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Urinary cathepsin L is predictive of changes in albuminuria and correlates with glucosepane in patients with type 2 diabetes in a closed-cohort study

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ABSTRACT

Aims: Cathepsin D (CTSD) and L (CTSL) are lysosomal proteases which degrade and detoxify advanced glycation end product (AGE)-modified proteins which are predictive of the development of diabetic nephropathy. We aimed to quantify cathepsin levels in urine from patients with type 2 diabetes and to relate these to the amount of urinary free AGEs at baseline and with kidney function after four years of follow-up in this closed cohort study.
Methods: We established and validated a LC MS/MS method for the quantification of CTSD and CTSL in urine. Patients with type 2 diabetes were screened for diabetic kidney disease and 141 patients were seen at baseline and after four years. CTSD and CTSL and free AGEs were quantified in urine by LC MS/MS at baseline in these patients.
Results: The detection limit of CTSD and CTSL in urine was 2.4 ng/l and 19.1 ng/l, respectively. CTSD ($p < 0.0001$, $r = 0.555$) and CTSL ($p < 0.0001$, $r = 0.608$) correlated positively with albuminuria at time of recruitment. In addition levels of the proteases but not albuminuria correlated with urinary levels of the major cross-linking AGE glucosepane (CTSD: $p = 0.012$, $r = 0.225$; CTSL: $p < 0.001$, $r = 0.376$). A strong non-linear association between CTSD ($r = 0.568$), CTSL ($r = 0.588$) and change in albuminuria over four years was present. High levels of CTSL ($p = 0.007$, beta $= -0.366$) were associated with an improvement of albuminuria after four years.
Conclusions: A sensitive LC MS/MS assay for the quantification of CTSD and CTSL in urine was established. High CTSL baseline levels were associated with an improvement in albuminuria at follow-up. An increased excretion and thus detoxification of the free form of the pathogenic cross-linking AGE glucosepane could explain the positive predictive value of high CTSL levels on albuminuria.

1. Introduction

Cathepsin D (CTSD) and L (CTSL) are major lysosomal proteases responsible for the general protein turnover in soft tissue and are abundant in the distant- and proximal tubule cells of the kidney, respectively.[1–3] Both CTSD and CTSL are able to digest and protect from advanced glycation end product (AGE) modified substrates and it was previously shown that CTSL levels are elevated when AGE modified proteins increase in kidneys from diabetic rats.[4,5] Thus, elevated levels of CTSD and CTSL may reduce the amount of protein bound AGEs, resulting in an elevated excretion of free AGEs, meaning AGEs not bound to proteins. Nevertheless, the proteases may also contribute to kidney disease as overexpression of CTSL exacerbates markers of diabetic nephropathy while the inhibition of CTSD reduces fibrosis in mice.[6–9] It has also been shown that CTSL activity levels in serum correlate with albuminuria in patients with chronic kidney disease.[10] However, CTSD and CTSL levels have not yet been analysed in patients with diabetic nephropathy and the potential of these proteases as prognostic markers of kidney function has not been investigated.

Considering the current data on lysosomal proteases in experimental diabetic nephropathy we aimed to investigate whether urinary CTSD and CTSL levels are associated with traditional markers of kidney function in diabetic nephropathy and whether they can predict future

Abbreviations: AGEs, advanced glycation end-products; ARGP, argpyrimidine; ASA, acetylsalicylic acid; BMI, body mass index; CEL, carboxy ethyllysine; CML, carboxy methyllysine; CTSD, cathepsin D; CTSL, cathepsin L; CVD, cardiovascular disease; DTT, dithiotreitol; eGFR, estimated glomerular filtration rate; FL, fructosyllysine; FPG, fasting plasma glucose; GH1, glyoxal hydroimidazolone 1; HbA1C, glycated hemoglobin; hs-CRP, high sensitive C-reactive protein; LOD, limit of detection; LOQ, limit of quantification; MGH1, methylglyoxal hydroimidazolone 1; MOLD, methylglyoxal-lysine dimer; MRM, multiple reaction monitoring; RAAS, renin-angiotensin-aldosterone system; TFA, Trifluoracetic acid.

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development of these markers. Furthermore AGEs are associated with diabetic nephropathy while CTSD and CTSL can degrade AGE modified proteins possibly reducing the toxic effect of AGEs.[11] Thus, we aimed to quantify CTSD, CTSL as well as free-AGEs in urine by LC MS/MS to determine whether a connection between urinary AGE levels and urinary CTSD and CTSL levels exist and whether these are related to traditional measures of kidney disease.

A LC MS/MS method was established and validated for the parallel quantification of CTSD and CTSL in urine after trypsin digestion. In silico digestion was combined with a search of the proteomics database PeptideAtlas to identify suitable peptides. Stable isotopically labelled versions of the tryptic peptides were employed as internal standards. These provide a performance comparable to isotopically labelled proteins.[12] The quantification of a panel of eight urinary free AGEs was carried out using established methods as previously described.[13] Samples from 141 patients with type 2 diabetes which was previously described at time of recruitment and after four years of follow up were analysed in this closed cohort study.[14–16]

2. Methods

2.1. Patient selection

The procedure for the patient selection for the original trial (Clinicaltrials.gov-No: NCT00263419) was described in detail previously.[14] The main inclusion criteria was the presence of diabetic kidney disease with microalbuminuria in two separate urine samples defined as albumin-creatinine-ratio > 20 mg/g or albumin excretion rate > 30 mg/24 h. For the original study a total of 694 patients were screened of which 110 were randomised for the trial while 43 refused randomisation but were available for a longitudinal observational follow-up study. The samples used here are from this part of the study. Of the combined 153 people 12 were lost to follow-up after 1 year (too ill, declined or moved away) resulting in a total of 141 patients for the baseline measurements (1 year follow-up of stress relief study). Of the 141 patients 102 were seen again 4 years after the baseline measurement (5 year follow-up of stress relief study).

2.2. Clinical chemistry

Blood samples were taken on the day of visit and routine blood and urine parameters were analysed in the Clinical Laboratory of the University of Heidelberg using standardized and certified methods. The following parameters were quantified from blood samples: Fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), triglyceride, cholesterol and high sensitive c-reactive protein (hs-CRP). Estimated glomerular-filtration-rate (eGFR) was calculated from creatinine levels in plasma using the CKD-EPI-formula.[17] Albumin, alpha-1-microglobulin and creatinine were quantified in urine. As previously described 24-h urine samples were collected on 3 consecutive days for measurement of albumin and creatinine excretion and the mean value of the three measurements was calculated.[14,15]

2.3. Sample preparation and digestion for cathepsin analysis

Protein from 4 ml of urine from diabetic patients was precipitated on ice by addition of 80 μl of deoxycholic acid (2% *v*/v), followed by vortexing and addition of 1.3 ml of 40% TCA. Samples were incubated for 30 min on a shaker at 4 °C and centrifuged for 30 min at 4 °C. The precipitate was washed with ice cold acetone once and the pellet was dried at room temperature.

Samples were re-suspended in 80 μl of 8 M urea/0.5 M Tris HCl pH 8.0. Protein concentration was determined by BCA assay which correlates well with the gold standard of amino acid quantification in urine samples.[18] 100 μg of each sample was spiked with 10 picomol of isotopically labelled proteotypic peptide for CTSD (sequence P-CTSD: LVDQNIFSFYLSR, sequence isotopically labelled P-CTSD-H: LVDQNIFSFYLSR[^{13}C6^{15}N4]) and CTSL (sequence P-CTSL: AVATVGPISVAIDAGHES-FLFYK, sequence isotopically labelled P-CTSL-H: AVATVGPISVAIDAGHESFLFYK[D9]). Proteins were reduced with DTT and alkylated with iodoacetamide as this improves the digestion efficiency.[19] Reduction of samples was carried out in 60 μl by addition of 2.5 μl of DTT (40 mg/ml; final concentration 1.5 mg/ml) for 60 min at 37 °C followed by alkylation with 10 μl of Iodoacetamide (40 mg/ml; final concentration 5 mg/ml) for 30 min at 37 °C and quenching of remaining alkylating reagent for 30 min at 37 °C by addition of DTT (40 mg/ml; final concentration 6 mg/ml).

Ammonium bicarbonate (1 ml, 50 mM) was added to yield a final concentration of <1 M urea. Samples were digested over night at 37 °C by addition of trypsin (Promega; V5117) at a protease/protein ratio of 1/30. Samples were acidified by addition of 150 μl of 5% trifluoracetic acid (TFA)/water (*v*/v). Spin columns (C18 based MonoSpin GL Sciences, Cat. No.: 5010–21,700) were washed and equilibrated with 0.1% TFA/acetonitrile (ACN) and 0.1% TFA/water and samples were loaded onto the columns, washed with 0.1% TFA/water, eluted by addition of 0.1% TFA/ACN and freeze dried.

2.4. LC MS/MS analysis of cathepsins

A 9 point standard curve ranging from 0 to 250 nM (1/2 dilution factor) with the internal standard at 200 nM was created. Samples were re-suspended in 50 μl of 20% of ACN/water (0.1% formic acid) and 3.0 μl of standard and sample were injected. Chromatography was carried out on an Acquity UPLC I equipped with a C18 column (Waters, Acquity UPLC BEH C18, 1.7 μm 2.1x100mm) and a pre-column (Waters, Acquity HSS T3 1.8 μm VanGuard, 2.1 × 5 mm, 1.7 μm) at 30 °C and a flow rate of 0.2 ml/min. Solvents used for UPLC were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A linear gradient from 20% to 50% B for 10 min was run. Flow was switched two 0% B for 2 min followed by a 10 min gradient to 100% B and 3 min equilibration at 20%. Analysis was carried out on a triple quadrupole tandem mass spectrometer using electrospray ionization (Xevo TQ-XS, Waters, USA). The following parameters were used for LC MS/MS analysis. Ionization source temperature and the desolvation temperature were set to 150 °C and 250 °C, respectively. Cone gas flow was 150 l/h while desolvation gas flow was 600 l/h. Capillary voltage was set to 2.70 kV. Fragmentation patterns, cone voltage and collision energy were as follows:

P-CTSD: Quantifier, 801.7 > 919.6, 58, 26 Qualifier, 801.7 > 1032.7, 58, 26

P-CTSD-H: Quantifier, 806.8 > 929.6, 2, 24 Qualifier, 806.8 > 1400.0, 2, 24

P-CTSL: Quantifier, 798.3 > 976.4, 4, 18 Qualifier, 798.3 > 1112.0, 4, 14

P-CTSL-H: Quantifier, 801.2 > 980.8, 2, 20 Qualifier, 801.2 > 1116.4, 2, 18

2.5. Urinary analysis of AGEs

Urinary free AGEs were determined by isotope dilution, tandem mass spectroscopy, as previously described.[13] Briefly, an aliquot of urine (50 μl) was diluted to 500 μl with water and filtered by microspin ultrafiltration (10 kDa cut-off) at 14000 rpm for 30 min at 4 °C. The ultrafiltrate was then retained for the free adduct analysis. An aliquot of the ultrafiltrate (ca.25 μl) was spiked with an equal volume of 0.2% TFA in water containing the isotopic standards (5–25 pmol). Normal and isotopic standards were either purchased (Cambridge Isotope, Polypeptide Laboratories, Iris Biotech) or prepared in-house, as described previously.[20,21] Samples were then analysed by LC MS/MS using an ACQUITY ultra-high-performance liquid chromatography system with

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a Xevo-TQS LC MS/MS spectrometer (Waters). Two 5 μm HypercarbTM columns (Thermo Scientific) in series were used: 2.1 × 50 mm, fitted with a 5 × 2.1 mm pre-column, and 2.1 × 250 mm. The mobile phases were 0.1% TFA in water and 0.1% TFA in 50% water. The column temperature and flow rate were 30 °C and 0.2 ml/min, respectively. Analytes were eluted using a two-step gradient and the columns washed after each sample with 0.1% TFA in 50% THF, as described previously.[13] AGEs, including oxidation and nitration markers, were detected by electrospray positive ionization with multiple reaction monitoring (MRM). The ionization source temperature was 150 °C and the desolvation temperature was 500 °C. The cone gas and desolvation gas flows were 150 and 1000 l/h, respectively. The capillary voltage was 0.5 kV. Molecular ion and fragment ion masses, as well as cone voltage and collision energizes were optimized to ±0.1 Da and ± 1 eV for MRM detection of the analytes. Acquisition and quantification was completed with MassLynx 4.1 and TargetLynx 2.7 (Waters®).

2.6. Statistical analysis

Multivariable linear regression analysis was carried out using SPSS (SPSS Statistics, IBM, Version 24). Patients lost to follow-up ($n = 39$) were included in the baseline parameter measurements. In order to determine whether bias was introduced due to this loss we compared eGFR (ml/min/1.73m^2) and urinary albumin levels (mg/g) at baseline between those patients lost to follow-up and those present. Neither eGFR levels nor albumin levels differed significantly (data not shown).

Since values for alpha-1-microglobulin were below the detection limit for a large amount of the patients the parameter was excluded from multivariable analysis. Univariable linear regression analysis was carried out for significant correlations to confirm the result. A threshold of $p < 0.05$ was considered to be significant.

3. Results

3.1. Establishment of quantification of CTSD and CTSL by LC MS/MS analysis

The peptides chosen to be quantified representative for CTSL and CTSD (proteotypic peptide) were derived from the heavy chain region of CTSD and CTSL. Thus all active forms of the proteases were quantified. As cysteine and methionine oxidize easily, peptides containing these amino acids were avoided.[22]The proteotypic peptides from amino acid 223–235 of CTSD and amino acids 238–260 for CTSL fulfil requirements given above. In addition these peptides represent the most frequently observed peptide in proteomics experiments for urine according to the database at peptideatlas.org.

3.2. Validation of method

The method showed good linearity over the range of the standard curve. The limit of detection (LOD) and limit of quantification (LOQ) were determined as a signal to noise ratio of 3 for LOD and 10 for LOQ. Levels for LOD were 2.4 ng/l (CTSD) and 19.1 (CTSL) and LOQ levels were 8.0 (CTSD) and 63.8 ng/l (CTSL). The sensitivity is superior to currently available ELISAs for cathepsin D and cathepsin L.

Reproducibility was determined by repeated injection from the same vial and a CV of 0.01 was obtained. Precision was determined on a urine sample with six repetitions carried out per sample each of which had undergone the entire sample preparation procedure starting with protein precipitation. The intraassay CV was 0.08 and 0.11 while the interassay CV was 0.12 and 0.13 for CTSD and CTSL, respectively.

Samples were spiked with pure CTSD (abcam, ab91123) and CTSL (abcam, ab174030) into albumin solution at three concentrations to determine accuracy. Recovery lay between 84.6% and 106.7% for CTSD and 86.8% to 92.8% for CTSL. In order to test the accuracy in the actual matrix CTSD and CTSL was spiked into urine. Recovery in urine sample was at 96.6% (CTSD) and 118.1% (CTSL).

3.3. Cathepsins correlate strongly with albuminuria at baseline

Urinary CTSD, CTSL and AGE levels were quantified in a cohort of 141 patients with type 2 diabetes and diabetic kidney disease. All urinary values were corrected for creatinine levels. Multivariable linear regression analysis was carried out with CTSD and CTSL set as dependent variable. Baseline characteristics (Table 1), except for alpha 1-microglobuline including levels of 6 AGEs (argpyrimidine (ARGP), CEL (carboxy ethyllysine), CML (carboxy methyllysine), G-H1 (glyoxal hydroimidazolone 1), glucosepane, MG-H1 (methylglyoxal hydroimidazolone 1), MOLD (methylglyoxal-lysine dimer)) and the AGE precursor fructosyllysine (FL) were chosen as dependent variables. The marker alpha-1-microglobulin was quantitated as an indicator for damage of the proximal tubule. It was not included in the multivariable analysis as levels were below the detection limit for approximately half of the cohort ($n = 66$). When employing univariable linear regression analysis alpha-1-microglobulin were positively and more strongly associated with CTSD ($r = 0.316$; $p = 0.01$) and CTSL ($r = 0.502$; $p < 0.0001$) than with albuminuria ($r = 0.26$; $p = 0.0253$). Both, CTSD and CTSL correlated strongly and positively with albuminuria and glucosepane levels in multivariable analysis (Table 2). Weak correlations were seen with hs-CRP for both proteases while CML levels correlated with CTSL, only. Urinary levels of CTSD (Fig. 1 A) and CTSL (Fig. 1 B) were significantly higher in patients with albumin levels >300 mg/g while CTSL levels were also significantly higher in patients present with albuminuria between 30 and 300 mg/g.

The correlations of CTSD and CTSL with albuminuria (Fig. 1 C and D) as well as the correlations with glucosepane and hs-CRP were confirmed by univariable linear regression analysis (Fig. 1 E-H). Neither glucosepane nor hs-CRP correlated with albuminuria in univariable or multivariable analysis (data not shown). Also, no correlation of glucosepane with hs-

Table 1
Baseline characteristics ($n = 141$).

Patient characteristics	Mean ± SEM or n
Age (years)	59.7 ± 0.6
Gender f/m (n)	29/110
BMI (kg/m^2)	33.5 ± 0.5
Diabetes duration (years)	12.0 ± 0.7
Cardiovascular disease (n, yes (%))	35 (25.2)
Clinical chemistry	Mean ± SEM
FPG (mmol/l; mg/dl)	8.5 ± 0.3; 153 ± 4.4
HbA1c (mmol/mol; %)	56.9 ± 1.1; 7.4 ± 0.1
Albumin (mg/g)	159.5 ± 44.8
Alpha 1-microglobulin (mg/mmol)	2.0 ± 0.1
eGFR (ml/min/1.73m^2)	86.0 ± 1.7
Triglycerides (mg/dl)	197.4 ± 11.3
Cholesterol (mg/dl)	187.78 ± 3.1
hs-CRP (mg/l)	3.8 ± 0.4
Medication	n, yes (%)
Oral antidiabetics	101 (73)
Insulin therapy	88 (64)
RAAS Inhibitors	128 (92.1)
ASS therapy	81 (58.3)
Statin therapy	83 (59.7)
AGEs	Mean ± SEM
ARGP (nmol/mg)	0.14 ± 0.01
CEL (nmol/mg)	14.5 ± 0.4
CML (nmol/mg)	7.7 ± 0.3
FL (nmol/mg)	42.2 ± 1.5
GH1 (nmol/mg)	1.0 ± 0.03
Glucosepane (nmol/mg)	2.0 ± 0.06
MGH1 (nmol/mg)	42.5 ± 2.4
MOLD (nmol/mg)	0.2 ± 0.01
Protease levels	Mean ± SEM
CTSD (nmol/g)	1.4 ± 0.1
CTSL (nmol/g)	1.0 ± 0.1

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Table 2
Multivariable linear regression analysis with CTSD (left columns) and CTSL (right columns) set as dependent variable. Data sets labelled with # were log transformed for analysis.

	CTSD (nmol/g)			CTSL (nmol/g)		
	Beta	t	p	Beta	t	p
Age (years)	−0.093	−0.775	0.440	−0.042	−0.407	0.685
Gender (f/m)	0.052	0.531	0.596	−0.038	−0.475	0.635
BMI (kg/m2)	−0.134	−1.479	0.142	−0.038	−0.473	0.637
CVD (y/n)	−0.175	−2.227	0.028	−0.107	−1.639	0.104
Diabetesduration (years)	−0.123	−1.340	0.183	−0.146	−1.894	0.061
HbA_{1C} (mmol/mol)	0.002	0.021	0.983	0.090	1.021	0.309
FPG (mmol/l)	−0.005	−0.051	0.959	0.036	0.409	0.683
eGFR (ml/min/1.73 m2)	−0.036	−0.282	0.779	−0.049	−0.467	0.642
Albumin (mg/g) #	0.488	5.699	0.000	0.515	6.973	0.000
hs-CRP (mg/l) #	0.203	2.374	0.020	0.165	2.231	0.028
Triglycerides (mg/dl)	0.053	0.556	0.580	0.041	0.511	0.611
Cholesterol (mg/dl)	−0.016	−0.177	0.860	−0.111	−1.444	0.152
Oral antidiabetics (y/n)	−0.039	−0.366	0.715	−0.087	−0.974	0.332
Insulin therapy (y/n)	0.089	0.852	0.397	0.019	0.209	0.835
RAAS inhibitors (y/n)	0.080	0.968	0.336	0.085	1.191	0.236
ASA therapy (y/n)	0.097	1.175	0.243	0.078	1.094	0.276
Statin therapy (y/n)	0.103	1.134	0.259	−0.039	−0.516	0.607
ARGP (nmol/mg)	−0.048	−0.460	0.647	0.069	0.785	0.434
CEL (nmol/mg)	0.158	1.410	0.162	0.124	1.290	0.200
CML (nmol/mg)	−0.210	−1.874	0.064	−0.295	−2.993	0.003
FL (nmol/mg)	−0.070	−0.597	0.552	0.050	0.506	0.614
GH1 (nmol/mg)	0.005	0.040	0.968	0.026	0.227	0.821
Glucosepane (nmol/mg) #	0.256	2.699	0.008	0.308	3.727	<0.001
MGH1 (nmol/mg)	0.158	1.064	0.290	0.022	0.184	0.854
MOLD (nmol/mg)	−0.069	−0.680	0.498	0.028	0.332	0.740

CRP was present (data not shown). The association of CTSL with CML was not present in univariable regression analysis. Several of the urinary AGEs correlated with each other (data not shown). In particular, the cross-link glucosepane correlated positively with the glucose lysine adduct FL in univariable linear regression analysis ($r = 0.39$; $p < 0.001$) giving support to an enhanced formation of the cross-link under high glucose conditions in vivo.

3.4. Urinary cathepsins as a prognostic marker for kidney function

Next we used multivariable linear regression analysis to determine whether any of the variables were associated with changes in the glomerular filtration rate between the baseline visit and the four year follow-up. Albuminuria did not correlate with changes in eGFR (ΔeGFR) in multivariable or in univariable linear regression analysis. Statin therapy (beta = −0.390, $p = 0.006$) and CTSD (beta = −0.588, $p = 0.007$) displayed the strongest association with ΔeGFR in multivariable analysis. However, CTSD levels did not correlate with ΔeGFR in univariable linear regression analysis. Also when statin therapy was removed from multivariable regression analysis no correlation between CTSD and change in filtration rate was seen, confirming that the interaction with ΔeGFR is dependent on statin therapy.

The change in eGFR was significantly higher in patients receiving statins compared to those not on statin therapy (Fig. 2 A). Only weak associations were seen for hs-CRP, CML and Argpyr with ΔeGFR in multivariable analysis and these were not present in univariable regression analysis (data not shown). In order to validate the connection between the use of statins and ΔeGFR multivariable regression analysis was repeated. Only those patients were included whose medication status with regard to statin use did not change over four years. Significance dropped (beta = −0.450, $p = 0.069$) possibly in connection to the decrease in sample size ($n = 102$ vs. $n = 61$). Nevertheless statin use remained the strongest predictor for a decline of eGFR (data not shown).

No linear correlation was seen with changes in albumin excretion rates when both CTSD and CTSL were analysed together in the multivariable model (data not shown). However, when proteases were analysed separately from each other (Table 3), weak and strong inverse correlations with changes in albumin excretion were seen for CTSD and CTSL, respectively. The association between CTSL and delta albuminuria was also present in univariable linear regression ($p = 0.012$; $r = 0.252$). The association with albuminuria was much stronger, both for CTSD and CTSL when a quadratic function rather than a linear function was fitted (Fig. 2 B and C). Levels of CTSD and CTSL were higher in those patients where albuminuria levels improved over four years rather than worsened. In support of this observation CTSL levels are higher in those patients in which albuminuria improves significantly (-Alb >30 mg/g) after four years compared to those in which albuminuria worsened (+Alb >30 mg/g) (Fig. 2 D). A similar trend was also observed for CTSD. Correlations for other parameters significant in multivariable analysis were not significant in univariable analysis except for CML which was weakly associated with changes in albuminuria.

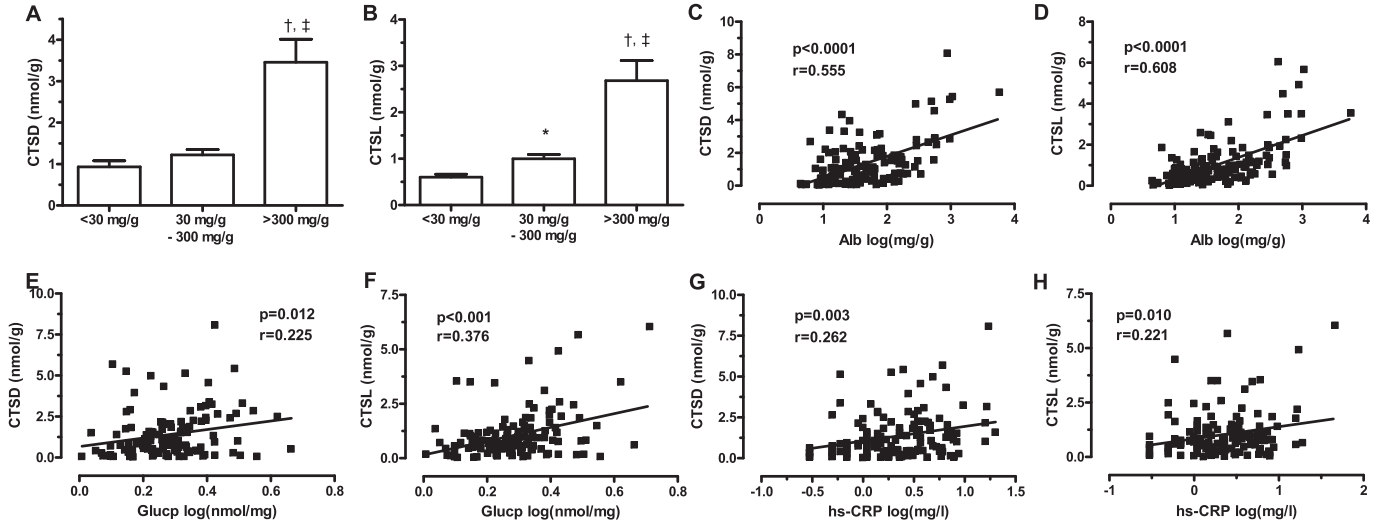

Fig. 1. Urinary cathepsin levels in diabetic patients were separated according to albumin excretion. CTSD (A) and CTSL levels (B) are higher in patients with albuminuria between 30 and 300 mg/g vs. <30 mg/g (†, $p < 0.001$) and those with albuminuria ≥30 mg/g – 300 mg/g (‡, $p < 0.001$). CTSL levels were also higher for patients with urinary albumin levels <30 mg/g vs. those with albuminuria of ≥30 mg/g – 300 mg/g (*, $p < 0.001$). Levels of CTSD (C) and CTSL (D) correlate strongly with albuminuria. CTSD- (A) and CTSL levels (B) correlate with glucosepane levels. Significance (top) and Pearson correlation coefficient r (bottom) are indicated. Similarly a correlation for CTSD (C) and CTSL levels (D) with hs-CRP levels was present. Values are given +/− SEM for A and B. Significance (top) and Pearson correlation coefficient r (bottom) are indicated for C—H.

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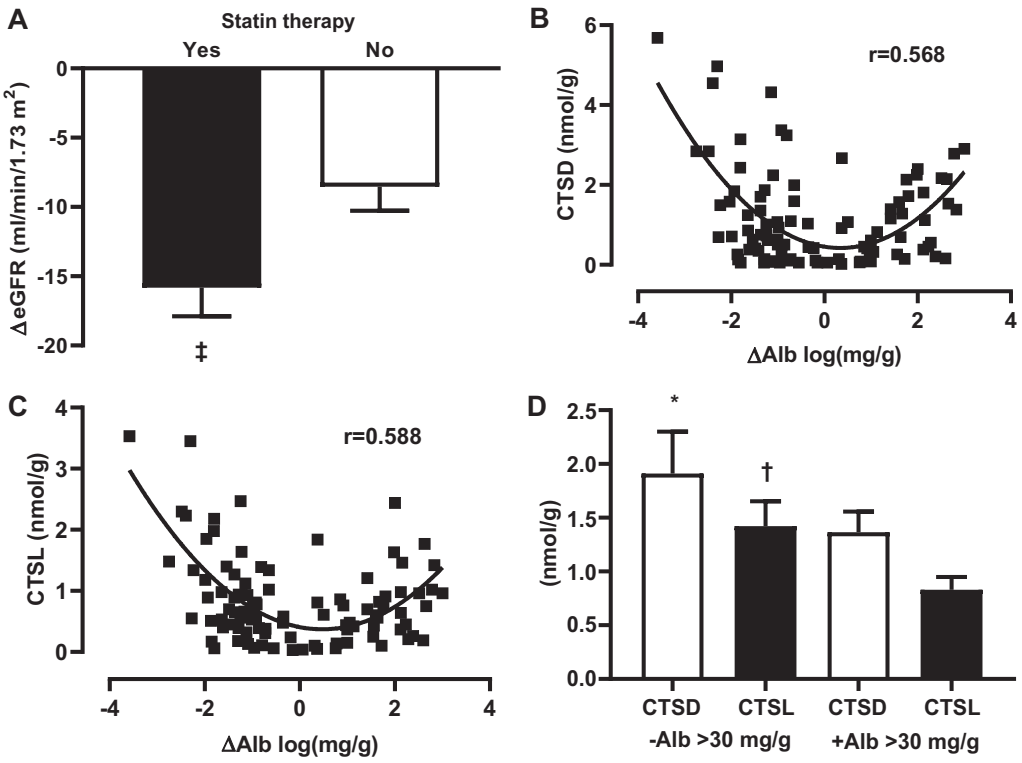

Fig. 2. The decrease in eGFR over four years is higher in patients receiving statins (A, ‡, $p < 0.01$). The association of CTSD (B) and CTSL (C) with changes in albuminuria describes a quadratic function. Levels of CTSL (†, $p < 0.05$) and CTSD (*, $p = 0.10$) were higher in those patients with significant improvements of albuminuria vs. those with worsening of albuminuria (D).

4. Discussion

We developed a sensitive and specific assay for the quantification of CTSL and CTSD in urine. The assay features higher sensitivity than currently available ELISAs and is more specific than the application of activity assays. The assay was applied to investigate the association of urinary CTSD and CTSL with kidney function in patients with type 2 diabetes as well as their value for the prognosis of kidney function. Cathepsin levels correlated strongly and positively with albuminuria at baseline. Previous cross-sectional observational studies reported an association of CTSL with proteinuria in patients with chronic kidney disease and acute kidney disease.[10,23]

No previous studies have investigated the prognostic properties of CTSD and CTSL on kidney function. Interestingly high urinary CTSL levels at baseline were the strongest predictor of improvement of albuminuria in the four year follow-up and a similar trend was seen for CTSD. In previous studies in mice a mechanistic involvement of CTSD and CTSL in the development of fibrosis and albuminuria was proposed.[7–9] Here we see a positive effect of high urinary CTSL and CTSD levels on albuminuria. A potential explanation for the positive effect of CTSD and CTSL on albuminuria is the positive association of the proteases with the free, meaning not protein bound, form of the major cross-linking AGE glucosepane. Elevated levels of CTSD and CTSL may directly contribute to an elevated excretion of free glucosepane since these proteases are able to digest AGE modified proteins.[5] This leads to the urinary excretion of free AGEs and could reduce the amount of AGEs bound to proteins. The finding that the excretion of free levels of glucosepane did not correlate with albuminuria underlines the specificity of the effect for the proteases.

Protein bound AGEs have previously been associated with the development of diabetic complications including diabetic nephropathy, diabetic neuropathy and diabetic retinopathy.[11] In contrast to the previous study only some urinary free AGEs displayed correlations with albuminuria and eGFR in multivariable analysis but these were not confirmed in univariable analysis. This underlines the importance of the type of AGEs studied. While the previous study looked at protein-bound AGEs, indicative of the glycation burden over time, free AGEs in urine were quantified in the current study. These can be considered to be indicators of the amount of toxic AGEs released from the organism.[24]

Table 3
Multiple linear regression analysis with Δalbumin set as dependent variable and CTSD (left columns) and CTSL (right columns) included in the analysis. Data sets labelled with # were log transformed for analysis.

	ΔAlbumin (mg/g) #					
	Beta	t	p	Beta	t	p
Age (years)	0.021	0.127	0.899	−0.019	−0.122	0.903
Gender (f/m)	−0.026	−0.168	0.867	−0.033	−0.241	0.810
BMI (kg/m2)	−0.038	−0.275	0.784	−0.059	−0.463	0.645
Diabetesduration (years)	−0.093	−0.692	0.491	−0.081	−0.650	0.518
CVD (y/n)	0.024	0.205	0.839	0.032	0.302	0.763
HbA1C	0.154	0.931	0.355	0.109	0.713	0.479
FPG (mmol)	0.029	0.173	0.863	0.110	0.693	0.490
eGFR (ml/min*1.73 m2)	−0.035	−0.206	0.838	−0.102	−0.655	0.515
Albumin (mg/g) #	−0.190	−10.345	0.183	−0.122	−0.887	0.378
hs-CRP (mg/l) #	0.332	2.502	0.015	0.369	3.003	0.004
CTSD (nmol/g)	−0.267	−10.924	0.059	–	–	–
CTSL (nmol/g)	–	–	–	−0.366	−2.790	0.007
Triglycerides (mg/dl)	−0.066	−0.484	0.630	−0.044	−0.361	0.719
Cholesterol (mg/dl)	0.020	0.171	0.865	0.007	0.059	0.953
Oral antidiabetics (y/n)	−0.212	−1.321	0.191	−0.192	−1.298	0.199
Insulin therapy (y/n)	0.041	0.274	0.785	−0.051	−0.371	0.712
RAAS inhibitors (y/n)	0.025	0.223	0.824	0.036	0.335	0.739
ASA therapy (y/n)	0.068	0.567	0.573	0.134	1.183	0.241
Statin therapy (y/n)	−0.326	−2.422	0.018	−0.299	−2.402	0.019
ARGP (nmol/mg)	0.207	1.299	0.199	0.147	0.971	0.335
CEL (nmol/mg)	0.548	3.205	0.002	0.492	3.069	0.003
CML (nmol/mg)	−0.444	−2.505	0.015	−0.549	−3.130	0.003
FL (nmol/mg)	−0.209	−1.240	0.219	−0.138	−0.894	0.374
GH1 (nmol/mg)	0.584	2.807	0.007	0.495	2.456	0.017
Glucosepane (nmol/mg) #	−0.003	−0.024	0.981	0.010	0.073	0.942
MGH1 (nmol/mg)	−0.571	−2.342	0.022	−0.417	−1.879	0.065
MOLD (nmol/mg)	−0.205	−1.427	0.158	−0.139	−1.058	0.294

It is unknown whether high urinary levels of CTSD and CTSL are indicative of higher expression levels, an elevated release from tissue or impaired tubular function. The latter may be the case since neither CTSD (43 kDa/38 kDa; +/−propeptide) nor CTSL (36 kDa/24 kDa; +/− propeptide) are expected to be retained by the glomerulus so that they are a marker of tubular function. In line with this both CTSD and CTSL were positively associated with the marker of tubular function alpha 1-mikroglobulin. However, in previous studies higher plasma activity levels of CTSL were also associated with albuminuria suggesting that the increased activity in urine does not solely reflect altered tubular function.[10] One potential mechanism leading to elevated CTSD and CTSL expression may be a low grade inflammation as both CTSD and CTSL levels also correlated with hs-CRP levels.[25] While such low-grade inflammation has been associated with incident diabetic nephropathy we do not see such a connection in the current study.[26,27]

The strongest predictor of worsening eGFR was the use of statins at baseline with users showing a decrease in eGFR twice as high as non-users. The significance of the association between statins and change in eGFR became weaker when only patients with unaltered medication status over four years were analysed. This can be explained by a decreased statistical power due to the reduction of the sample size ($n = 102$ vs. $n = 61$). Pre-existing cardiovascular disease and cholesterol level were not associated with changes in eGFR. However, it cannot be fully excluded that the association with statins was due to selection criteria for statin treatment in the current study. The effect of statin use reported in previous studies was mixed. One reported no effect while another reported potential harmful effects on kidney function.[28,29] Negative effects on kidney function were also seen for the use of high potency vs. low potency statins while one study reported an increased incidence of acute and chronic kidney disease with statin use.[30,31] A positive effect of statins was detected in a subgroup of patients with chronic kidney disease of stage 3b-5 and proteinuria ≥1000 mg/day.[32]

The study presented here was carried out in a well characterised cohort of patients with a reasonable follow-up time of four years. The low number of participants limits the overall impact of the current study. Thus the findings need to be reproduced in a larger patient cohort. Furthermore no kidney biopsies were obtained so that no statement about morphological changes of the kidney and the underlying kidney disease can be made. In addition it would be of interest to quantify AGE levels in urine from non-diabetic and diabetic individuals to determine the impact of glucose levels. Certain AGEs like the cross-link glucosepane do form more extensively under high glucose conditions in vitro. The current finding that the AGE precursor FL, which is a glucose lysine adduct, correlates with the cross-link glucosepane supports that AGE levels do depend on glucose levels in vivo as well.

In summary CTSD and -L levels were strongly associated with albuminuria at baseline while high levels were the strongest predictor for an improvement of albuminuria after four years of follow-up. The positive association of CTSD and CTSL with glucosepane could be indicative of an elevated release of protein bound AGEs upon proteolytic cleaved of modified proteins. The usefulness of CTSD and CTSL as prognostic marker for diabetic kidney disease will need to be evaluated further in future studies.

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Contribution statement

Sebastian Brings conceived the study, established and carried out the biochemical analysis, carried out statistical analysis and drafted the manuscript.

Thomas Fleming carried out biochemical analysis.

Stefan Kopf was responsible for the recruitment and assessment of patients and the collection of blood and urine samples and carried out statistical analysis.

Peter P Nawroth and Stephan Herzig conceived, designed and supervised the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Saftig P, Hetman M, Schmahl W, Weber K, Heine L, Mossmann H, et al. Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. EMBO J 1995;14:3599-608.
2. Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 2000;1477:98-111.
3. Goto M, Mizunashi K, Kimura N, Furukawa Y. Decreased sensitivity of distal nephron and collecting duct to parathyroid hormone in pseudohypoparathyroidism type I. J Am Soc Nephrol 2001;12:1965-70.
4. Brings S, Zhang S, Choong YS, Hogl S, Middleditch M, Kamalov M, et al. Diabetes-induced alterations in tissue collagen and carboxymethyllysine in rat kidneys: association with increased collagen-degrading proteinases and amelioration by Cu(II)-selective chelation. Biochim Biophys Acta 2015;1852:1610-8.
5. Grimm S, Horlacher M, Catalgol B, Hoehn A, Reinheckel T, Grune T. Cathepsins D and L reduce the toxicity of advanced glycation end products. Free Radic Biol Med 2012;52:1011-23.
6. Reiser J, Oh J, Shirato I, Asanuma K, Hug A, Mundel TM, et al. Podocyte migration during nephrotic syndrome requires a coordinated interplay between cathepsin L and alpha3 integrin. J Biol Chem 2004;279:34827-32.
7. Sever S, Altintas MM, Nankoe SR, Moller CC, Ko D, Wei C, et al. Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease. J Clin Invest 2007;117:2095-104.
8. Garsen M, Rops AL, Dijkman H, Willemsen B, van Kuppevelt TH, Russel FG, et al. Cathepsin L is crucial for the development of early experimental diabetic nephropathy. Kidney Int 2016;90:1012-22.
9. Fox C, Cocchiaro P, Oakley F, Howarth R, Callaghan K, Leslie J, et al. Inhibition of lysosomal protease cathepsin D reduces renal fibrosis in murine chronic kidney disease. Sci Rep 2016;6:20101.
10. Cao Y, Liu X, Li Y, Lu Y, Zhong H, Jiang W, et al. Cathepsin L activity correlates with proteinuria in chronic kidney disease in humans. Int Urol Nephrol 2017;49:1409-17.
11. Genuth S, Sun W, Cleary P, Gao X, Sell DR, Lachin J, et al. Skin advanced glycation end products glucosepane and methylglyoxal hydroimidazolone are independently associated with long-term microvascular complication progression of type 1 diabetes. Diabetes 2015;64:266-78.
12. Bronsema KJ, Bischoff R, van de Merbel NC. High-sensitivity LC-MS/MS quantification of peptides and proteins in complex biological samples: the impact of enzymatic digestion and internal standard selection on method performance. Anal Chem 2013;85:9528-35.
13. Rabbani N, Shaheen F, Anwar A, Masania J, Thornalley PJ. Assay of methylglyoxal-derived protein and nucleotide AGEs. Biochem Soc Trans 2014;42:511-7.
14. Hartmann M, Kopf S, Kircher C, Faude-Lang V, Djuric Z, Augstein F, et al. Sustained effects of a mindfulness-based stress-reduction intervention in type 2 diabetic patients: design and first results of a randomized controlled trial (the Heidelberger Diabetes and Stress-study). Diabetes Care 2012;35:945-7.
15. Kopf S, Oikonomou D, Hartmann M, Feier F, Faude-Lang V, Morcos M, et al. Effects of stress reduction on cardiovascular risk factors in type 2 diabetes patients with early kidney disease - results of a randomized controlled trial (HEIDIS). Exp Clin Endocrinol Diabetes 2014;122:341-9.
16. Kopf S, Oikonomou D, von Eynatten M, Kieser M, Zdunek D, Hess G, et al. Urinary excretion of high molecular weight adiponectin is an independent predictor of decline of renal function in type 2 diabetes. Acta Diabetol 2014;51:479-89.
17. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro 3rd AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-12.
18. Mistry HD, Bramham K, Weston AJ, Ward MA, Thompson AJ, Chappell LC. Urine protein concentration estimation for biomarker discovery. Pregnancy Hypertens 2013;3:211-4.
19. Kettenbach AN, Rush J, Gerber SA. Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. Nat Protoc 2011;6:175-86.
20. Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, et al. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 2003;375:581-92.
21. Ahmed N, Thornalley PJ. Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and intrinsic fluorescence. Biochem J 2002;364:15-24.

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22. Ren D, Pipes GD, Liu D, Shih LY, Nichols AC, Treuheit MJ, et al. An improved trypsin digestion method minimizes digestion-induced modifications on proteins. Anal Biochem 2009;392:12-21.
23. Haase M, Bellomo R, Albert C, Vanpoucke G, Thomas G, Laroy W, et al. The identification of three novel biomarkers of major adverse kidney events. Biomark Med 2014;8:1207-17.
24. Brings S, Fleming T, Freichel M, Muckenthaler MU, Herzig S, Nawroth PP. Dicarbonyls and advanced glycation end-products in the development of diabetic complications and targets for intervention. Int J Mol Sci 2017:18.
25. Kramer L, Turk D, Turk B. The future of cysteine cathepsins in disease management. Trends Pharmacol Sci 2017;38:873-98.
26. Sinha SK, Nicholas SB, Sung JH, Correa A, Rajavashisth TB, Norris KC, et al. hs-CRP is associated with incident diabetic nephropathy: findings from the Jackson heart study. Diabetes Care 2019:dc182563.
27. Sinha SK, Shaheen M, Rajavashisth TB, Pan D, Norris KC, Nicholas SB. Association of race/ethnicity, inflammation, and albuminuria in patients with diabetes and early chronic kidney disease. Diabetes Care 2014;37:1060-8.
28. Hanai K, Babazono T, Uchigata Y. Effects of statins on the kidneys in patients with type 2 diabetes. Clin Exp Nephrol 2017;21:633-42.
29. Nielsen SF, Nordestgaard BG. Statin use before diabetes diagnosis and risk of microvascular disease: a nationwide nested matched study. Lancet Diabetes Endocrinol 2014;2:894-900.
30. Dormuth CR, Hemmelgarn BR, Paterson JM, James MT, Teare GF, Raymond CB, et al. Use of high potency statins and rates of admission for acute kidney injury: multicenter, retrospective observational analysis of administrative databases. BMJ 2013;346:f880.
31. Acharya T, Huang J, Tringali S, Frei CR, Mortensen EM, Mansi IA. Statin use and the risk of kidney disease with long-term follow-up (8.4-year study). Am J Cardiol 2016;117:647-55.
32. Hu PJ, Wu MY, Lin TC, Chen TT, Wu YC, Su SL, et al. Effect of statins on renal function in chronic kidney disease patients. Sci Rep 2018;8:16276.

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