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*Article*

# Urine Nephrin and Podocalyxin Reflecting Podocyte Damage and Severity of Kidney Disease in Various Glomerulonephritis. A Cross-Sectional Study

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**Abstract: Introduction:** Glomerulopathy is a term used to describe a broad spectrum of renal diseases, characterized by dysfunction of glomerular filtration barrier, especially of podocytes. Several podocyte-associated proteins have been found and proved their usefulness as urine markers of podocyte dysfunction. Two of them are nephrin (NEP) and prodocalyxin (PDC). **Purpose:** This study aims to evaluate the association of podocyte damage, as it is demonstrated via the concentrations of urinary proteins, with clinical and histological data from patients with several types of glomerulonephritis. **Methods:** We measured urine levels of two podocyte-specific markers, NEP and PDC (corrected for urine creatinine levels), in patients with a wide range of glomerulopathies. Serum and urine parameters as well as histological parameters from renal biopsy were recorded. **Results:** In total, data from 37 patients with glomerulonephritis and 5 healthy controls were analyzed. PDC and NEP concentrations correlated between them and with serum creatinine levels (p=0.001 and p=0.013 respectively), and with histological lesions associated with chronicity index of renal cortex, such as severe interstitial fibrosis, severe tubular atrophy and hyalinosis (for PDC/NEP, all p<0.05). In addition, the PDC and NEP demonstrated statistically significant correlations with interstitial inflammation, (p=0.018/p=0.028). Regarding electron microscopy evaluation, PDC levels correlated with distinct characteristics, such as fibrils and global podocyte foot processes fusion, while the NEP/CR ratio was uniquely significantly associated with podocyte fusion only in non-immune-complex mediated glomerulonephritis (p=0.02). Among the other clinical and histological parameters included in our study, a strong correlation of proteinuria>3gr/24h with diffuse fusion of podocyte foot processes (p=0.016) was identified. **Conclusions:** Podocalyxin and nephrin concentrations in urine represent markers of podocyte dysfunction and in our study they were associated both with serum creatinine and histological chronicity indices.

**Keywords:** Podocalyxin; nephrin; glomerulonephritis; biomarkers

## 1. Introduction

Glomerulopathies represent a quite heterogeneous group of renal diseases, which affect approximately 10-15 per 10, 000 of adults and they are associated with significant morbidity and

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mortality [1]. Even though they may be triggered by different stimuli, and they may be associated with various pathophysiological mechanisms, they all seem to share as a common pathogenetic feature the impairment of the glomerular filtration barrier (GFB) [1] .More precisely, podocytes represent the key structural and functional component of the GFB and their ability to perform their crucial functions depends on the integrity of their postmitotic phenotype and especially of their foot processes[2] . Podocytes' foot processes along with the glomerular basement membrane (GBM) and the endothelial cells of the glomerular capillaries create a slit diaphragm, which allows selective permeability to different molecules, based on a combination of their size and ion charge[2]. This complex role of the podocytes depends on the coordinated functions of multiple different proteins, which regulate their interaction with the other components of the slit diaphragm [2]. Moreover, these complex intercellular interactions enable podocytes to adapt to conditions of excessive stress that may arise, due to a wide range of stimuli, such as hemodynamic, toxic or immune mediated factors. In such cases, podocyte injury leads to detachment of foot processes from the GBM and subsequently to various clinical manifestations[2].

Two podocyte proteins, with well-established roles in the maintenance of the GFB homeostasis, are nephrin (NEP) and podocalyxin (PDC). The first one, NEP, is a cellular adhesion protein, localized at the basal area and in intercellular regions, where it controls interactions between adjacent podocytes[3]. It regulates signal transduction that modulates the cytoskeleton structure, which under physiological circumstances maintains cell polarity, while in stress-conditions facilitates the maintenance of their integrity [3]. On the other hand, PDC is a negatively charged transmembrane protein, which helps maintain the selectivity of the slit diaphragm to charged molecules [3].

The purpose of this study was to determine the association of the urine levels of these two proteins, NEP and PDC, with several parameters, both clinical and histological, and to assess their potential use as biomarkers for the severity of kidney disease in patients with a wide spectrum of glomerular diseases.

## 2. Patients and Methods

### *2.1. Study Participants*

This is a prospective cross-sectional study in adult patients with glomerulonephritis (GN), who were submitted to a kidney biopsy, based on clinical indication, at the Nephrology Department of the Hippokration General Hospital of Athens, in one year period. Exclusion criteria were active malignancy, active infection and non-compliance of the patient. Five subjects were defined as healthy controls, as they had no hypertension, no diabetes mellitus, eGFR> 90ml/min/1.73m$^2$, no active urine sediment or proteinuria and they were not submitted to kidney biopsy, only serum and urine measurements were made.

### *2.2. Clinical and Histological Data*

Demographic data were collected for each patient, as well as blood samples to determine serum creatinine (CR) levels (reference range in our laboratory 0.57-1.2 mg/dL). Moreover, urine samples of the participants were used to determine spot urine CR levels (reference range in our laboratory 20-320 mg/dl) and proteinuria measured in a 24hour urine collection. Furthermore, we collected the first-morning urine sample from all the participants prior to the kidney biopsy and measured Podocalyxin and Nephrin using ELISA method. The following ELISA kits (Exocell Inc.,Philadelphia, PA) were used: **1.** E-EL-H1901, Human NPHN (for Nephrin) and 2. E-EL-H2360, Human PCX (for Podocalyxin). Dilutions of 1:1 and 1:2 of the urine were used for the measurement of urinary nephrin and podocalyxin, respectively. The values were expressed as ng/ml. We calculated urine protein concentrations by the Brad-ford method [4] . Urine creatinine was measured by the Jaffe reaction on the same aliquot of urine to calculate the ratio of urinary podocalyxin to creatinine (PED/CR), urinary nephrin to creatinine (NEP/CR)[5].

Renal biopsy was performed by ultrasound guidance, with an automated Pro Magnum (Bard, Covington, GA, USA) biopsy system using a 16-G needle. The biopsy samples were examined with light microscopy, immunofluorescence and electron microscopy by the same pathologist in the 1st Department of Pathology, National and Kapodistrian University of Athens. The histopathological diagnosis included assessment of the total number of glomeruli, percentage of totally sclerosed

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glomeruli, segmental glomerulosclerosis, as well as Periodic acid-Schiff, Hematoxylin and eosin, Masson, Silver, Congo red staining and for DNAJB9 &C4d. The percentage of interstitial fibrosis and tubular atrophy were quantified in the renal cortex, based on Banff term as follows: absent (≤5), mild (6-25%), moderate (26-50%), severe (>50%) and absent (0%), mild (≤25%), moderate (26-50%), severe (>50%), respectively[27]

Immune-complex mediated GN are defined as IgA nephropathy, IgG4 related GN, membranous nephropathy, lupus nephritis [28].

### *2.3. Statistical Analysis*

Categorical variables were described by absolute and relative frequencies based on non-missing data, while the description of continuous was based on mean and standard deviation. The association of PDC, NEP, PDC/CR, NEP/CR, all of which are continuous variables, was evaluated based on the Pearson's r. Comparisons of PDC, NEP, PDC/CR, NEP/CR across several groups of interest were conducted by t-test. Multivariate linear regression was employed to adjust for potential confounders using PDC, NEP, PDC/CR, NEP/CR as dependent variables and as independent all variables presenting p-value < 0.20 at the univariate analysis. Entire statistical analysis was carried out using STATA 15.1; all tests were 2-sided, and the level of statistical significance was set to $\alpha = 0.05$.

## 3. Results

Data from 37 participants with GN and 5 healthy controls were included in the analysis and their characteristics are presented in Table 1. The histological diagnoses from the renal biopsies performed are summarized in Table 2. There was a significant correlation between PDC and NEP concentration in urine (r=0.55, p<0.01), as well as for the ratios between PDC/CR and NEP/CR (r=0.71, p<0.01). In addition, urine PDC and NEP concentrations demonstrated significant correlation with serum CR levels (r=0.56, p=0.001 and r=0.39, p=0.013, respectively) and a significant correlation with serum CR levels was also found for PDC/CR (r=-0.41, p=0.009) and NEP/CR (r=-0.42, p=0.030). We also found that PDC/CR was significantly lower in non-diabetics (coef ±SE [95% CI], -1.539 ± 0.711 [-2.983 to -0.095], p=0.037) and NEP/CR levels were moderately lower in normotensive patients (coef ±SE [95% CI], -0.59± 0.314 [-1.228 to 0.048], p<0.069). No statistically significant differences were found between males and females concerning the PDC/CR and NEP/CR concentrations.

**Table 1.** Characteristics of the Study Participants.

| Variable | Healthy controls N=5 | Mean±SD N=37 |
|---|---|---|
| Age, y | 39±5 | 58 ±15 |
| Females, % | 3 (60) | 13 (35) |
| Positive urine sediment, % | 0 | 21 (56) |
| Nephrotic range proteinuria, % | 0 | 17 (45) |
| Hypertension, % | 0 | 20 (54) |
| Diabetes mellitus, % | 0 | 10 (27) |
| Serum creatinine, mg/dl | 0.8±0.3 | 2.2±1.8 |
| Proteinuria, gr/24h | 0 | 4.1±3.4 |
| Nephrin, ng/ml | 25.3±1.3 | 45.4±31.7 |
| Podocalyxin, ng/ml | 30.8±6.2 | 70.9±63 |
| NEP/CR , ng/mg | 0.22±0.2 | 1.05±0.99 |
| PDC/CR, ng/mg | 0.33±0.4 | 1.84±2 |

**Table 2.** The diagnoses of the patients according to kidney biopsy (n=37).

| Diagnosis | N |
|---|---|
| FSGS (primary/secondary/ tip lesion) | 4/8/1 |
| Alport syndrome | 1 |
| IgA nephropathy | 5 |
| IgG4 related GN | 2 |

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| | |
|---|---|
| MCD | 1 |
| MIDD | 1 |
| Hypertensive nephropathy | 2 |
| Pauci-immune GN | 3 |
| Diabetic nephropathy | 1 |
| Membranous GN | 2 |
| SLE | 2 |
| TMA | 3 |
| Crescentic GN | 1 |

FSGS: Focal segmental glomerulosclerosis, GN; Glomerulonephritis, SLE; Systemic lupus erythematosus, TMA; Thrombotic microangiopathy.

### *3.1. Correlations of Urine Nephrin and Podocalyxin with Histological Parameters*

#### 3.1.1. Light Microscopy

PDC was positively correlated to tubular atrophy (p=0.075), severe fibrosis (p= 0.001) and GBM thickening (p=0.017). More precisely, the patients with tubular atrophy>50% presented higher levels of PDC with a mean value of 73.649 ng/ml (p=0.016), compared to patients with atrophy <50%. The ratio of PDC/CR not only demonstrated significant correlation with chronicity parameters such as severe tubular atrophy, arterial hyalinosis and interstitial fibrosis, but also interstitial inflammation (Table 3).

**Table 3.** Significant correlations of urine Nephrin (a) and Podocalyxin (b) (corrected for urine CR) with histological findings.

**a).**

| NEP/CR | Coef. | SE | t-value | p-value | [95% Conf. Interval] |
|---|---|---|---|---|---|
| Tubular Atrophy >50% | 1.272 | 0.36 | 3.53 | 0.001 | 0.54 to 2.003 |
| Intestitial Fibrosis >50% | 1.425 | 0.343 | 4.15 | <0.001 | 0.728 to 2.122 |
| Interstitial inflammation>25% | 0.673 | 0.291 | 2.31 | 0.028 | 0.076 to 1.27 |
| Segmental podocyte foot processes fusion | -0.702 | 0.274 | -2.56 | 0.016 | -1.264 to -.141 |

**b).**

| PDC/CR | Coef. | SE | t-value | p-value | [95% Conf. Interval] |
|---|---|---|---|---|---|
| Tubular Atrophy >50% | 2.721 | 0.725 | 3.75 | 0.001 | 1.248 to 4.193 |
| Interstitial fibrosis >50% | 2.603 | 0.738 | 3.53 | 0.001 | 1.106 to 4.1 |
| Arterial Hyalinosis | 1.906 | 0.751 | 2.54 | 0.016 | 0.382 to 3.43 |
| Interstitial inflammation >25% | 1.55 | 0.622 | 2.49 | 0.018 | 0.287 to 2.813 |

NEP was also positively correlated to severe tubular atrophy, severe interstitial fibrosis and GBM thickening (p=0.019, p=0.002, p=0.039, respectively). NEP/CR was also associated with tubular atrophy, interstitial fibrosis and interstitial inflammation and (Table 3) segmental glomerulosclerosis (p=0.016).

### *3.2. Electron Microscopy*

Patients with global podocyte foot processes fusion (n=11) had a higher mean urine PDC value, 99.331 ng/ml, compared to those with segmental fusion (n=26), with PDC value 58.889ng/ml. Moreover, in patients with fibrillary structures (n=3), we recorded significantly higher PDC levels (p=0.009) and a statistically significant correlation of PDC/CR ratio with the presence of fibrils

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(p=0.01). In non-immune complex mediated glomerulonephritis, such as minimal change disease, NEP/CR (coef ±SE [95% CI], -0.697±0.331[-1.369 to -0.026], p 0.042). NEP/CR was also associated with segmental podocyte foot processes fusion (Table 3).

In addition, we observed that there was a strong correlation between proteinuria >3 gr/24h and diffuse fusion of podocyte foot processes (p=0.016).

In multivariable analysis for NEP/CR interstitial inflammation and segmental podocyte fusion were found as determinants (Table 4).

**Table 4.** Multivariate regression analysis for NEP/CR.

| Variable | Coef. | SE | t-value | p-value | [95% Conf. Interval] |
|---|---|---|---|---|---|
| Segmental glomerulosclerosis | 1.778 | 0.951 | 1.87 | 0.071 | -0.162 to 3.718 |
| Normotension | -0.14 | 0.293 | -0.48 | 0.636 | -0.738 to 0.458 |
| Interstitial inflammation >25% | 0.796 | 0.26 | 3.06 | 0.005 | 0.266 to 1.327 |
| Segmental fusion | -0.718 | 0.282 | -2.54 | 0.016 | -1.293 to -0.142 |
| Immune-complex mediated GN | -0.493 | 0.291 | -1.70 | 0.1 | -1.087 to 0.1 |
| Constant | 1.156 | 0.349 | 3.31 | 0.002 | 0.444 to 1.868 |

GN: Glomerulonephritis.

## 4. Discussion

Both PDC and NEP play a significant role in the establishment and maintenance of podocyte identity and differentiation and thus, they are pivotal for the structural and functional integrity of the GFB[6]. Hence, an impairment of the podocytes' normal function can lead to a disruption of the slit diaphragm and to several pathophysiological consequences, characterized by loss of proteins and erythrocytes in the urine and a compromise of the glomerular filtration rate. [12–15]

Their crucial role of podocyte-associated proteins for normal renal physiology means that they could serve as biomarkers for the non-invasive detection and quantification of podocyte damage. Indeed theoretically, estimation of their urine concentration could be a very useful measure of the severity of podocyte dysfunction and reflect the integrity of GFB.

In the present study we found that there is a significant correlation between the levels of nephrin and podocalyxin in urine and interestingly with the levels of serum creatinine. This is an important finding given that there are no available studies exploring the importance of urinary podocyte-associated proteins in terms of prognosis and kidney disease progression.

The findings of our study are in line with those of previous studies, regarding the increased urinary levels of PDC and NEP in several GNs such as, IgA nephropathy [9]. Several studies in IgA nephropathy have revealed a statistically significant association of the levels of urinary PDC with podocyte injury. The same stands for membranous nephropathy, lupus nephropathy and diabetic nephropathy [5,8].

In our study both nephrin and podocalyxin levels were associated with severe tubular atrophy, interstitial fibrosis and interstitial inflammation. There are several studies affirming this correlation as well as the fact that NEP levels are correlated also with albuminuria while changes in NEP excretion have been linked to podocyte injury [19]-[21]. In our study, we also reported that segmental glomerulosclerosis was significantly associated with NEP/CR ratio. As far as the PDC/CR urine level is concerned, our study interestingly validated the correlation with activity parameters, such as severe interstitial inflammation. To the best of our knowledge few studies have assessed both these markers (PDC/CR, NEP/CR ratio) simultaneously in patients with GN. A previous study [22] assessed the impact of systemic lupus erythematosus (SLE) on urinary levels of podocalyxin as well as its correlation to renal biopsy histological parameters, proteinuria, and disease activity. They found a significant correlation of the estimated ratios with the lupus nephritis class as well as with the BILAG scores (especially with PDC/CR ratio). Another study also found that podocalyxin is associated with GN disease activity [26]. However, our study highlights that the increased urine levels of PDC and NEP are mainly associated with chronicity indices, such as tubular atrophy, severe

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fibrosis and GBM thickening not only in podocytopathies, but also interestingly in non-podocytopathies.

Similarly, patients with diabetic nephropathy in our study displayed elevated urine PDC/CR, which is in line with existing bibliographical data and support its potential utility as a non-invasive marker for the early detection of podocyte damage in these patients. Two studies, from Hara et al. [16] and Petrica et al [17] have already demonstrated the ability of PDC to identify early podocyte injury in patients with diabetes and have showcased its correlation with proximal tubule dysfunction. Moreover, urine PDC in diabetic patients increases before the onset of microalbuminuria and, meaning that it could be more sensitive for the early detection of diabetic kidney disease [18]. Concerning our findings in hypertensive patients, it is another interesting field for further evaluation, since GNs and in general kidney diseases are strongly associated with hypertension.

Moreover, we found that PDC concentration in urine was associated with distinct histological parameters available only after examination of the biopsy tissue on electron microscopy, such as podocyte foot processes fusion and fibrils. Some would think that PDC urine levels could be used as a biomarker when electron microscopy is not available. Of course, our data face multiple limitations and safe conclusion could not be made. However, this point could be a target field for future studies.

Our findings must be interpreted considering the study's limitations. The main drawback of our work is the heterogeneity of our cohort, comprising of a small number of cases from every distinct category of glomerulopathies, which imposes severe restrictions to the statistical power of our results. Moreover, the small number of healthy controls could not allow for statistical associations, the only result would be the significantly lower levels of NEP an PDC in their urine samples.

## 5. Conclusions

Podocalyxin and nephrin concentrations in urine of patients with GNs are associated with serum CR levels and with indices of chronicity in renal biopsy. However, larger prospective studies are needed to validate and extend our results and define how these markers can be used in terms with the response to therapy and follow up of kidney survival of these patients.

Taking into consideration the results of our study, having of course in mind the limitations, probably it would be of great interest to investigate urine levels of PDC and NEP and their associations not only in podocytopathies, but also in non-podocytopathies.

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| HISTOLOGICAL PARAMETERS. | VALID | N=37 (%) |
|---|---|---|
| TUBULAR ATROPHY | 0% | 10.8 |
| | <25% | 43.2 |
| | 25-50% | 27.0 |
| | >50% | 18.9 |
| HYALINOSIS | ABSENT | 51.4 |
| | 1 SEGMENTAL | 10.8 |
| | >1 SEGMENTAL | 16.2 |
| | 1 GLOBAL | 21.6 |
| ARTHRIOSCLEROSIS | 0% | 13.5 |
| | 1-25% | 32.4 |
| | 26-50% | 24.3 |
| | >50% | 29.7 |
| INERSTITIAL INFLAMMATION | ABSENT | 37.8 |
| | MILD | 16.2 |
| | MODERATE | 27.0 |
| | SEVERE | 18.9 |
| INTESTITIAL FIBROSIS | <5% | 10.8 |
| | <25% | 35.1 |
| | 26-50% | 35.1 |
| | >50% | 18.9 |
| GLOBAL FUSION | NO | 70.3 |
| | YES | 29.7 |
| SEGMENTAL FUSION | NO | 29.7 |
| | YES | 70.3 |
| PODOCYTE SWELLING | YES | 100 |
| MICROCYSTIC PODOCYTE DEGENARATION | NO | 2.7 |
| | YES | 97.3 |
| AUTOPHAGIC BODIES | NO | 70.3 |
| | YES | 29.7 |
| DENSE DEPOSITS | NONE | 51.4 |
| | MINOR | 24.3 |
| | MAJOR | 24.3 |
| FIBRILS | NO | 91.9 |
| | YES | 8.1 |
| GB MEMBRANE THICKENING | NO | 8.1 |
| | SEGMENTAL | 54.1 |
| | GLOBAL | 37.8 |
| MICROVILLUS TRANSFORMATION | NO | 43.2 |
| | YES | 56.8 |