URINARY PODOCYTES ARE ASSOCIATED WITH PROXIMAL TUBULE DYSFUNCTION IN TYPE 2 DIABETES MELLITUS PATIENTS: A CROSS-SECTIONAL STUDY

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Background

- podocytes are highly specialized epithelial cells which cover the outer aspect of the glomerular basement membrane, playing an important role in the function of the glomerular filtration barrier
- detection of podocytes in the urinary sediment of various glomerular diseases has been shown to indicate severe injury to the podocytes
- urinary podocytes may be a useful marker of disease activity in diabetic nephropathy (DN)
- the tubular theory concerning albuminuria in the course of diabetes mellitus states that albuminuria is caused primarily by impaired tubular uptake of intact albumin rather than by an increased leakiness of the glomerular filtration barrier
- in previous studies we showed that in type 2 diabetes there is an association of proximal tubule (PT) dysfunction with podocyte damage biomarkers, even in the normoalbuminuria stage
- this observation suggests a potential role of the PT in urinary nephrin and urinary VEGF processing in early DN, a fact which could be related to advanced glycation end- products (AGE) intervention

Aim of study

- to evaluate a potential association of urinary podocytes with PT dysfunction
- we queried if this association could be related to AGE intervention, which may impact both the PT and the podocytes

Methods

- 86 patients with type 2 DM attending the Department of Diabetes and Metabolic Diseases (34-normoalbuminuria; 30-microalbuminuria; 22-macroalbuminuria) and 28 healthy control subjects
- a cross-sectional study
- inclusion criteria
  - long-standing DM (>5 years)
  - normoalbuminuria (urine albumin-to-creatinine ratio (UACR) <30 mg/g) or microalbuminuria (UACR between 30 and 300 mg/g),
  - patients were on oral antidiabetic medication, angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs), and statins

All p were assessed concerning:
- GFR
- C-reactive protein (CRP)
- plasma advanced glycation end-products (pAGEs)
- serum cystatin C
- urine albumin/creatinine ratio (UACR)
- urinary alpha1-microglobulin
- urinary kidney injury molecule-1 (KIM-1)
- urinary nephrin
- urinary vascular endothelial growth factor (VEGF)
- urinary advanced glycation end-products (uAGEs)

Statistical analysis

- clinical, biological and cerebral haemodynamics indices are presented as means, Standard Deviations(SD) and proportions
- Man-Whitney test and Kruskal-Wallis test for the differences among the groups were used to compare the means among the three groups.
- simple and multiple linear regression analyses were carried out to evaluate the significance of the relation between continuous variables for all groups together (pooled data).
- only significant variables yielded by univariate regression analysis were introduced in the models for multivariable regression analysis (Cox & Snell R square).
- the P values for all hypothesis tests were two-sided, and statistical significance was set at P<0.05.
- All analyses were conducted with Stata 9.2 (Statacorp, Texas, USA).

- urinary podocytes were examined in cell cultures by immunofluorescence utilizing a monoclonal antibody against podocalyxin
- cultures of urinary podocytes were performed as follows: Midstream urine samples of 15ml were collected in sterile tubs and centrifuged at 700g for 5 minutes. The pelleted cellular material was washed twice with PBS without Ca and Mg (Gibco-Life Technologies, Catalog No. 16120-036) suspended in appropriate medium [RPMI 1640 with glutamine supplemented with 10% fetal bovin serum, insulin-transferrin-selenium G (Gibco- Life Technologies, Catalog No. 41400-045) and 1% penicillin/streptomycin (Gibco-Life Technologies, Catalog No. 15140-122)], and cultured on cell culture flasks coated with type I collagen (from rat tail- Gibco-Life Technologies, Catalog No. 15379-021). After 12hours, the cells were detached with trypsin, suspended in PBS, and cytocentrifuged at 700g for 5 minutes. The slides were fixed with the primary antibody [Podocalyxin Mouse Monoclonal Antibody (Clone 3D3), Invitrogen- Life Technologies, Catalog No. 39-3800] for 60 minutes. After washing, the slides were incubated with the secondary antibody [fluorescein goat anti-mouse IgG-(H+L), F2761, Invitrogen-Life Technologies, Catalog No. 15667-139] and examined by immunofluorescence microscopy. Urinary podocytes were expressed as cells/ml.

- urinary KIM-1, urinary nephrin, urinary VEGF, plasma AGEs, and urinary AGEs were evaluated by the ELISA method
- serum cystatin C, urinary alpha1 microglobulin and albuminuria were assessed by means of particle-enhanced immunonephelometry using the BN ProSpec System
- CKD was defined and the stages(1-5) of CKD were established according to the KDIGO Guidelines 2012 (estimated GFR- CKD-EPI equation formula)

Results

- Podocytes were detected in the urine of 10% of the healthy controls, 24% of the normoalbuminuric, 40% of the microalbuminuric, and 82% of the macroalbuminuric patients
- The demographic, clinical and laboratory data of the patients and control subjects are presented in Table 1, Table 2, Table 3, Fig 1: A, B,C

Parameter	Group 1 (healthy controls)	Group 2 (normoalbuminuria)	Group 3 (microalbuminuria)	Group 4 (macroalbuminuria)	p*	p**	p***	p
Number of subjects	28	34	30	22	–	–	–	–
Age (years)	57.5 (52.5; 63.5)	58 (53; 60)	61 (52; 65)	60 (54; 62)	0.438	0.225	0.9187	0.767
DM duration (years)	–	8.5 (7; 10)	8 (5; 13)	10.5 (8; 12)	0.455	0.028	0.042	0.052
BMI	24.68 (23.84; 28.79)	32.41 (29.24; 35.06)	32.32 (28.08; 37.78)	36.49 (31.05; 40.75)	0.935	0.010	0.047	0.0001
SBP (mmHg)	120 (117.5; 135)	130 (120; 140)	132.5 (125; 135)	155 (150; 165)	0.742	<0.0001	<0.0001	0.0001
DBP (mmHg)	70 (60; 77.5)	75 (70; 80)	75 (70; 80)	90 (85; 95)	0.632	<0.0001	<0.0001	0.0001
Hb (g/dl)	13.8 (12.9; 14.7)	12.95 (12.1; 13.7)	13.45 (12.6; 14)	10.67 (10.33; 11.25)	0.230	<0.0001	<0.0001	0.0001
Serum creatinine (mg/dl)	0.92 (0.81; 1.03)	0.94 (0.82; 1.06)	1 (0.91; 1.12)	1.52 (1.45; 1.99)	0.123	<0.0001	<0.0001	0.0001
eGFR (ml/min/1.73m^2)	77.42 (71.45; 85.40)	80.22 (61.32; 90.01)	73.40 (58.05; 85.28)	39.27 (31.54; 45.58)	0.112	<0.0001	<0.0001	0.0001
Glycaemia (mg/dl)	100 (92.5; 108)	143.5 (118; 200)	135.5 (115; 210)	171 (138; 275)	0.957	0.033	0.038	0.0001
HbA_{1c} (%)	5.2 (4.9; 5.5)	6.9 (6.4; 7.4)	7 (6.4; 8.4)	8.6 (7.9; 9.7)	0.156	<0.0001	0.0002	0.0001
HbA_{1c} (mmol/mol)	33 (30; 37)	52 (46; 57)	53 (46; 68)	70 (63; 83)	0.156	<0.0001	0.0002	0.0001
Serum cholesterol (mg/dl)	162.5 (135; 184)	213 (183; 246)	239 (199; 288)	275 (244; 343)	0.104	0.0001	0.010	0.0001
Triglycerides (mg/dl)	106.5 (86.5; 133.5)	153.5 (108; 193)	151.5 (126; 194)	178.5 (144; 218)	0.509	0.028	0.091	0.0001
hsCRP (mg/dl)	1.48 (1.15; 2.81)	3.74 (3.22; 5.13)	11 (9.52; 16.24)	24.43 (20.50; 36.74)	<0.0001	<0.0001	<0.0001	0.0001
UACR (mg/g)	17.48 (16.02; 19.26)	27.04 (21.48; 28.25)	80.71 (44.28; 115.38)	886.65 (527.92; 1267.15)	<0.0001	<0.0001	<0.0001	0.0001
Cystatin C (mg/L)	0.68 (0.63; 0.76)	0.88 (0.63; 0.98)	0.88 (0.78; 0.99)	1.71 (1.47; 2.00)	0.290	<0.0001	<0.0001	0.0001
Alpha1/creat (mg/g)	3.22 (2.92; 3.53)	3.74 (3.52; 6.25)	6.94 (4.6; 9.08)	50.8 (33.38; 66.47)	<0.0001	<0.0001	<0.0001	0.0001
Nephrin/creat (mg/g)	0.08 (0.03; 0.09)	0.11 (0.09; 0.15)	0.85 (0.41; 1.46)	6.09 (3.79; 10.06)	<0.0001	<0.0001	<0.0001	0.0001
Podocytes	0 (0; 0)	0 (0; 0)	3 (0; 7)	5.5 (0; 10)	0.035	0.0001	0.027	0.0001
VEGF/creat (ng/g)	38.1 (19.42; 47.75)	83.4 (60.4; 114.6)	103.45 (87.69; 200.8)	716.02 (555.63; 1317.24)	0.008	<0.0001	<0.0001	0.0001
KIM-1/creat (ng/g)	48.52 (42.02; 61.64)	74.66 (47.89; 98.68)	107.84 (66.08; 134)	686.36 (408.06; 853.52)	0.001	<0.0001	<0.0001	0.0001
Urinary AGE (pg/ml)	33.55 (32.13; 35.40)	37.99 (32.90; 63.60)	57.36 (36.46; 108.204)	487.34 (248.93; 765.00)	0.009	<0.0001	<0.0001	0.0001
Plasma AGE (pg/ml)	304.10 (274.83 370.25)	373.41 (304.77; 674.20)	652.85 (551.33; 752.14)	4718.16 (3754.90; 6184.69)	<0.0001	<0.0001	<0.0001	0.0001

Table 1. Clinical and biological data of the patients studied
DM: diabetes mellitus; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; hsCRP: high-sensitive C-reactive protein; UACR: urinary albumin:creatinine ratio; Alpha1/creat: urinary alpha$_1$-microglobulin:creatinine ratio; Nephrin/creat: urinary nephrin:creatinine ratio; VEGF/creat: urinary vascular endothelial growth factor:creatinine ratio; KIM-1/creat: urinary kidney injury molecule-1:creatinine ratio; AGE: advanced glycation end-products; p*: group 2 vs. group 3; p**: group 2 vs. group 4; p***: group 3 vs. group 4; p: group 1 vs. group 2 vs. group 3 vs. group 4.

Table 2. Univariable regression analysis for podocytes
eGFR: estimated glomerular filtration rate; hsCRP: high-sensitive C-reactive protein; UACR: urinary albumin:creatinine ratio;
Alpha1/creat: urinary alpha$_1$-microglobulin:creatinine ratio;
KIM-1/creat: urinary kidney injury molecule-1:creatinine ratio;
Nephrin/creat: urinary nephrin:creatinine ratio;
VEGF/creat: urinary vascular endothelial growth factor:creatinine ratio;
AGE: advanced glycation end-products.

Variable	Parameter		
	Podocytes		
	R^2	Coef β	p
eGFR	0.205	-0.083	<0.001
Cystatin C	0.252	3.187	<0.001
HbA_{1c}	0.032	0.488	0.054
Cholesterol	0.037	0.010	0.039
Triglycerides	0.035	0.012	0.045
hsCRP	0.051	0.019	0.053
UACR	0.184	0.003	<0.001
Alpha1/creat	0.367	0.117	<0.001
KIM-1/creat	0.260	0.007	<0.001
Nephrin/creat	0.534	0.909	<0.001
VEGF/creat	0.535	0.007	<0.001
Urinary AGE	0.217	0.007	<0.001
Plasma AGE	0.187	0.0008	<0.001

Table 3. Multivariable regression analysis for urinary podocytes
UACR: urinary albumin:creatinine ratio; Alpha1/creat: urinary alpha$_1$-microglobulin:creatinine ratio;
KIM-1/creat: urinary kidney injury molecule-1:creatinine ratio; VEGF/creat: urinary vascular endothelial growth factor:creatinine ratio;
Nephrin/creat: urinary nephrin:creatinine ratio; eGFR: estimated glomerular filtration rate;
hsCRP: high-sensitive C-reactive protein; AGE: advanced glycation end-products.

Parameter	Variable	Coef β	p	95% CI	F	Prob>F	R^2
Podocytes	Constant	0.674	0.013	0.143 to 1.206	61.57	<0.0001	0.6268
	UACR	-0.003	0.001	-0.0055 to -0.0015			
	Alpha1/creat	-0.003	0.001	-0.0051 to -0.0013			
	KIM-1/creat	-0.012	0.048	-0.0041 to -0.0065			
	VEGF/creat	0.011	<0.001	0.0096 to 0.0138			
	Nephrin/creat	0.038	0.059	0.0057 to 0.0118			
	Cystatin C	0.005	0.016	0.0028 to 0.0037			
	eGFR	-0.019	0.050	-0.0066 to -0.0027			
	hsCRP	0.319	0.477	-0.5698 to 1.2094			
	Cholesterol	0.008	0.180	-0.0209 to 0.0039			
	Triglycerides	0.010	0.151	-0.0038 to 0.0243			
	HbA_{1c}	-0.141	0.570	-0.6335 to 0.3509			
	Urinary AGE	-0.0004	0.050	-0.0005 to -0.0061			
	Plasma AGE	-0.0004	0.050	-0.0008 to -8.03e-0.7			

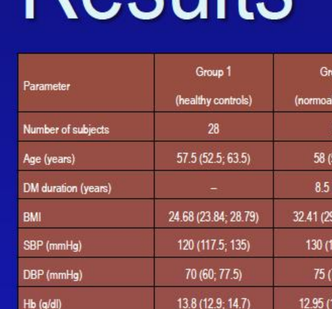

Fig 1. A. Small-sized urinary cell colonies with typical epithelial cell morphology; these cells display small buds of foot processes. Phase contrast microscopy, x40

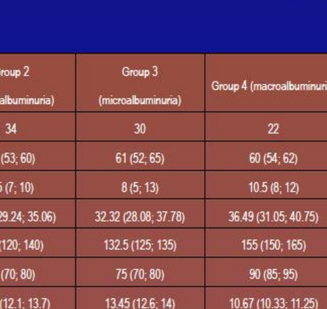

Fig 1. B. Morphology of urinary podocytes and expression of podocyte -specific marker-podocalyxin; Immunofluorescence microscopy, x20

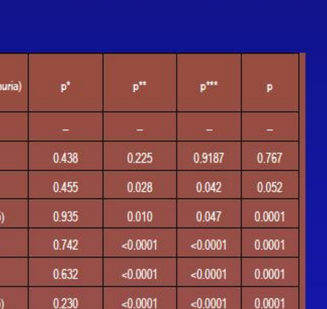

Fig 1. C. Morphology of urinary podocytes and expression of podocyte -specific marker podocalyxin; Immunofluorescence microscopy, x40

Discussion

- to the best of our knowledge this is the first study to demonstrate an association between PT dysfunction and urinary podocytes, as assessed in cell cultures, and their damage biomarkers, nephrin and VEGF.
- we found urinary podocytes in patients with type 2 diabetes even in the normoalbuminuria stage
- podocyte detachment may also occur in healthy subjects, as shown by our results in the control group
- the number of urinary podocytes correlated with UACR and with the podocyte damage biomarkers, nephrin and VEGF, even in the normoalbuminuria stage
- we assume that podocyturia, in conjunction with nephrinuria could be utilized for an accurate diagnostic of early DN
- this hypothesis is reinforced by the observation in our study of healthy subjects who displayed a few number of urinary podocytes, but they did not present with increased levels of nephrinuria
- podocyturia correlated with the levels of urinary VEGF, which were increased even in normoalbuminuric patients
- urinary podocytes correlated with the levels of urinary alpha$_1$-microglobulin and urinary KIM-1, even in patients with high-to-normal levels of albuminuria
- this observation raises the possibility of a concomitant podocyte injury and PT dysfunction, the latter phenomenon showing the role of major importance of the PT in albumin processing in early DN

Conclusion

- in patients with type 2 diabetes urinary podocytes are found even in the normoalbuminuria stage
- there is an association between PT dysfunction and urinary podocytes and their damage biomarkers, nephrin and VEGF
- AGE could be involved in podocyturia, as well as in PT dysfunction
- podocyturia may be a useful marker of early DN in conjunction with biomarkers of PT dysfunction and of podocyte injury

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