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URINARY TRANSFERRIN AS AN EARLY BIOMARKER OF DIABETIC NEPHROPATHY.

Urinarni transferrin kao rani marker dijabetesne nefropatije

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Apstrakt

Uvod /Cilj: Dijabetesna nefropatija predstavlja jedan od vodećih uzroka hronične bubrežne bolesti i terminalne bubrežne insuficijencije. Zastupljena je kod 20-40% pacijenata sa dijabetes melitusom, a kao prvi znak dijabetesne nefropatije još uvek se smatra mikroalbuminurija. Niska senzitivnost i specifičnost mikroalbuminurije su doveli do ispitivanja novih urinarnih biomarkera koji bi mogli biti rani pokazatelji postojanja dijabetesne nefropatije. Ova studija je spovedena da bi se utvrdilo da li urinarni transferin može biti rani marker dijabetesne nefropatije

Metode: U našu studiju je bilo uključeno 80 pacijenata sa tipom 2 dijabetesa, podeljenih u dve grupe: grupa 1 - normoalbuminurični pacijenti (ekskrecija albumina do 30 mg/dan), grupa 2 - mikroalbuminurični (ekskrecija albumina od 30-300 mg/dan) i 10 zdravih kontrola. Svi pacijenti su bili stariji od 18 godina, imaju dijabetes melitus duže od jedne godine i jačinu glomerulske filtracije veću od 60 ml/min/1.73m². Svim pacijentima je određivan nivo serumskog kreatinina, glikozilovanog hemoglobina i transferina u urinu. Koncentracija transferina je analizirana u 24h uzorku urina i u prvom jutarnjem urinu. Urinarni transferin je određen primenom visoko senzitivnog ELISA kita.

Rezultati: Koncentracija urinarnog transferina je bila značajno veća kod pacijenata koji su imali mikroalbuminuriju u poredjenju sa pacijentima koji su bili normoalbuminurični i zdravim kontrolama a Pearson’s koečijent korelacije je r=0.584 (p<0.001). Nismo dobili povezanost izmedju nivoa urinarnog transferina i glikoregulacije kao ni nivoa transferina i dužine trajanja dijabetesa.

Zaključak: Rezultati ove studije pokazuju da bi urinarni transferin mogao biti rani marker dijabetesne nefropatije

Ključne reči: Dijabetes melitus, mikroalbuminurija, biomarker, transferin
Abstract

Background/Aim: Diabetic nephropathy is one of the leading cause of chronic kidney disease and end-stage renal disease. It occurs in 20-40% patients with diabetes mellitus, and microalbuminuria is still considered as the first sign of diabetic nephropathy. Low sensitivity and specificity of microalbuminuria leads to more sensitive biomarkers that may be used to detect diabetic nephropathy at an earlier stage with higher accuracy. This study was carried out to determine whether urinary transferrin can serve as an indicator of diabetic nephropathy.

Methods: Our study included 80 type 2 diabetic patients who were classified into two groups: group 1 - normoalbuminuric patients (albumin excretion up to 30 mg/d), group 2 - microalbuminuric patients (albumin excretion from 30 – 300 mg/d), and 10 healthy control. All patients were older than 18, with diabetic disease more than one year, glomerular filtration rate more than 60ml/min/1.73m². Serum creatinine, glycosylated hemoglobin (HbA1c), and concentration of transferrin in the 24h urine samples as well as in spot urine were measured. The urinary concentrations of transferrin were measured using a highly sensitive one-step sandwich enzyme immunoassay kit.

Results: Urinary transferrin was significantly higher in microalbuminuric patients than in the normoalbuminuric and healthy control subject. By comparing these groups according to urinary transferrin concentration we found statistically significant positive correlation r=0.584 (p<0.001). There was no correlation between level of urinary transferrin and glycoregulation, and no correlation was found between transferrin and duration of diabetes.

Conclusions: The results from this study provide the evidence that urinary transferrin could be used as an early marker of diabetic nephropathy.

Key words: Diabetes mellitus, microalbuminuria, biomarker, transferrin
Introduction

Diabetes mellitus (DM) is a chronic disease whose incidence and prevalence show a steady increase. According to the International Diabetes Federation about 415 million people suffer from diabetes around the world, and it is estimated that by 2040 the number of people with diabetes will be around 642 million, with prevalence of 10%. (1) An increasing number of diabetic patients, mostly with type 2 diabetes (90%) is associated with enhanced rate of diabetic complications, including diabetic kidney disease. (2) Diabetes is considered as the leading cause of chronic kidney disease (CKD) and end stage renal disease (ESRD). Costs of care for patients with diabetic kidney disease (DKD) are extremely high, especially after they enter ESRD, and it is necessary to establish the diagnosis of diabetic nephropathy as soon as possible. (3,4) Microalbuminuria is generally considered as the earliest non-invasive marker of kidney damage and it was described for the first time in 1960s. (5) Microalbuminuria (MA) is defined as persistent elevation of albumin in the urine, of 30-300 mg/day, and it is generally considered as the earliest non-invasive marker for the development of diabetic nephropathy (DN), even though the specificity and sensitivity of microalbuminuria are limited. (6,7) Some patients with diabetes mellitus progress to diabetic nephropathy (DN) even if urinary albumin levels are in the normal range, indicating that albuminuria is not the perfect marker for the early detection of DN. (8,9) Recent studies have shown that some relevant biomarkers associated with diabetic nephropathy have been found and they potentially could be used to predict DN or progression of the disease. (10) Several different markers of tubular and glomerular damage have been investigated to discover DN in its early phase, and to start therapy as soon as possible. (11)

Urinary transferrin is considered as an early marker of glomerular injury in diabetic patients. It is a protein, slightly higher molecular weight than albumin (76.5kDa). Due to its low molecular weight and its less ionic load it filters easily through the glomerular membrane. (12) Some previous studies have shown that increased urinary transferrin excretion can be reported before microalbuminuria in normoalbuminuric patients with DM type 2. Because of that, urinary transferrin is considered as a more sensitive marker of glomerular damage in diabetic patients. (13) Excretion of transferrin was not associated
with glycemic control (hemoglobin A1c), but some studies have shown that urinary transferrin concentration was higher in patients with diabetic retinopathy. (14) The aim of this study was to determine if urinary transferrin can be classified into a group of early biomarkers of diabetic nephropathy.

Method

This cross-sectional prospective observational study was carried out between September 2015 and December 2016, to investigate the correlation between microalbuminuria and urinary transferrin in diabetic nephropathy. Study was approved by the Ethical Committee of the Military Medical Academy, Belgrade, Serbia and written informed consent was taken from all the patients. Eighty patients with type 2 diabetes mellitus with disease duration one year or more, estimated glomerular filtration rates more than 60ml/min/1.73m², and without albuminuria were included in the study. Patients with overt albuminuria (>300 mg/day), previous renal diseases, urinary tract infection in the last 4 weeks, the use of nephrotoxic drugs, systemic disease, malignant diseases except of basocellular skin carcinoma were excluded. The selected patients were studied in detail with history and physical examination, including ultrasonography of the kidney. Age, gender, duration of diabetes mellitus, weight, height, blood pressure and smoking habit were noted too. Body mass index (BMI) was calculated according to formula based on the height and weight measurements of the patients. Blood samples were taken after overnight fasting, at least 8 hours, and the following parameters were analyzed: serum level of glycaemia, urea, creatinine, glycated hemoglobin (HbA1C). Glomerular filtration rate (GFR) was calculated based on CKD-EPI formula. (GFR = 141 × min (Scr /κ, 1) α × max (Scr /κ, 1) - 1.209 × 0.993 years × 1.018 [for women]) = ml/min/1.73 m². (15) Transferrin concentration (ng/ml) and transferrin to creatinine ratio (mg/g of creatinine) were determined in a spot morning urine sample, and albuminuria (30mg/day or greater) measured in a 24h urine collected on the subsequent day. All samples of urine were immediately processed within four hours of collection to ensure optimal protein stability. Urine was centrifuged (1000xg, 20 min), then dividing into 1.5 mL aliquots and frozen at -80°C until analysis. The levels of urinary transferrin were
determined by commercially available ELISA kits from Elabscience Biotechnology Co., Ltd. Minimum and detectable dose for urinary transferrin was 1.56 ng/mL.

Statistics

Statistical analyses were performed using Statistical Package for the Social Science (SPSS) version 19.0. Basic descriptive statistical parameters were presented by measures of central tendency (mean and median), a measure of variability (standard deviation and variation interval) and were expressed in percentages. To compare continuous variables Student's t-test was used for independent samples or Mann Whitney test, depending on the normality of distribution, which was checked by Kolmogorov-Smirnov test. For comparison of frequencies for categorical variables, χ²-test was used. Statistical hypothesis was tested at 0.05 level of significance, and probability (p) value less than 0.05 was regarded as statistically significant.

Results

Our study included 80 type 2 diabetic patients, 44 (55%) males and 36 (45%) females, mean age 59.85± 8.87 years, (range 38-73 years). Prevalence of microalbuminuria was 41.25% (33 patients) and 58.75% were normoalbuminuric (47 patients). Among the patients with microalbuminuria 17 were males (51.52%) and 16 were females (48.48%). The average duration of diabetes was 13.29±7.69 years, and the average estimated GFR was 86.86±14.18. There were no significant differences in baseline clinical characteristics between examined groups. (Table 1)

Urinary transferrin concentration in spot samples and urinary transferrin concentrations in 24h urine samples showed significant linear correlation, therefore, we used results from spot urine samples for further analyses.

The mean concentration of urinary transferrin in microalbuminuric patients was 85.07±56.54 µg/gCr, and for normoalbuminuric patients it was 25.63 ± 29.85 µg/gCr. We
found statistically significant correlation in the transferrin concentration between these two groups. (Table 2) Correlation analysis for concentration of urinary transferrin with independent variables is shown in the table 3. Among all variables, we found significant correlation only with microalbuminuria. (Table 3)

Diabetic retinopathy was found in 24 (30%) patients. Those patients had significantly higher urinary transferrin levels, albumin excretion and duration of diabetes. Sensitivity and specificity of urinary transferrin concentrations expressed as an area under the ROC curve (AUC), and It was 87.1%, with the sensitivity 81.8%, and specificity 80.9% (95% confidence interval (CI) – from 0.796 up to 0.945; p<0.001). (Figure 1)

Discussion

Albuminuria is considered as a marker of kidney (glomerular) damage, and the first clinical indicator of diabetic nephropathy presence. (16) Even today it is a clinically useful tool for predicting prognosis and for monitoring response to therapy. (11) Discordance between the presence of albuminuria and the decline in renal function is crucial point of clinically significance of albuminuria. The presence of albuminuria is not mandatory in all patients with reduced GFR. Perkins et al reported the development of advanced CKD (GFR < 60 mL/ min per 1.73 m2) without concomitant progression of albuminuria in type 1 diabetic patients. (9) Chen et al compared several studies from 1977 to the present and shown that a portion of diabetic patients with normoalbuminuria have progressive decline in renal function, referred to nonalbuminuric diabetic nephropathy. (17) In different studies the number of nonalbuminuric diabetic kidney disease was from 21.8% reported by Boronat et al (18) to 56.6% reported in 2014 by Penno. et al.(19) Nonalbuminuric renal impairment was not associated with HbA1c and retinopathy, but some study found that gender is correlated with nonalbuminuric renal impairment. That's why we need a new biomarker with higher sensitivity and specificity for an earlier detection of diabetic nephropathy and more accurate prediction of the progression to ESRD. Therefore, we analyzed urinary transferrin as a biomarker of glomerular injury implicated in early diabetic nepropathy, nonalbuminuric diabetic patients.

The results of our study showed that increased urinary excretions of transferrin was higher in patients with microalbuminuria than in normoalbuminuric type 2 diabetic patients. We
found statistically significant correlation between concentration of urinary transferrin and microalbuminuria. This is in accordance with the results obtained by Narita et al who reported that increased urinary transferrin found in diabetic patients independently of microalbuminuria could also predict development of microalbuminuria in normoalbuminuric DM2 patients. (13) In 24 months follow-up study with type 2 diabetic patients, Kazumi et al found that 31% of patients who had transferrinuria at baseline subsequently developed microalbuminuria, compared with 7% of patients without transferrin excretion. They conclude that in patients with type 2 diabetes without microalbuminuria, increased urinary transferrin excretion may predict the development of microalbuminuria. (20) The same results found Kanauchi et al in the group of 60 type 2 diabetic patients. They presented a significant correlation between the urinary excretion of transferrin and albumin. Their findings indicate that urinary transferrin may be useful in detecting diabetic nephropathy at an early stage. (21) Al-Rubeaan et al described similar results in a cross-sectional study in the group of 467 type 2 diabetic patients. (22) Similarly to our results, O’Donnell et al found no correlation between urinary transferrin levels and glycemic control in the group of 40 type 2 diabetic patients at first day of diagnosis disease and after 6 and 12 weeks of treatment. Urinary excretion rates of transferrin were measured, and they showed that urinary transferrin was not correlated with glycemic control. (23) We found no correlation between urinary transferrin levels and duration of diabetes, as well. Several studies found relationship between excretion of urinary albumin and diabetic retinopathy, and one of the famous is the Japanese study of Moriya et al, which included 2205 diabetic type 2 patients aged 40-70. (24) We found similar results. Patients with retinopathy had significantly higher values of urinary transferrin excretion as well as higher levels of microalbuminuria. Our sensitivity and specificity analysis of urinary transferrin excretion showed that it could be more sensitive indicator of early glomerular damage in diabetes mellitus than microalbuminuria.

Conclusion
In summary, urinary transferrin was significantly increased in type 2 diabetic patients with microalbuminuria. It was independent of diabetes duration and glycemic control
coregulation. According to our results, the level of urinary transferrin excretion could be used as an early biomarker of diabetic nephropathy.

REFERENCES


### Table 1
Baseline clinical characteristics of 80 type 2 diabetic patients according to levels of urinary albumin

<table>
<thead>
<tr>
<th>Characteristic of the patients</th>
<th>All Patients (n=80)</th>
<th>Normoalbuminuric (n=47)</th>
<th>Microalbuminuric (n=33)</th>
<th>Healthy (n=10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>44/36</td>
<td>27/20</td>
<td>17/16</td>
<td>5/5</td>
<td>0.276</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>59.85±8.871</td>
<td>60.49±8.73</td>
<td>58.94±9.13</td>
<td>54±10.59</td>
<td>0.014</td>
</tr>
<tr>
<td>Duration of DM (Years)</td>
<td>13.29±7.69</td>
<td>13.34±7.74</td>
<td>13.21±7.73</td>
<td>n/a</td>
<td>0.942</td>
</tr>
<tr>
<td>BMI (Kg/m2)</td>
<td>27.36±4.42</td>
<td>26.64±3.56</td>
<td>28.38±5.31</td>
<td>25.73±4.77</td>
<td>0.325</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>39 (48.8 %)</td>
<td>26 (55.3%)</td>
<td>13 (39.4%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>134.60±14.08</td>
<td>133.47±12.87</td>
<td>136.21±15.71</td>
<td>122±17.02</td>
<td>0.411</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.56±7.53</td>
<td>80.96±7.42</td>
<td>82.42±7.72</td>
<td>75.5±10.39</td>
<td>0.389</td>
</tr>
<tr>
<td>Creatinine(µmol/L)</td>
<td>75.38±15.04</td>
<td>76.64±15.97</td>
<td>73.58±13.64</td>
<td>73.6±7.6</td>
<td>0.360</td>
</tr>
<tr>
<td>GFR (mL/min/1.73m²)</td>
<td>86.86±14.18</td>
<td>85.78±13.55</td>
<td>88.39±15.12</td>
<td>92.84±9.06</td>
<td>0.430</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>7.59±1.34</td>
<td>7.25±1.15</td>
<td>8.07±1.45</td>
<td>4.93±0.3</td>
<td>0.074</td>
</tr>
</tbody>
</table>

All data are expressed as means ± SD except smoking habit. DM – diabetes mellitus; BMI – body mass index; HbA1c – hemoglobin A1c; GFR – glomerular filtration rate.
### Table 2

**Correlation of transferinuria and microalbuminuria**

<table>
<thead>
<tr>
<th>Transferin concentration (µg/gC₆)</th>
<th>Microalbuminuric ≥30mg/24h (n=33)</th>
<th>Normoalbuminuric &lt;30mg/24h (n=47)</th>
<th>Pearson’s r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>x̄ ±SD</td>
<td>x̄ ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferin concentration 24h urine</td>
<td>91.76± 68.45</td>
<td>22.56± 31.46</td>
<td>0.489</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Transferin concentration 24h urine</td>
<td>85.07± 56.54</td>
<td>25.63± 29.85</td>
<td>0.354</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Pearson’s test, r – correlation coefficient; x̄ – mean; SD – standard deviation
Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n=80)</th>
<th>Pearson's r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.85±8.87</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3±4.42</td>
<td>0.053</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>7.59±1.34</td>
<td>0.132</td>
</tr>
<tr>
<td>Duration of DM (Years)</td>
<td>13.29±7.69</td>
<td>0.127</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>40.42±40.89</td>
<td>0.584</td>
</tr>
</tbody>
</table>

Pearson’s test, \( r \) – correlation coefficient; \( \bar{x} \) – mean; SD – standard deviation