URINARY PODOCYTE-ASSOCIATED MESSENGER RNA LEVELS CORRELATE WITH PROXIMAL TUBULE DYSFUNCTION IN EARLY DIABETIC NEPHROPATHY OF TYPE 2 DIABETES MELLITUS

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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 quantification of messenger RNA (mRNA) of podocyte-associated molecules expression in urinary sediments represents a reliable modality for the diagnosis of early DN and for monitoring its activity and progression
- 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 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 assess the mRNA expression of podocyte-associated molecules in urinary sediment of patients with type 2 diabetes mellitus (DM) in relation to urinary podocytes, and to biomarkers of podocyte injury and of proximal tubule (PT) dysfunction.
- we queried if this association could be related to AGE intervention, which may impact both the PT and the podocytes

Methods

- 76 patients with type 2 DM attending the Department of Diabetes and Metabolic Diseases (28-normoalbuminuria; 27-microalbuminuria; 21-macroalbuminuria) and 20 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)
- urinary podocytes were examined in cell cultures by immunofluorescence utilizing monoclonal antibodies against podocalyxin and synaptopodin; 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 creat-cystatin C equation formula)

- urinary mRNA podocyte-associated genes assessed nephrin, podocin, alpha-actinin-4, CD2-associated protein (CD2AP), glomerular epithelial protein 1 (GLEPP1), ADAM 10, and NFkB
- for RNA preparation and RT-PCR Assay, the total urine pellet RNA was isolated using the TaqMan Gene Expression kit. Relative quantification of the CD2AP, podocin NPHS2, nephrin NPHS1, ADAM 10, alpha actinine 4, GLEPP1, and NFkB was realised using the 7900 HT Fast Real-Time PCR System. GAPDH mRNA was the internal control. The relative amounts of the gene were expressed as $2^{-\Delta CT}$ (ΔCT =CT value target gene - CT value internal control)

Statistical analysis

- clinical and biological data are presented as medians and IQR, as for variables with skewed distribution
- differences between groups were analysed with the Mann Whitney U test for comparison of 2 groups and the Kruskal Wallis test for comparison of 4 groups.
- simple linear regression analysis was 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).

Results

- The demographic, clinical and laboratory data of the patients and control subjects are presented in Table 1
- all 7 target podocyte-associated genes showed higher levels of expression in patients with type 2 DM as compared to controls
- we found significant differences between urinary mRNA of podocyte-associated proteins in relation with albuminuria stage
- changes in urinary podocyte-associated mRNA levels increased with disease progression

Parameter	Group 1 (healthy controls)	Group 2 NA	Group 3 MI	Group 4 MA	p*	p**	p***	p
Number of subjects	20	28	27	21	–	–	–	–
Age (years)	56.5 (51; 63.5)	60 (56.5; 63.5)	59 (53; 62)	60 (56; 62)	0.50	0.7	0.75	0.74
DM duration (years)	–	8 (7; 10)	8 (5; 13)	11 (8; 12)	0.72	0.01	0.08	0.06
BMI	24.68 (23.84; 29.41)	32.02 (28.74; 34.80)	32.6 (28.08; 38.09)	36.56 (31.05; 40.72)	0.48	0.005	0.08	0.0001
SBP (mmHg)	120 (120; 135)	130 (120; 140)	135 (125; 135)	155 (150; 165)	0.97	<0.0001	<0.0001	0.0001
DBP (mmHg)	70 (60; 80)	75 (70; 80)	75 (70; 80)	90 (85; 95)	0.67	<0.0001	<0.0001	0.0001
Hb (g/dl)	13.8 (13.2; 14.6)	12.95 (12.05; 14.1)	13.4 (12.4; 14)	10.67 (10.33; 11.25)	0.44	<0.0001	<0.0001	0.0001
Serum creatinine (mg/dl)	0.83 (0.74; 0.92)	0.93 (0.81; 0.99)	1.06 (1; 1.24)	1.48 (1.34; 1.99)	<0.0001	<0.0001	<0.0001	0.0001
eGFR (ml/min/1.73m^2)	101.6 (99.61; 110.69)	91.3 (82.45; 97.7)	64.9 (60.1; 76.1)	37.05 (29.57; 44.20)	0.112	<0.0001	<0.0001	0.0001
Glycaemia (mg/dl)	100 (95; 108.5)	149 (116.5; 204)	137 (115; 210)	176 (138; 275)	0.96	0.07	0.06	0.0001
HbA_{1c} (%)	6.2 (5.95; 6.35)	6.75 (6.3; 7.3)	7.17 (6.8; 8.4)	8.68 (7.93; 9.67)	0.008	<0.0001	0.0007	0.0001
Cholesterol (mg/dl)	163 (132.5; 185.5)	215 (194; 246)	238 (191; 288)	274 (244; 343)	0.42	0.0004	0.01	0.0001
Triglycerides (mg/dl)	107.5 (875; 139)	153.5 (109.5; 183.5)	157 (121; 205)	169 (144; 218)	0.63	0.04	0.13	0.0001
hsCRP (mg/dl)	1.2 (0.83; 2.81)	3.95 (2.86; 6.56)	10.25 (9.21; 17.44)	24.2 (19.87; 35.73)	<0.0001	<0.0001	0.0001	0.0001
UACR (mg/g)	17.97 (16.02; 21.36)	27.28 (21.71; 28.26)	79.83 (44.02; 116.29)	908 (527.9; 1267.15)	<0.0001	<0.0001	<0.0001	0.0001
Cystatin C (mg/L)	0.61 (0.55; 0.65)	0.81 (0.73; 0.87)	1.01 (0.95; 1.11)	1.68 (1.49; 2.00)	0.290	<0.0001	<0.0001	0.0001
Alpha1/creat (mg/g)	2.79 (2.62; 3.18)	3.78 (3.56; 4.41)	6.94 (4.6; 9.08)	51.53 (33.38; 65.15)	<0.0001	<0.0001	<0.0001	0.0001
Nephrin/creat (mg/g)	0.08 (0.037; 0.084)	0.12 (0.1; 0.15)	0.8 (0.39; 1.08)	5.92 (3.8; 8)	<0.0001	<0.0001	<0.0001	0.0001
Podocytes	0.10+/-0.30****	0.32+/-0.81****	3.14+/-3.50****	6.38+/-4.20****	0.001	<0.0001	0.01	0.0001
VEGF/creat (ng/g)	34.3 (23.5; 38.05)	84.65 (67; 92.2)	150 (111.4; 210.6)	701.26 (555.63; 1120.45)	<0.0001	<0.0001	<0.0001	0.0001
KIM-1/creat (ng/g)	42.27 (35.75; 44.41)	70.32 (59.5; 82.6)	125.33 (107.87; 137.66)	687.9 (408.06; 853.52)	<0.0001	<0.0001	<0.0001	0.0001
Urinary AGE (pg/ml)	31.3 (30.84; 32.02)	35.78 (34.27; 38.71)	65.29 (77.75; 108.2)	479.5 (248.93; 731.31)	<0.0001	<0.0001	<0.0001	0.0001
Plasma AGE (pg/ml)	282.53 (267.98 302.43)	426.43 (356.98; 501.06)	730.26 (673.56; 851.88)	4875.5 (3922.4; 6184.7)	<0.0001	<0.0001	<0.0001	0.0001
NFkB	0.1 (0.09; 0.14)	0.36 (0.28;0.42)	0.5(0.48;0.53)	0.7 (0.67; 0.72)	<0.0001	<0.0001	<0.0001	0.0001
GLEPP 1	0.1 (0.08; 0.12)	0.35 (0.28;0.4)	0.5 (0.45;0.52)	0.67 (0.63; 0.7)	<0.0001	<0.0001	<0.0001	0.0001
CD2-AP	0.15 (0.12;0.175)	0.4 (0.32;0.45)	0.52 (0.49;0.55)	0.61 (0.6; 0.7)	<0.0001	<0.0001	<0.0001	0.0001
NPHS1	0.15 (0.11;0145)	0.3 (0.24; 0.32)	0.45 (0.35; 0.5)	0.65 (0.55; 0.7)	<0.0001	<0.0001	<0.0001	0.0001
ADAM 10	0.13 (0.095;0.155)	0.35 (0.32; 0.36)	0.57 (0.42; 0.6)	0.67 (0.65; 0.75)	<0.0001	<0.0001	<0.0001	0.0001
NPHS 2	0.1 (0.06; 0.14)	0.25 (0.2; 0.3)	0.42 (0.35; 0.5)	0.65 (0.62; 0.7)	<0.0001	<0.0001	<0.0001	0.0001
Alpha actinin- 4	0.145 (0.1; 0.16)	0.22 (0.19; 027)	6.93 (6.37; 8.9)	0.68 (0.63; 0.78)	<0.0001	<0.0001	<0.0001	0.0001

Table 1. Clinical and biological data of the patients studied

Table 1. 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; NPHS1- nephrin, NPHS2- podocin, CD2AP-CD2-associated protein, GLEPP 1- glomerular epithelial protein 1, NFkB- nuclear factor kB; Clinical and biological data are presented as medians and IQR, as for variables with skewed distribution; podocytes****. expressed as means+/-SD; 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; NA- normoalbuminuria; MI- microalbuminuria; MA- macroalbuminuria

- After adjustment for potential confounders in multivariable regression analysis, such as lipid profile, HbA_{1c}, and high-sensitive C-reactive protein, urinary mRNA of podocyte-associated molecules nephrin, podocin, alpha-actinin-4, CD2AP, GLEPP1, ADAM 10, and NFkB correlated directly with urinary alpha$_1$-microglobulin, uKIM-1, UACR, uAGE, nephrinuria, uVEGF, serum cystatin C, and indirectly with eGFR (Table 2)

Parameter	Variable	Coef β	p	95% CI	Prob>F	R^2
NPHS1	Constant	0.0816	0.347	-0.0897 to 0.2529	<0.0001	0.962
	VEGF/creat	0.0005	<0.0001	0.0007 to 0.0004		
	KIM-1/creat	0.0003	<0.0001	0.0001 to 0.0004		
	UACR	0.0001	0.002	0.0001 to 0.00003		
	Cystatin C	0.566	<0.0001	0.4383 to 0.6942		
	eGFR	-0.002	<0.0001	-0.0035 to -0.0016		
NPHS2	Constant	0.0269	0.568	0.1202 to 0.0663	<0.0001	0.991
	Podocyte	0.0023	0.028	0.0002 to 0.0044		
	Nephrin	0.0218	0.002	0.0357 to 0.0079		
	Alpha1/creat	0.0074803	<0.0001	0.0044 to 0.0105		
	KIM-1/creat	0.0005	0.001	0.0008 to 0.0002		
	UACR	0.00005	0.023	7.12e-06 to 0.00009		
	Cystatin C	0.2260	0.050	0.1403 to 0.3117		
	eGFR	-0.0009	0.004	-0.0015 to -0.0003		
	Urinary AGE	0.0001	0.006	0.0002 to 0.00004		
Alpha actinin- 4	Constant	0.1808	0.029	0.0191 to 0.3425	<0.0001	0.960
	KIM-1/creat	0.00009	0.006	0.0001 to 0.00002		
	eGFR	-0.0019	0.004	-0.0032 to -0.0006		
CD2AP	Constant	0.1434	<0.0001	0.1976 to 0.0892	<0.0001	0.976
	Podocytes	0.0039	0.009	0.0069 to 0.001		
	Nephrin	0.0569	<0.0001	0.0763 to 0.0375		
	Alpha1/creat	0.0090	<0.0001	0.0132 to 0.0049		
	KIM-1/creat	0.0009	<0.0001	0.0005 to 0.0013		
	UACR	0.0001	<0.0001	0.0001 to0.00005		
	Cystatin C	0.3560	<0.0001	0.2482 to 0.4638		
ADAM10	Constant	0.0194	0.007	0.0333 to 0.0055	<0.0001	0.985
	KIM-1/creat	0.0001	<0.0001	0.0002 to 0.0001		
GLEPP-1	Constant	0.1821	<0.0001	0.2325 to 0.1316	<0.0001	0.982
	Nephrin	0.0442	<0.0001	0.0619 to 0.0265		
	KIM-1/creat	0.0002	0.001	0.00008 to 0.0003		
	Cystatin C	0.3386	<0.0001	0.2341 to 0.4432		
	Urinary AGE	0.0002	0.001	0.0004 to 0.0001		
NFkB	Constant	0.2219	<0.0001	0.2758 to 0.1680	<0.0001	0.981
	Nephrin	0.0556	<0.0001	0.0745 to 0.0366		
	KIM-1/creat	0.0003	<0.0001	0.0001 to 0.0004		
	Cystatin C	0.4651	<0.0001	0.3534 to 0.5768		
	Urinary AGE	0.0003	<0.0001	0.0005 to 0.0002		

Table 2. Multivariable regression analysis for mRNAs of podocyte-associated molecules

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; AGE: advanced glycation end-products; NPHS1- nephrin, NPHS2- podocin, CD2AP- CD2-associated protein, GLEPP 1- glomerular epithelial protein 1, NFkB- nuclear factor kB

Discussion

- the incresed levels of podocyte-associated genes were observed in all 7 podocyte markers, translating into increased excretion of podocytes into the urine of patients with type 2 DM, even in the normoalbuminuria stage
- urinary mRNA profiles of podocyte-associated genes increased with disease severity and progresion staged by albuminuria and eGFR, in conjunction with biomarkers of podocyte damage (nephrin, VEGF), and of proximal tubule dysfunction (alpha1-microglobulin, KIM-1)
- three more podocyte-specific mRNAs with high gene expression levels were identified, serving as potential additional candidate molecules of urinary podocyte biomarkers (GLEPP1, ADAM10, NFkB), even in normoalbuminuric patients

Conclusion

- in patients with Type 2 DM there is an association between urinary mRNA of podocyte-associated molecules, biomarkers of podocyte damage, and biomarkers of PT dysfunction
- this association holds true even in normoalbuminuric patients
- GLEPP1, ADAM10, and NFkB may be considered additional candidate molecules highly indicative of early DN
- AGE could be involved in this association

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