.46	12	30
AAV	69	F	22.94	440	0.21	9.5	200
SLE IV	31	F	79.48	330	0.34	12	50
AAV	69	F	25.86	510	0.17	0.5	60
SLE IV	36	F	56.43	290	0.38	5.5	44
AAV	59	M	25.81	350	0.28	9	500
AAV	58	F	190.73	220	0.48	2.5	250
AAV	61	M	42.49	390	0.24	4.5	100
HSPN	32	F	147.12	160	0.77	7.3	360
AAV	69	F	74.45	190	0.54	6.4	222
SLE IV	23	F	264.69	110	1.28	5.9	80
IgAN	76	F	84.88	200	0.50	7	600
HSPN	32	F	270.00	199	0.60	7.9	500
AGBM	58	F	192.56	600	0.15	12.3	432
AAV	74	M	457.81	500	0.18	3.9	222
MPGN	60	M	32.27	309	0.32	4.6	193
SLE IV	20	F	52.59	600	0.19	1.5	30
SLE IV	19	F	37.50	80	1.92	0.38	494
SLE IV	31	F	81.45	100	1.34	5.5	27

Scientists in many other laboratories (Petermann et al. in Germany, or Lemley et al., 2007, in the USA) also tried to use the FACS method, but they had problems with the intepretation of their results (Petermann et al., 2007). FACS gives only the proportional representation of single elements; thus, the absolute number depends on actual urine composition, which is very variable. We tried to solve this problem by using the TruCount method (conversion of the number of elements to calibrated elements in the test tube) providing the result as the absolute number of elements in a microlitre of urine.

The next difficulty with FACS was a rather high variability of fluorescence background which interfered with the monoclonal antibody fluorochrome signal. The key to this problem was evaluation of the absolute number of elements before and after addition of the anti-podocalyxin monoclonal antibody, which gave us the possibility to subtract the background. With these simple and smart modifications the FACS method became really quick and not demanding in preparation and performance.

We divided the patients with active glomerulonephritis into two groups as described above. Our results are similar to the finding by M. Hara (Hara et al., 2005), who also found lower counts of podocytes or podocalyxin in patients with FSGS, MCD (podocytopathies – Barisoni et al, 2007) than in patients with LN, HSPN, IgAN (i.e. more active glomerulonephritis with crescents). Urinary PCX+EL excretion was not increased in our patients in long-term remission compared to healthy

controls. The significant difference in the urinary levels of PCX+EL between the patients with active disease and patients in long-term remission suggests that this method could be useful in monitoring the activity of glomerular disease. The very low counts of PCX+EL we found in the urine of patients with membranous nephropathy are perhaps caused by only sublethal podocyte damage caused by C5b-9 (Shankland et al., 1999). Based on the available data there is no podocytopoenia in membranous nephropathy, so no PCX+EL are expected to appear in the urine (Hara et al., 2005).

We didn't find any correlation between PCX+EL and proteinuria, erythrocyturia and renal function. Whether the PCX+EL urinary levels correlate with the histologic changes in the renal biopsy should be a matter of further investigation and could not be assessed in this study (PCX+EL examination in urine was not performed immediately before renal biopsy, there were different time intervals between renal biopsy and PCX+EL examination).

Some patients in remission with type IV LN had proteinuria, but no immunological activity (as assessed by the titres of antinucleosomal and anti-dsDNA antibodies). In five patients with active type IV LN, PCX+EL decreased to less than 10/µl in parallel with the evolution of clinical and immunological remission. Laboratory findings in five patients with hypertensive nephropathy (also verified by renal biopsy) with increased serum creatinine level, proteinuria and erythrocyturia, but low count of PCX+EL (less than 10/µl) are also in keeping with this view.

The results of our pilot study presented here are only preliminary due to the limited and heterogenic sample of patiens. Hopefully, using strictly the first voided morning urine miminized the effect of somewhat different concentration of the urine on our results. To obtain more conclusive data, in our next studies we should correct the PCX+EL levels to the urinary creatinine concentration. We also should expand the number of examined patients and study the PCX+EL in individual patients repeatedly during follow-up. If our preliminary data are confirmed in larger prospective studies, this simple method should represent a more useful tool in monitoring the activity of the disease than proteinuria and erythrocyturia.

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