ONLINE APPENDIX

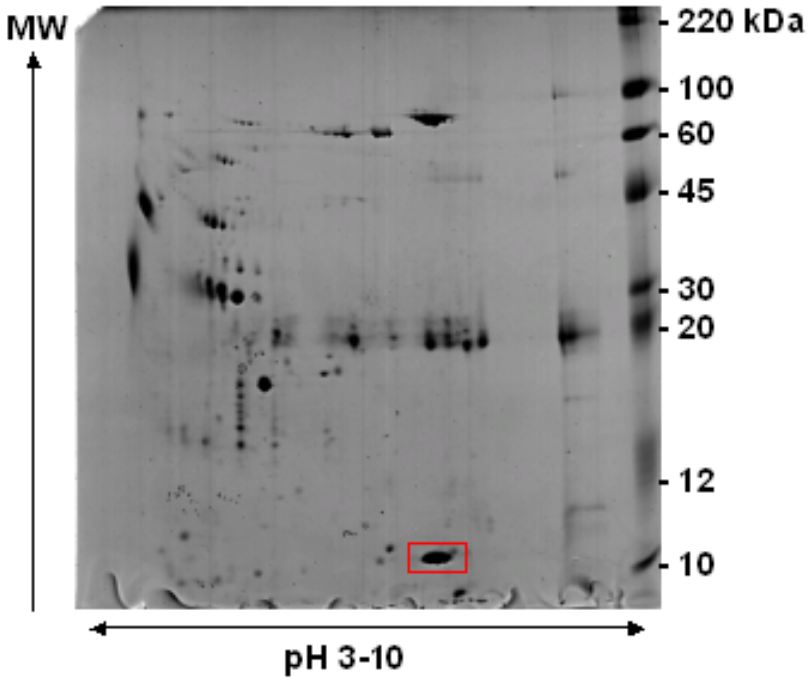
Table A1 Clinical and laboratory features of healthy subjects, type 2 diabetic patients and ND-CKD patients enrolled in the present study.

	Healthy Subjects N= 20	Normoalbuminuric Diabetic patients N=20	Microalbuminuric Diabetic patients N=18	Diabetic Nephropathy Patients N= 65	ND-CKD Non-diabetic Patients N=57	ND-CKD Diabetic patients N=10
Sex (M/F)	18/2	19/1	15/3	44/21**	39/18	7/3
Age (years)	51.3 ± 5.5	52.8 ± 7.0	55.9 ± 7.5	58.0 ± 12.2	50.5 ± 15.9 †	52.0 ± 17.8
Duration of diabetes (years)	-	7.9 ± 10.3	10.6 ± 7.6	15.4 ± 8.6**	-	7.25 ± 7.74
BMI (kg/m^2)	22.2 ± 1.7	28.4 ± 6.0	30.7 ± 11.9	27.3 ± 3.5	28.9 ± 6.8	32.6 ± 12.4
Waist circumference (cm)	88.4 ± 6.8	101.2 ± 13.2	101.3 ± 13.1	97.3 ± 13.2	100.2 ± 15.1	109.6 ± 29.5
SBP (mmHg)	113.9 ± 6.8	122.6 ± 15.8	133.8 ± 20.2	140.5 ± 17.9**	133.8 ± 16.3 †	135.5 ± 15.0
DBP (mmHg)	72.3 ± 7.4	76.3 ± 8.7	79.7 ± 5.5	81.4 ± 10.5	83.3 ± 8.5	78.8 ± 7.4
Triglycerides (mg/dl) *	134 (88-149)	122 (41-259)	161 (70-470)	175 (12-721)	155 (57-381)	240 (146-382) ‡
Total cholesterol (mg/dl)	167.7 ± 12.5	181.9 ± 29.8	191.2 ± 46.9	181.1 ± 53.8	205.1 ± 53.8 †	174.0 ± 50.6
HDL cholesterol (mg/dl)	45.0 ± 3.6	46.1 ± 9.6	43.1 ± 15.3	43.2 ± 12.7	52.7 ± 17.4 †	39.6 ± 17.8
LDL cholesterol (mg/dl)	91.3 ± 11.6	109.3 ± 24.3	108.7 ± 38.1	99.0 ± 41.5	119.5 ± 36.0 †	88.9 ± 40.8
Glycated hemoglobin (%)	4.6 ± 0.8	8.1 ± 2.2	8.6 ± 2.4	7.8 ± 1.6	-	6.5 ± 0.76
ACR (mg/mmol) *	0.5 (0.25-2.4)	0.58 (0.23-2.33)	6.9 (2.6-24.5)	99.6 (3.21-3771)***	17.4 (0.02-714) #	74.7 (4.63-714)‡
e-GFR (ml·min $^{-1}$·1.73 m^{-2})	93.0 ± 5.0	96.6 ± 28.4	80.0 ± 29.4	42.8 ± 25.3***	67.9 ± 35.9 #	54.0 ± 34.8
Smoking habit n (%)	5 (25.0)	5 (25.0)	5 (27.8)	25 (38.4) ***	9 (15.7) †	0 (0)
Antidiabetic Therapy	-					
Diet alone n (%)	-	6 (30.0)	0 (0)	9 (13.8) } **	52 (91.2) } #	3 (30) } §
OHA n (%)	-	11 (55.0)	8 (44.4)	13 (20.0) }	2 (3.5) }	3 (30) }
Insulin ±OHA n (%)	-	3 (15.0)	10 (55.5)	43 (66.1) }	3 (5.3) }	4 (40) }
Arterial Hypertension n (%)	-	10 (50.0)	15 (83.3)	52 (80.0) **	41 (71.9)	8 (80)
Treatment with ACE inhibitor	-					
/ARBs n (%)	-	7 (35.0)	13 (72.2)	48 (73.8) **	40 (70.2)	7 (70)
Dyslipidemia n (%)	-	12 (60.0)	10 (55.6)	45 (69.2)	37 (64.9)	4 (40)
Treatment with hypolipidemic drugs n (%)	-	7 (35.0)	7 (38.9)	28 (43.0)	13 (23.0)	4 (40)
Retinopathy n (%)	-	4 (20.0)	10 (55.6)	41 (63.0) **	1 (1.8) #	0 (0)

Data are presented as number (percentage), mean ± standard deviation (SD), or * median (range), as appropriate.
**p < 0.05 (comparison among NAD, MICRO and DN patients); *** p < 0.0001 (comparison among NAD, MICRO and DN patients)
# p <0.0001 (comparison between DN and ND-CKD patients); † p <0.05 (comparison between DN and ND-CKD patients)
§ p < 0.0001 (comparison between ND-CKD patients and ND-CKD in patients with Diabetes); ‡ <0.05 (comparison between ND-CKD patients and ND-CKD in patients with Diabetes).
GFR, glomerular filtration rate; OHA, oral hypoglycemic agents; ACE inhibitors, angiotensin Converting Enzyme inhibitors; ARB, Angiotensin II Receptor Blockers.

FIGURE A1. B2-MG SEPARATION BY TWO DIMENSIONAL ELECTROPHORESIS AND IDENTIFICATION BY TANDEM MASS SPECTROMETRY

1) Two mg of urine proteins pooled from 5 DN patients were denaturated (8 M urea, 2% CHAPS, 0.5% Ampholine pH 3-10, 18 mM DTT, 0.002% bromophenol blue) and loaded onto rehydrated IPG strips (13 cm immobiline DryStrip, pH 3-10 non linear range, Amersham Biosciences) and isoelectrofocusing (IEF) was performed at 40kVolt hour total producted by overnight run. After IEF, IPG strips were equilibrated in 130 mM DTT for 15 min, then for further 15 min in 270 mM iodoacetamide (IAA). The second dimension was carried out on polyacrylamide/PDA (12,5% T/ 2.6% C) slab gels in SDS-PAGE running buffer. Gels were stained by Colloidal Coomassie Blue G-250 and scanned with a flat-bed ImageScanner (Amersham Pharmacia Biotech) to generate digitized images. In figure below is shown a representative 2DE gel: MW =molecular weight. The gel spot identified as B2MG outlined in the red box.



2) MALDI-TOF/MS/MS analysis: The protein spots on 2-DE gels were manually excised, and underwent in-gel tryptic digestion by an adaptation of the procedure of Shevchenko et al [Shevchenko, A., Wilm, M., Vorm, O., Mann, M. et al. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996, 68, 850-858.].
Prior to mass spectrometry analysis, the tryptic peptide mixture was desalting and concentrated by using ZipTip® Pipette Tips packed with C_{18} resin (Millipore, Billerica-USA). The peptides were bound to ZipTips by repeated aspiration of the reaction solution, desalted by repeated aspiration with water followed by 0.1% aqueous TFA, and eluted directly onto the a Prespotted Anchor Chip™ (PAC, Bruker Daltonics, Germany) a MALDI sample carrier with readly spotted matrix (α-ciano-4-hydroxycinnaminic acid) positions besides the prespotted calibration point. After spotting the peptide mixture on the MALDI target plate it was dried under ambient conditions. The MALDI mass spectra were acquired on Autoflex III™ TOF/TOF200 instrument with smartbeam™ laser technology. All spectra were acquired in reflecting mode with 200 Hz

laser frequency, a delayed extraction time of 10, in the 500-3500m/z range. LIFT^{TM} MS/MS spectra were externally calibrated using abundant fragment ion peaks derived from bradykinin(1-7), angiotensin I, angiotensin II, substance P, bombesin, ACTH 1-17, and ACTH 18-39, ACTH1_24, Insulin_B. Precursor ions for MS/MS analysis were selected with a timed ion selector at a resolution of approximately 450. All mass values are reported as monoisotopic masses. The program used to create the ”peak list” from the raw data acquired from the FlexControl 3.3 was FlexAnalysis 3.3 with the default parameters. Protein identification was achieved by database search via Biotools 3.2 and MASCOT search algorithm (http://www.matrix.science.com) against the MSDB, NCBInr and Swissprot databases using the following parameters: Homo Sapiens as taxonomic category, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues, oxidation of methionine as variable modification, and one missing cleavage and 25ppm as mass tolerance for the monoisotopic peptide masses and 0,5Da mass tolerance for MS/MS analysis.

Information for β2-microglobulin protein sequence identified by Mascot:

Accession No. (Swiss-prot)	**MASCOT score (Swiss-prot)**	**Sequence Coverage (%)**	**No. of peptides matched**	**Peptide sequence of the peptides matched**
P61769	54	14%	2	IQVYSR VNHVTLSQPR

(Mascot Search Results below)

Mascot search results for **β2-microglobulin (B2MG)**

MATRIX SCIENCE Mascot Search Results

Protein View

Match to: **gi|34616** Score: **54**

beta-2 microglobulin [Homo sapiens]

Found in search of DATA.TXT

Nominal mass (M_r): **12905**; Calculated pI value: **5.77**

NCBI BLAST search of gi|34616 against nr

Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **14%**

Matched peptides shown in **Bold Red**

1 LALLSLSGLE AIQRTPK**IQV YSR**HPAENGK SNFLNCYVSG FHPSDIEVDL

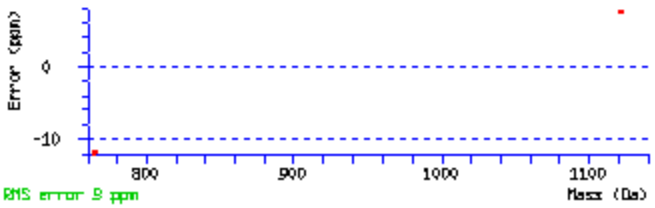
51 LKNGERIEKV EHSDLSFSKD WSFYLLYYTE FTPTEKDEYA CR**VNHVTLSQ**

101 **PK**IVKWDRDM

Show predicted peptides also

Sort Peptides By ☐ Residue Number ☐ Increasing Mass ☐ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
18 - 23	765.4163	764.4090	764.4181	-12	0	K.IQVYSR.H	(Ions score 38)
93 - 102	1122.6350	1121.6277	1121.6193	7	0	R.VNHVTLSQPK.I	(Ions score 17)



Mascot search results for **MS/MS** of **764.4** m/z peptide of **B2MG**

Mascot Search Results

Peptide View

MS/MS Fragmentation of **IQVYSR**

Found in **gi|34616**, beta-2 microglobulin [Homo sapiens]

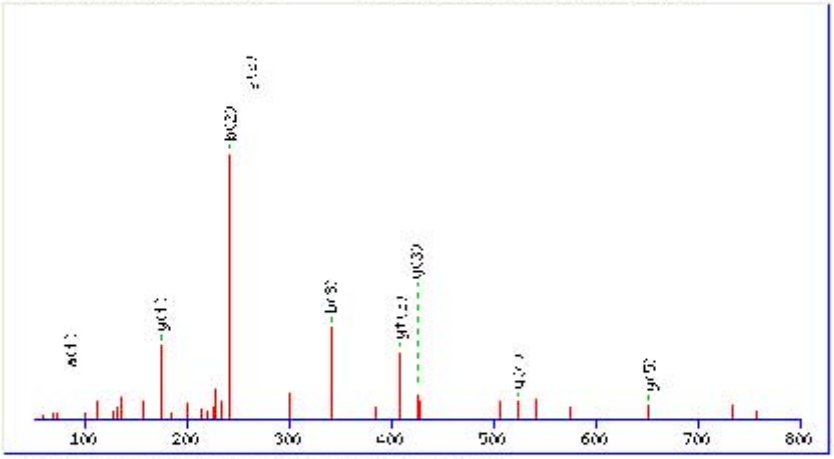
Match to Query 1: 764.409030 from(765.416306,1+) intensity(0.0000)

Data file DATA.TXT

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 50 to 800 Da Full range

Label all possible matches Label matches used for scoring



Monoisotopic mass of neutral peptide Mr(calc): 764.4181

Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)

Ions Score: 38 **Expect:** 0.044

Matches : 9/28 fragment ions using 14 most intense peaks (help)

#	a	a*	b	b*	Seq.	y	y*	#
1	86.0964		114.0913		I			6
2	214.1550	197.1285	242.1499	225.1234	Q	652.3413	635.3148	5
3	313.2234	296.1969	341.2183	324.1918	V	524.2827	507.2562	4
4	476.2867	459.2602	504.2817	487.2551	Y	425.2143	408.1878	3
5	563.3188	546.2922	591.3137	574.2871	S	262.1510	245.1244	2

Mascot search results for **MS/MS** of **1121.6** m/z peptide of **B2MG**

Mascot Search Results

Peptide View

MS/MS Fragmentation of VNHVTLSQPK

Found in gi|34616, beta-2 microglobulin [Homo sapiens]

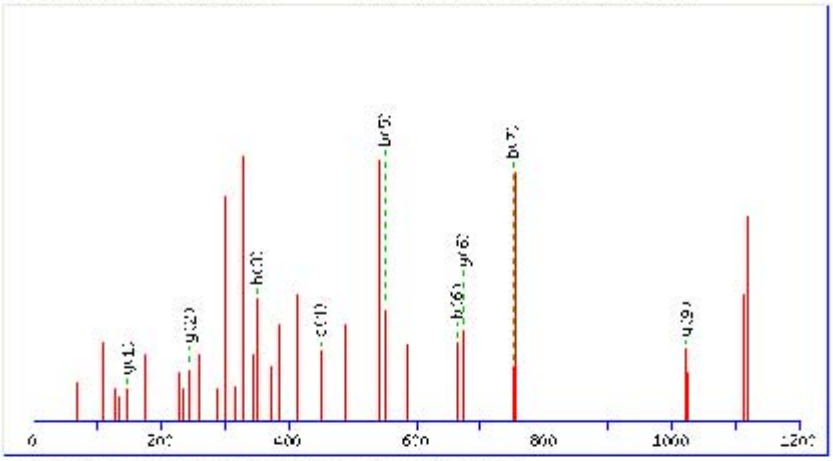
Match to Query 4: 1121.627710 from(1122.634986,1+) intensity(0.0000)

Data file DATA.TXT

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 0 to 1200 Da Full range

Label all possible matches Label matches used for scoring



Monoisotopic mass of neutral peptide Mr(calc): 1121.6193

Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)

Ions Score: 17 **Expect:** 4.5

Matches : 9/52 fragment ions using 34 most intense peaks (help)

#	a	a*	b	b*	Seq.	y	y*	#
1	72.0808		100.0757		V			10
2	186.1237	169.0972	214.1186	197.0921	N	*1023.5582*	1006.5316	9
3	323.1826	306.1561	*351.1775*	334.1510	H	909.5152	892.4887	8
4	422.2510	405.2245	*450.2459*	433.2194	V	772.4563	755.4298	7
5	523.2987	506.2722	*551.2936*	534.2671	T	*673.3879*	656.3614	6

EVALUATION OF B2-MG URIBNARY EXCRETION BY ELISA

Urinary B2-MG ha been measured by ELISA kit (Alpha Diagnostic International, San Antonio, Tex, USA) according to manufacturers' instructions. Briefly urine pH was firstly adjusted to 8 by adding, if necessary, 1N Na-OH then 10 µl of B2-MG standards (0-150 ng/ml) and urine samples were loaded in appropriate wells in duplicate and, after the addition of 100 µl antibody-enzyme

conjugate, the incubation was carried out for 60 minutes at room temperature. At the end of the reaction, the plate was washed five times with 1x wash buffer then 100 µl horseradish peroxidase solution was added to each well and incubated at room temperature for 15 minutes. Fifty µl stop solution was further added to each well and the adsorbance at 450 nm was finally measured using an ELISA reader within 30 minutes.

FIGURE A2. SUPPORTING INFORMATION FOR THE PURIFICATION AND IDENTIFICATION OF URINE UBIQUITIN

One mg Lyophilized ubiquitin standard (Sigma Aldrich, USA) was resuspended in 1ml ultrapure water (Milli Q- Millipore, Bellerica,USA) then 10 µl were diluted 2:3 (v/v) with denaturing buffer solution (9 M Urea, 2% CHAPS and 100 mM DTT) and analysed by CM10 ProteinChip array (BIORAD)according to manufacturer's instructions. After the spectra acquisition, ubiquitin mass peak was detected by DataManager 3.5 software (BIORAD, Hercules, CA, USA) and its molecular weight and shape was used to identify the corresponding peak within the mass spectra of the patients enrolled in the present study.
Furthermore, urinary ubiquitin was immunoprecipitated from 500 µg urine proteins of 6 DN and 8 nd-CKD patients by means of 50 µg ubiquitin monoclonal antibody (Abcam, Cambridge, UK) coupled to µl 200 protein G resin (Pierce Crosslink IP Kit, Thermo Scientific, Rockford, USA) following manufacturer's instruction.
At the end of the procedure, the eluted (IP) ubiquitin of each patient was diluted 2:3 (v/v) in denaturing buffer solution and analysed by CM10 ProteinChip array.
The figure below shows the correspondence of the ubiquitin standard with the ubquitin peak in the whole urine profile and the ubiquitin IP of the same patient. Of note, the ubiquitin peak nearly disappeared in the IP flowthurough after immunoprecipitation procedure.