



**MEASUREMENT OF ALBUMINURIA WITH SIZE-EXCLUSION
CHROMATOGRAPHY
CHARACTERIZATION AND NEW PERSPECTIVES**

PHD THESES

SUMMARY

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ABBREVIATIONS

ACN	acetonitrile
ACR	albumin-creatinine ratio
AusDiab	Australian Diabetes, Obesity and Lifestyle
CD	Crohn's disease
CV	coefficient of variation
DMR	dimeric to monomeric ratio of urinary albumin
DTNB	5, 5'-dithio-bis (2-nitrobenzoic acid)
FDA	Food and Drug Administration
GSA	glycated human serum albumin
GSH	reduced glutathione
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IN	immunonephelometry
ir-uAlb	immunoreactive urinary albumin
IT	immunospectrophotometry
LDL	low-density lipoprotein
MALDI-TOF/MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MGO-HSA	human serum albumin modified with methylglyoxal
MM	patients microalbuminuric using both IN and HPLC methods
MS	mass spectrometry
NM	patients normoalbuminuric by IN, microalbuminuric by HPLC method
NN	normoalbuminuric patients using both IN and HPLC methods
PMF	peptide mass fingerprinting
RF	relative fluorescence
RP	reversed-phase
SD	standard deviation of mean
SDS-PAGE	sodiumdodecylsulphate polyacrylamide gel-electrophoresis
SE	size-exclusion
TFA	trifluoroacetic acid
TFSG	total free sulfhydryl groups
t-uAlb	total urinary albumin
UAC	urinary albumin concentration
uAlb	urinary albumin

1. INTRODUCTION

Accurate measurement of the urinary excretion of albumin (albuminuria) is of great importance to be able to identify those at risk in order to be able to start treatment. Recently, a new method has been developed to measure albuminuria and some aspect of this method has been investigated in this thesis. This introduction aimed to give a short overview about albumin, its role as a risk marker and its measurement.

1.1. DEFINITION AND PROPERTIES OF ALBUMIN

Albumin is one of the longest known and probably the most studied of all proteins. By definition, the term “albumin” refers to any proteins that are soluble in water and in moderately concentrated salt solution, and that are coagulable on heating. The human serum albumin (further referred as albumin) is the most abundant protein in human blood plasma, synthesized by the liver. Constituting almost 60% of the total plasma protein, albumin is responsible for approximately 70% of the colloid osmotic pressure, and binds a variety of ligands such as fatty acids, metal ions, pharmaceuticals, and metabolites, playing a significant role in drug delivery, efficacy and detoxification. Because of its only free cysteine residue albumin is the major extracellular source of thiols and acts as scavenger of reactive oxygen and nitrogen species.

1.2. ALBUMINURIA AS A WELL-ESTABLISHED RISK MARKER

Under physiological conditions albumin is excreted in the urine in very small amounts of less than 30 mg per day. Persistent albuminuria in the range of 30-300 mg/day (microalbuminuria) is recognized as one of the earliest indicators of nephropathy in patients with type 1 or type 2 diabetes mellitus and a marker of progressive kidney disease. Moreover, it has been recognized as a powerful marker and predictor for cardiovascular disease and overall mortality in diabetes and in the general population, as well.

Given the fact that diabetes mellitus and cardiovascular disease are the leading cause of death in industrialized countries, accurate measurement of albuminuria is of great importance.

1.3. MEASUREMENT OF ALBUMINURIA

The very first laboratory tests developed to detect urinary albumin (dipstick tests) could only estimate concentrations of 300 mg/24 hour and above. The first analytical test that

could measure lower albumin concentrations was a radioimmunoassay, using ^{125}I labeled albumin which is based on immune reaction. Unfortunately, this method was time-consuming and too expensive for routine laboratory measurement. Therefore other immuno-based (immunonephelometry (IN) and immunoturbidimetry (IT)) automatic assays have been developed where the albumin containing sample (serum or urine) is mixed with albumin-antibody, resulting in small aggregates. These aggregates will scatter light and the amount of scatter is measured. In the clinical setting, assessment of microalbuminuria (30-300 mg/day by immuno-based methods) has been established as a valuable risk marker.

Recently, a high-performance liquid chromatography (HPLC) method based on size-exclusion has been developed to detect albuminuria. The very first study using this new method has shown that urinary albumin concentration in diabetic patients is significantly higher compared with conventional assays. Urinary albumin measured by HPLC is referred as total urinary albumin. The fraction of albumin which is not detectable by conventional immunochemical methods, but which can be measured by HPLC is referred as immuno-unreactive, nonimmunoreactive or immunochemically nonreactive albumin.

2. AIMS

2.1. MEASUREMENT OF MODIFICATION AND INTERFERENCE RATE OF URINARY ALBUMIN DETECTED BY SIZE-EXCLUSION HPLC (PART I OF THIS THESIS)

After the introduction of the new HPLC method for the measurement of albuminuria some authors proposed that oxidative stress-induced modification of albumin could be one of the reasons for immuno-unreactivity, while other authors proposed that the size-exclusion HPLC method does not have sufficient resolution to separate albumin from other similar molecules of similar size. First aim of the PhD thesis was to address these questions.

- Therefore a HPLC-based method has been worked out and applied for studying the relation between the proposed oxidative stress-induced modification and the immuno-unreactivity.
- The role of interference with other substances affecting the detection has also been considered.

- Our aim has also been to measure glycooxidative modifications of total urinary albumin in samples of patients with diabetes mellitus and reveal possible connection with clinical parameters.

2.2. HPLC-MEASURED ALBUMINURIA AND STORAGE OF SPECIMENS (PART II OF THIS THESIS)

Since the introduction of the new HPLC-based urinary albumin measurement, several studies proved that HPLC detects more albumin (firstly only in diabetic patients, later in the general population, as well) than the immuno-based methods. However, the clinical significance of the measurement of the total albumin remained unclear. The first paper which aimed to address this question was the reevaluation of the longitudinal Australian Diabetes, Obesity and Lifestyle (AusDiab) study. The authors tested the hypothesis whether HPLC-detected albuminuria identifies more patients at risk of mortality than IN and they found that each test has a similar ability to predict mortality. For the calculation they used the data for IN-measured albuminuria what were measured in fresh urine at the time of the original collection (1999-2000) and for HPLC what were measured in stored urine (at first thaw after storage at -80°C) in 2007.

However, it was already questioned by conventional immuno-based assays whether storage of samples at -20°C , but also at -80°C , is permissible for the correct assessment of albumin in the urine. Moreover, it was not even known how HPLC-detected total albumin affected by long-term storage and if so what factors could play a role. Therefore the second aim of the PhD thesis was to elucidate these open questions.

- We aimed to determine changes of HPLC-detected albuminuria - regarding both HPLC-detectable dimeric and monomeric albumin forms - in 2.5 years deep-frozen (-80°C) urine samples.
- Since it has been suggested that urinary pH is a determinant of urinary albumin decrease we aimed to examine possible pH-dependency of decline of albumin concentration.
- And since it was also proposed that non-immunoreactive form of albumin is a partially cleaved form of albumin which is maintained in an intact relative molecular mass (66 kDa) by the help of the disulfide bonds we hypothesized that the reduction of these disulfide bonds could also play a role in the measurement of total urinary albumin by HPLC. Therefore we aimed to assess the reducing

capacity of stored and fresh urines by measuring the total sulfhydryl groups of the urine samples

2.3. NEW POTENTIAL BIOMARKERS DISCOVERED BY MEASURING ALBUMINURIA WITH HPLC IN A CROHN'S DISEASE PATIENT (PART III OF THIS THESIS)

Although clinical application of albuminuria is still largely limited to the area of diabetes it has been shown in several other clinical disorders that measurement of albuminuria can be a valuable marker. Measurement of albuminuria by immuno-based methods has been shown to have the potential to be an objective marker in the monitoring of disease activity and response to treatment in inflammatory bowel diseases. However, the HPLC-measured total albuminuria was not yet addressed. As a third part of this thesis we followed up a young Crohn's disease patient with frequent exacerbation phases.

- We aimed to measure the changes of the concentration of total albumin in the course of his disease compared to the measured concentration by immuno-based methods.
- The surprising high difference between the two methods led us to further analyze the albumin peak of the size-exclusion chromatography of the Crohn's disease patient. Therefore we further aimed to apply techniques (reversed-phase HPLC, sodiumdodecylsulphate polyacrylamide gel-electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)) that allow us the identification of possible biomarkers.

3. METHODS

3.1. MEASUREMENT OF MODIFICATION AND INTERFERENCE RATE OF URINARY ALBUMIN DETECTED BY SIZE-EXCLUSION HPLC (PART I OF THIS THESIS)

3.1.1. PREPARATION OF THE DIFFERENT FORMS OF ALBUMIN IN VITRO

In order to decide whether glycoxidative modification alters albumin immunoreactivity we used in our experiments different forms of albumin, namely human serum albumin (HSA; A9511, Sigma-Aldrich Co., St. Louis, MO, USA), glycated human serum

albumin (GSA; A8301, Sigma-Aldrich Co., St. Louis, MO, USA) and human serum albumin modified with methylglyoxal (MGO-HSA). We applied MGO since it is proven to be the most important advanced glycation end product forming agent. MGO-HSA was prepared as follows: 6.6 mg/ml HSA was incubated with 1 mM methylglyoxal (M0252, Sigma-Aldrich Co., St. Louis, MO, USA) in sodium phosphate buffer, pH=7.4, at 37°C for 24 hours, under aseptic conditions. After the incubation time MGO-modified albumin was dialyzed against ammonium bicarbonate buffer (pH 7.9) at 4 °C for 72 hours to remove excessive MGO. A solution of 6.6 mg/ml of HSA and GSA were prepared, as well. The solutions of HSA, GSA and MGO-HSA were 50-fold diluted, then serially diluted to get the following concentrations: 132, 66, 33, 16.5 and 8.25 mg/l.

3.1.2. PREPARATION OF THE URINE OF PATIENTS WITH DIABETES MELLITUS

The procedures used were approved by the Ethical Committee of the Medical Faculty of the University of Pécs, Hungary. Seventy-nine patients with type 1 (n=20) or type 2 (n=59) diabetes mellitus with previously IN diagnosed normoalbuminuria (n=59) and microalbuminuria (n=20) were enrolled in a cross-sectional study. Patients with acute diseases, fever and/or suffering haemodynamic stress as well as pregnant or menstruating woman were excluded from the study.

The first morning urine specimen was collected from each patient. Urine samples were stored at -80°C for a maximum of 2 weeks before measurement. They were thawed to room temperature, vortexed and centrifuged (2500 x g) for 10 minutes before use. Supernatant of the urine was used for further examination.

Age, gender, type of diabetes mellitus, type of medications, smoking habits, systolic and diastolic blood pressure and body mass index were recorded from patient histories. Urine pH was measured with a microprocessor-based pH meter (HI 9024 pH-meter, Geo Scientific Ltd., Vancouver, British Columbia, Canada). All other clinical parameters such as plasma glucose, fructosamine, haemoglobin A_{1c}, total-, low-density lipoprotein- (LDL), high-density lipoprotein- (HDL) cholesterol, total blood count, serum creatinine were determined with routine laboratory diagnostic at the Department of Laboratory Medicine of the University of Pécs. The estimated glomerular filtration rate was calculated using the Cockcroft-Gault formula.

Because of the fact that first morning urine samples were used, urinary creatinine levels were measured as well as part of routine laboratory work by buffered kinetic Jaffé reaction without deproteinization. (Cobas Integra 400, Roche, Germany), and albumin-creatinine ratios were calculated for both IN and HPLC-measured albumin concentrations.

3.1.3. MEASUREMENT OF THE CONCENTRATION OF ALBUMIN

The in vitro prepared different forms of albumin as well as urinary albumin concentrations were measured in duplicate by means of IN (IMMAGE Immunochemistry Systems, Beckman Coulter Inc., Fullerton, CA, USA, sensitivity (quantitation limit): 2 mg/l, linearity: 2-8640 mg/l, inter-assay and intra-assay precision (percentage coefficient of variation) 8 % and 5 % respectively) in the routine laboratory diagnostic, and by means of the size-exclusion HPLC method (Shimadzu SPD 10AVvp, Shimadzu Corp., Japan) using a Food and Drug Administration (FDA) approved AccuminTM kit (Accumin Diagnostics Inc., New York, NY, USA, sensitivity (quantitation limit): 3 mg/l, linearity: 3-2000 mg/l, inter-assay and intra-assay precision (percentage coefficient of variation) 5.8 % and 2.5 % respectively). The AccuminTM kit contained a Zorbax Bio-Series GF 250 column and Zorbax Diol guard column (both from Agilent Technologies Inc., Santa Clara, CA, USA). The mobile phase was phosphate buffer saline (pH=6.93, provided with the kit). The HPLC system used for the measurements was consisted of DGU-14A four-line vacuum membrane degasser, a FCV-10ALvp solvent proportioning valve, a LC-10ADvp solvent delivery unit, a SIL-10ADvp autosampler, a SPD-10AVvp UV-VIS detector and a SCL-10Avp system controller (all parts purchased from Shimadzu Corp., Kyoto, Japan). During the HPLC measurements 25 µl of the samples (in vitro prepared albumin or centrifuged urine) were used. Absorbance was measured at 214 nm. The time program included 6 min at flow rate of 0.5 ml/min, then a ramp up to 2 ml/min and washing time of 6.5-11.5 min. Then ramping down to 0.5 ml/min in 0.5 min and washing were employed until a steady baseline was observed (usually until 22 min). The peak retention time of albumin was within ± 2 % of the elution time of the monomer albumin under the circumstances recommended by the manufacturer. Data acquisition was carried out with LCSolution software (Ver.: 1.11 SP1, Shimadzu, Japan).

3.1.4. MEASUREMENT OF THE MODIFICATION RATE OF ALBUMIN

To be able to measure the modification rate of the albumin in the same run of the same sample the UV detector of the size-exclusion HPLC was coupled to the fluorescent detector (Shimadzu RF 10AXL, Shimadzu Corp., Japan). Fluorescence was recorded at characteristic wavelengths of glycoxidative modification (370 nm of excitation and 440 nm of emission). Sensitivity and gain of the fluorescent detector was set to the maximum for the first 6 min, then set to medium until the end of the sample running. Integration of the chromatograms was carried out to baseline using LCSolution software (version 1.11 SP1, Shimadzu, Japan). In order to calculate the modification rate of albumin we have introduced the concept of relative fluorescence (RF) which was calculated as follows:

$$\frac{\text{Fluorescence peak area of albumin}}{\text{UV peak area of albumin}} = \text{RF}$$

3.1.5. ASSESSMENT OF THE INTERFERENCE RATE OF ALBUMIN PEAK OF SIZE-EXCLUSION HPLC

The purity of albumin peak was assessed in a separate experiment carried out with reversed-phase (RP) HPLC. For these studies eight urine samples of the diabetic patients were randomly chosen. Albumin fraction of size-exclusion HPLC was collected from each urine sample of three consecutive runs. The collected fraction was desalted and concentrated with Ultracel YM-3 Centricon centrifugal filter devices (Millipore, MA, USA) to a final volume of 150 μ l. These samples were analysed further using a RP-HPLC method.

For the separation a lately developed non-porous Kovalis MS C18 column (particle size: 1.5 μ m, 33 \times 4.6 mm, Zeochem AG, Uetikon, Switzerland) was used, which enables a short analysis time and sensitive separation of complex samples. A gradient consisting of eluent "A" (0.1% trifluoroacetic acid (TFA) and 5 % acetonitrile in water) and eluent "B" (0.1% TFA and 5 % water in acetonitrile) was employed at 1 ml/min flow rate. The applied gradient was the following: 0-20 min: ramp up from 0 % "B" to 60% "B" , 20-25 min: ramp up from 60 % "B" to 100% "B". The HPLC instrument was built up from a Dionex P680 gradient pump and a Dionex UVD170U UV-VIS detector (Germering, Germany). Data analyses were carried out by Chromeleon software (version 6.60 SP3, Sunnyvale, CA, USA).

Chromatograms obtained during RP-HPLC presented two to three peaks with a very small elution time difference. The albumin peak was identified in each case with external albumin standard. Due to the small elution time difference of the peaks interference could be assessed by calculating the ratio of non-albumin peak area to the total peak area.

3.1.6. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and MedCalc (MedCalc Software, Mariakerke, Belgium) programs. The Bland-Altman bias plot was used to compare the IN and HPLC methods. Data of normal distribution were analyzed by one-way ANOVA and Pearson's correlation. Data of non-normal distribution were analyzed with the Kruskal-Wallis test, the Mann-Whitney U test and Spearman's rho correlation. Chi-square tests were used to compare categorical data. Data with normal distribution are presented as mean \pm SEM., while data with non-normal distribution are presented as median and interquartile ranges. P values <0.05 were considered to be statistically significant. Forward multivariate stepwise linear regression analyses were performed to determine the independent predictors of the RF of urinary albumin.

3.2. HPLC-MEASURED ALBUMINURIA AND STORAGE OF SPECIMENS (PART II OF THIS THESIS)

3.2.1. STUDY POPULATION

In 2005 patients with type 2 diabetes mellitus (n=30), attending the 2nd Department of Medicine and Nephrological Center, Pécs, Hungary with previously IN diagnosed normo- and microalbuminuria, were enrolled in a cross-sectional study. Patients with acute diseases, a fever and/or suffering haemodynamic stress as well as pregnant or menstruating woman were excluded from the study. To assess total free sulfhydryl groups (TFSG) of fresh urine samples, another 30 IN diagnosed normo- and microalbuminuric type 2 diabetic patients, attending the Department, were included in the study in 2008. The clinical characteristics of these patients did not differ from those patients with stored urine. Both studies were approved by the Ethical Committee of the Medical Faculty of the University of Pécs, Hungary.

3.2.2. LABORATORY METHODS

Urinary albumin concentration (UAC) of fresh urine (first morning urine, centrifuged at 2500xg for 10 min, separated in three polypropylene aliquots and kept at -80°C for a maximum of 2 weeks before use) was assessed by the previously in detail described (3.1.3), FDA-approved HPLC Accumin™ kit at the time of the original collection (2005) and in 2008. Routine laboratory parameters of patients were measured as well as urinary pH by a microprocessor-based pH meter (HI 9024 pH-meter, Geo Scientific Ltd., Vancouver, British Columbia, Canada) and both dimeric and monomeric forms of urinary albumin (assessed with Accumin™ kit according to the guidelines of the manufacturer as the peak immediately preceding the albumin peak is that of albumin dimer) and dimeric to monomeric ratio of urinary albumin (DMR) was calculated. Presence and accuracy of elution time of dimeric form were verified using the spike recovery method by adding external human albumin standard (containing both forms of albumin) to the samples.

After 2.5 years of -80°C storage one of the two never used aliquots of the patients' urine was thawed and UAC was measured by the same HPLC method. We have measured both dimeric and monomeric form of urinary albumin and DMR was calculated again.

3.2.3. MEASUREMENT OF THE CONCENTRATION OF THE TOTAL FREE SULFHYDRYL GROUPS

TFSG of the stored and of newly collected fresh urine samples were also measured. Urine preparation was the same as for the UAC measurements. Briefly, in excess (final concentration of 100 µM) 10 µl of colorimetric Ellman's reagent, 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, Schnellendorf, Germany) was added to 0.98 ml of urine in a 3 ml quartz cuvette. Maximum absorbance was measured against urine not containing DTNB at 412 nm with Hitachi U-2001 double-beam Spectrophotometer, Tokyo, Japan during a 3600 sec time scan. As baseline was reached (reaction completed) 10 µl (final concentration of 10 µM) of freshly prepared reduced glutathione (GSH) (Sigma-Aldrich, Schnellendorf, Germany) was added to the samples and absorbance elevation was measured again. From these data TFSG of urine (in GSH equivalent unit) could be calculated as follows: maximum absorbance with GSH minus maximum absorbance with DTNB (delta), then the maximum absorbance with DTNB divided by the delta and multiplied by 10 to get µM equivalent. Both stored and freshly

collected urine samples were measured at room temperature. Measurement of TFSG in the fresh urine samples was performed in 1 hour.

3.2.4. STATISTICAL ANALYSES

Statistical analysis was performed using the SPSS 13.0 (SPSS Inc., IE, USA) software. Wilcoxon tests were used to test changes in stored urine and paired-samples t-test to test changes in DMR. Independent samples t-tests were used to test differences between fresh and stored urine and to compare the clinical characteristics of the two study populations. Correlation analyses were carried out using Pearson's correlation. Chi-square tests were used to compare qualitative data. Data are presented as mean \pm SD. P values <0.05 were considered as statistically significant.

3.3. NEW POTENTIAL BIOMARKERS DISCOVERED BY MEASURING ALBUMINURIA WITH HPLC IN A CROHN'S DISEASE PATIENT (PART III OF THIS THESIS)

3.3.1. STUDY PATIENT

A 23-year-old non-smoker Hungarian male patient suffering frequent exacerbations from CD was involved in a pilot study. CD was previously (2006) diagnosed on the basis of endoscopy (Montreal classification A2, L1, B1) and histology. The patient attended the 2nd Department of Medicine and Nephrological Center, Pécs, Hungary and suffered from no other disease than CD. His regular medication included oral mesalamine (3x1000 mg/day) and azathioprine (2.5 mg/kg/day). During acute phase regular medication was supplemented with parenteral steroid (methylprednisolon 1 mg/kg/day). To assess disease activity, the Crohn's Disease Activity Index was used. Scores \geq 150 are defined as active.

First morning urine samples were obtained from the patient at the time of clinical visits. Urine samples were vortexed and centrifuged (2500xg for 10 min) and were used for analysis immediately. At the time of his clinical visits samples were taken for routine biochemistry. All routine laboratory measurements were carried out at the Institute of Laboratory Medicine of the University of Pécs. Aliquots of urine and serum samples were reserved at -80°C for later examinations, as well. The study was performed in accordance with the ethical standards as formulated in the Helsinki

Declaration and was approved by the Ethical Committee of the Medical Faculty of the University of Pécs, Hungary.

3.3.2. URINARY ALBUMIN ASSAYS

Concentrations of immunoreactive urinary albumin (ir-uAlb) were measured in duplicates by means of IT (Roche Diagnostics GmbH, Mannheim, Germany) using Roche/Hitachi 812 Modular P analyzer (sensitivity: 3 mg/l, linearity: 3-3000 mg/l, inter-assay and intra-assay precision 4.3% and 2.6% respectively). Concentrations of total urinary albumin (t-uAlb) were measured in triplicates by the previously described (3.1.3) SE-HPLC protocol.

3.3.3. REVERSED-PHASE HPLC ANALYSIS OF THE ALBUMIN PEAK OF SIZE-EXCLUSION HPLC

Central fractions of albumin peaks of SE-HPLC were collected and prepared as previously described (3.1.5). Eluted peaks were collected, evaporated to dryness and were analyzed with MALDI-TOF/MS directly after taken up in 5 µl bidistilled water or after in solution digestion according to Shevchenko.

3.3.4. GEL-ELECTROPHORETIC STUDIES

Central fractions of albumin peaks from SE-HPLC were collected and prepared as described earlier. Due to the high concentration of salt of the size-exclusion fraction, additional desalting prior to sodiumdodecylsulphate polyacrylamide gel-electrophoresis (SDS-PAGE) was performed. The salt-free sample was evaporated to dryness and the proteins were taken up in 5 µl bidistilled water.

Thus prepared samples were separated by SDS-PAGE according to Laemmli. Two µg protein per lane was analyzed in a 12.5 % gel. Detection of protein fractions was performed by silver post-intensification according to Willoughby following the traditional Coomassie brilliant blue R-250 staining. Proteins identified were excised from gel and after in-gel digestion according to Shevchenko were analyzed by MALDI-TOF/MS.

3.3.5. MALDI-TOF/MS MEASUREMENTS

An Autoflex II MALDI instrument (Bruker Daltonics, Bremen, Germany) was employed for the mass spectrometric measurements. For the measurement of the

digested proteins 8 mg of α -cyano-4-hydroxycinnamic acid was dissolved in 1 ml of 50 % ACN and 0.1 % TFA in water. For the measurement of intact proteins a saturated sinapinic acid matrix was prepared in 50 % v/v ACN and 0.1 % TFA in water. In each case 1 μ l of the matrix was deposited on a stainless steel target together with 1 μ l of the sample. All mass spectra were monitored in positive mode with pulsed ionization ($\lambda = 337$ nm; nitrogen laser, maximum pulse rate: 50 Hz; maximal intensity 20-30 % of the laser for peptides). Peptides of the digests were measured in reflectron mode using a delayed extraction of 120 nsec and proteins were measured in linear mode at a delayed extraction of 550 nsec. The accelerating voltage was set to +19 kV, the reflectron voltage was set to + 20 kV. Spectra of peptides and proteins were the sum of 1000 shots, external calibration has been implemented. Data processing was executed with Flex Analysis software packages (version: 2.4.). For the analysis of in solution digestion Sequence Editor software (Bruker Daltonics, Bremen, Germany) was used with the following criteria: 1. All cysteines were supposed to be treated with iodoacetamide 2. Monoisotopic masses were allowed 3. The maximum number of missed cleavage sites was two.

4. RESULTS

4.1. MEASUREMENT OF MODIFICATION AND INTERFERENCE RATE OF URINARY ALBUMIN DETECTED BY SIZE-EXCLUSION HPLC (PART I OF THIS THESIS)

4.1.1. CHARACTERIZATION OF THE UV-FLUORESCENT HPLC SYSTEM

To calculate between-day imprecision of the measurements with UV and fluorescent detectors five samples (concentrations: 8.25, 16.5, 33, 66 and 132 mg/l) of each kind of albumin form (HSA, GSA and MGO-HSA) were tested 5 times in one week. The between-day imprecision (expressed as the percent coefficient of variation (%CV)) of the lowest concentration (8.25 mg/l) were as follows: 3.5% and 11.8% for HSA, 3.7% and 11.6% for GSA and 5.9% and 5.6% for MGO-HSA respectively for the UV and fluorescent measurements. The %CVs of the highest concentration (132 mg/l) were as follows: 1.1% and 5.1% for HSA, 1.5% and 3.0% for GSA and 1.8% and 2.0% for MGO-HSA respectively for the UV and fluorescent measurements. To investigate reproducibility of the measurements over time of the UV and fluorescent detections, the same samples after 12 months of freezing at -80°C were thawed and were measured the

same way as for the between-day imprecision using a new kit. The total imprecision of the two between-day imprecision measurements of the lowest concentration (8.25 mg/l) were as follows: 10.7% and 13.9% for HSA, 12.5% and 10.9% for GSA and 11.7% and 11.5% for MGO-HSA respectively for the UV and fluorescent peak areas; and of the highest concentration (132 mg/l) were as follows: 2.6% and 8.9% for HSA, 7.5% and 9.1% for GSA and 3.4% and 7.8% for MGO-HSA respectively for the of the UV and fluorescent peak areas.

Between-day imprecision was calculated for the urine samples as well. To make the calculations, 10 samples were randomly chosen and measurements were repeated one week after the first measurement. The between-day imprecision expressed as the percent CV of UV and fluorescent peak areas of the urine samples of patients with diabetes mellitus were 6.1% and 8.8% respectively. To investigate reproducibility of the measurements over time the urine samples were also re-analyzed after 12 months. Interestingly, we have found a significant decrease in the UV signal of the albumin ($-25\pm 9\%$, $p<0.05$) and a non-significant increase in the fluorescent signal ($11\pm 20\%$, $\text{mean}\pm\text{SD}$, $p=0.093$).

4.1.2. COMPARISON OF THE CONCENTRATION OF THE DIFFERENT FORMS OF IN VITRO PREPARED ALBUMIN BY *IN* AND BY *HPLC*

The different forms of albumin (HSA, GSA and MGO-HSA) prepared in the concentrations of 8.25, 16.5, 33, 66 and 132 mg/l were measured by HPLC and IN in triplicate. Then the albumin concentrations measured by HPLC were divided by the concentrations measured by IN. These quotients of HSA, GSA and MGO-HSA were compared by one-way ANOVA. The test failed to find a significant difference ($p=0.210$, HSA: $132\pm 10\%$, GSA: $120\pm 8\%$ and MGO-HSA: $142\pm 8\%$).

4.1.3. RELATIVE FLUORESCENCE OF THE DIFFERENT FORMS OF IN VITRO ALBUMIN

To avoid any possible confounding effect of fluorescent measurement, such as non-linear changes in the peak area of fluorescence with concentration, correlation analysis of UV and fluorescence signal of the different albumin forms were tested in the examined concentration range and were as follow: HSA, $r=0.9998$, GSA= 0.9999 , MGO-HSA, $r=0.9997$.

Relative fluorescence (RF) of the in vitro prepared albumin forms was determined. The average RF of HSA was considered to be 100 %. RF of GSA and of MGO-HSA was higher ($p < 0.001$ for both) compared to HSA and RF of MGO-HSA was also higher ($p < 0.01$) compared to RF of GSA which indicates extensive changes in the albumin structure of both GSA and MGO-HSA.

4.1.4. CHARACTERISTICS OF THE PATIENTS WITH DIABETES MELLITUS

Using the conventionally accepted cut-offs for albumin-creatinine ratio (ACR) for microalbuminuria (male: 2.5-25 mg/mmol, female: 3.5-35 mg/mmol) the diabetic patients were grouped as follow: normoalbuminuric using both IN and HPLC (NN, $n=47$), normoalbuminuric by IN but microalbuminuric by HPLC (NM, $n=12$), and microalbuminuric by both methods (MM, $n=20$). Classical ACR cut-off values were used for HPLC measured urinary albumin concentrations as well, since there are no accepted ACR cut-off values for HPLC yet. Of the clinical characteristics of the groups of patients only serum creatinine was higher (and consequently eGFR lower) in NM and MM groups compared to NN; however there was no difference between the NM and MM groups. More patients took angiotensin converting enzyme inhibitors in the MM group than in the NN group. There was no further difference between the groups.

Bland-Altman bias plot for both assays showed that in the majority of cases HPLC measured a higher concentration of urinary albumin than IN and also that the amount of bias increases as urinary albumin decreases.

4.1.5. RELATIVE FLUORESCENCE OF URINARY ALBUMIN IN DIABETIC PATIENTS

We found a higher RF of albumin in the urine of the MM group compared to the NN and NM groups ($p < 0.001$ and $p = 0.007$, respectively) but there was no difference between the NN and NM groups ($p = 0.201$). RF of urinary albumin showed significant positive correlation with the serum creatinine levels ($r = 0.295$; $p = 0.009$) and significant negative correlation with the estimated glomerular filtration rate eGFR levels ($r = -0.255$; $p = 0.026$), but not with glycaemic parameters (concentration of plasma glucose, $p = 0.766$; concentration of fructosamine, $p = 0.979$; levels of hemoglobin A_{1c}, $p = 0.442$). By forward stepwise multivariate linear regression analyses, both serum creatinine and eGFR levels proved to be independent predictors of urinary albumin RF ($\beta = 0.397$; $p = 0.014$ and $\beta = -0.337$; $p = 0.039$, respectively). The first model included age, plasma

glucose, fructosamine, hemoglobin A_{1c}, systolic and diastolic blood pressure, triglycerides, LDL- and HDL-cholesterol, haemoglobin and serum creatinine; the second model included the same parameters with the exception of ln eGFR in place of serum creatinine.

4.1.6. INTERFERENCE RATE OF ALBUMIN PEAK OF SIZE-EXCLUSION HPLC

Carrying out our albumin peak purity test of size-exclusion HPLC using RP-HPLC it was found that non-albumin material (calculated as non-albumin peak area to total peak area) was present in 12.7±1.9% in the albumin peak of size-exclusion HPLC.

4.2. HPLC-MEASURED ALBUMINURIA AND STORAGE OF SPECIMENS (PART II OF THIS THESIS)

4.2.1. EFFECT OF STORAGE ON THE CONCENTRATION OF URINARY ALBUMIN

Mean decrease±SD in HPLC-detected albuminuria after 2.5 years at -80°C storage was 24±9% (UAC: 88±259 vs. 55±187 mg/l, p=0.002). When patients were categorized according to their decrease of UAC to higher and lower than interassay imprecision and their urinary pH (above and under mean pH), we found a significant relationship between under mean urinary pH and higher UAC-decrease (p=0.030).

On the other hand, a significant increase could be observed in the DMR (p<0.001). However, only peak areas of the monomeric form of albumin changed significantly (p<0.001), while peak areas of the dimeric form of albumin did not (p=0.275).

4.2.2. REDUCING CAPACITY OF URINE

We found an exponential correlation between urinary pH and the TFSG of fresh urine samples (r=-0.795; p<0.001 for linear correlation), but not in 2.5 year stored urine samples (r=-0.216; p=0.261 for linear correlation). Average TFSG was significantly lower in stored urine compared to the fresh urine (6.6±7.7 vs. 22.7±14.3 in µM GSH equivalent, p<0.001). Moreover, we found a significant correlation between increase of DMR and pH (r=-0.382, p=0.041).

4.3. NEW POTENTIAL BIOMARKERS DISCOVERED BY MEASURING ALBUMINURIA WITH HPLC IN A CROHN'S DISEASE PATIENT (PART III OF THIS THESIS)

4.3.1. ALBUMIN ASSAYS

Total uAlb measured by SE-HPLC showed a marked increase during active phase comparing with the measured value of IT. The difference between the uAlb concentrations measured by the two methods during active phase was almost 15-fold which difference decreased to 6-10-fold during inactive phase. This unexpectedly high difference between the t-uAlb and ir-uAlb led us to analyze further our results.

4.3.2. REVERSED-PHASE HPLC AND SDS-PAGE ANALYSIS OF THE ALBUMIN PEAK BY SIZE-EXCLUSION HPLC

Chromatogram of RP-HPLC of albumin fraction of SE-HPLC obtained during acute phase clearly showed the presence of co-eluted proteins. Two fractions were collected from the RP-separation. First fraction included those proteins eluted at 12.40 min and 12.69 min, being recognized as two partially resolved constituents, while the second fraction contained actually uAlb that was verified by spike recovery studies and later by MALDI-TOF/MS. Considerable decrease of first-fraction-proteins but not albumin could be observed in the urine obtained in remission. Presence of two co-eluting proteins was proven by SDS-PAGE, as well.

4.3.3. MALDI-TOF/MS MEASUREMENTS

Mass spectrum measured from the first fraction of RP-HPLC showed peaks appearing at 23.5 kDa, 34.7 kDa and at 70.3 kDa (which can be considered to be the dimer of the protein with a mass of 34.7 kDa). The resulted peptide mass fingerprinting (PMF) and all the peptides of the PMF recognized by Mascot data base search engine were analyzed. Three proteins, α 1-acid-glycoprotein-1, α 1-acid-glycoprotein-2 and Zn- α 2-glycoprotein have been identified with high scores and sequence coverage values of 39.3%, 56.2% and 48.1%, respectively. Identification of these proteins was also corroborated by post-source decay spectra of the corresponding tryptic peptides.

Proteins identified from the excised gel slabs also confirmed these results. Investigating control urine from healthy individual allowed only the identification of albumin.

5. DISCUSSION AND CONCLUSIONS

Conventional urinary albumin assays, used in every-day laboratory medicine, are based on immunochemical methods using antibodies raised against serum albumin rather than urinary albumin. These assays detect immunoreactive albumin and other albumin compounds such as albumin aggregates and albumin fragments with a molecular weight of >12kDa. In 2003 a new method has been introduced for the measurement of albumin in the urine, using size-exclusion high performance liquid chromatography. Early studies using this method have shown that concentration of albumin is higher as measured by conventional, immuno-based assays; with other words there is a portion of albumin which is not immunoreactive. As an expected consequence, the nature of albumin measured by high performance liquid chromatography has been addressed. Moreover, some authors proposed that the method simply does not have sufficient resolution.

As a **first part of this thesis** we wanted to address these questions. Firstly, we have established a high performance liquid chromatography method equipped with tandem UV and fluorescent detection to assess the changes of detectability of albumin with the rate of modification. For this measurement in-vitro differently modified forms of albumin were used. As a part of these measurements we have also aimed to measure the modification rate of the total urinary albumin of diabetic patients to find a potential connection between the modification rate and clinical parameters. We concluded that albumin modification does not affect immunoreactivity. Interestingly, we found that the modification rate of total urinary albumin in diabetic patients correlates with the renal function and not with the parameters of glycaemia. Secondly, we have established a reversed-phase high performance liquid chromatography method to assess the interference rate of the albumin peak of size-exclusion high performance liquid chromatography. With the help of this method the interference rate of the albumin peak was found to be 12.7% on average, which does not explain the measured concentration difference between the immuno-based and high performance liquid chromatography methods.

In only 4 years after the publication of this new method for the measurement of albuminuria, reevaluation of big studies such as the Australian Diabetes, Obesity and Lifestyle study has been published to address the question if there is any clinical significance of high performance liquid chromatography-measured albuminuria. They

found that both traditional immunonephelometry and the new high performance liquid chromatography method have the same power for predicting mortality. However, for the HPLC measurements stored urine was used.

Based on some publications which showed that storage could strongly decrease the concentration of immunoreactive urinary albumin as **a second part of this thesis** we wanted to investigate the effect of storage on the concentration of high performance liquid chromatography-detected urinary albumin and we aimed to find possible mechanisms for the results we have found. We found that measurement of the concentration of albumin by high performance liquid chromatography in urine, stored for long periods at -80°C gives unreliable results, as we have found a significant 24% decrease in urinary albumin concentration after 2.5 years of storage. We found this decrease pH-dependent. As it was suggested by one study, the nonimmunoreactive form of urinary albumin is a partially cleaved form of albumin which is maintained with an intact relative molecular mass by the help of the disulfide bonds and which form fragments into smaller parts to reducing agents. That is why we have measured total sulfhydryl groups of our urine samples, in an attempt to assess whether this free sulfhydryl group capacity could play a role in the decrease of high performance liquid chromatography-detected albuminuria, by reducing disulfide bonds of albumin. We found a strong correlation between free sulfhydryl groups and urinary pH in fresh urine samples, which could not be observed, in stored urine and concentration of free sulfhydryl groups significantly decreased during the storage. We interpreted these results as urine has a potentially high level of reducing activity which is pH-dependent, and so it may play a role in the decrease of high performance liquid chromatography-detected albuminuria by breaking up the cleaved nonimmunoreactive form of urinary albumin.

Although clinical application of albuminuria is still largely limited to the area of diabetes it has been shown in several other clinical disorders that measurement of albuminuria can be a valuable marker. For instance, measurement of albuminuria has been shown to have the potential to be an objective marker in the monitoring of disease activity and response to treatment in inflammatory bowel diseases. As **a third part of this thesis** we followed up a young Crohn's disease patient with frequent exacerbation phases to measure the changes of the concentration of total albumin in the course of his disease compared to the measured concentration by immuno-based methods. The

surprisingly high difference between the two methods led us to further analyze the albumin peak of the size-exclusion chromatography of the Crohn's disease patient using techniques that allowed us the identification of possible biomarkers. We concluded from this study that urinary albumin measured by size-exclusion chromatography method in acute phase of Crohn's disease is not reliable since it measures a high amount of other proteins. On the other side, the identified coeluting urinary proteins, the α -1 acid glycoprotein and the Zn- α -2 glycoprotein, showed a perfect association with the clinical status, which let them candidature as a novel, non-invasive, easy-to-access activity biomarkers in Crohn's disease.

6. LIST OF PHD THESES

- 1) Glycooxidative modification of the albumin does not affect immunoreactivity.
- 2) Glycooxidative modification rate of total urinary albumin in patients with diabetes mellitus reflects renal pathophysiology.
- 3) Coeluting proteins in the peak of albumin by size-exclusion chromatography are present less than 20% on average in the urine of diabetic patients. This interference rate does not explain the difference between the concentration of albumin measured by immuno-based and size-exclusion chromatography methods.
- 4) Concentration of albumin by high performance liquid chromatography in stored urine decreases despite storage at -80°C which decrease is pH dependent.
- 5) Fresh urine has a potentially high level of reducing activity. This reducing capacity is pH dependent and disappears with storage.
- 6) Urinary albumin measured by size-exclusion chromatography method in acute phase of Crohn's disease is not reliable.
- 7) The urinary α -1 acid glycoprotein and the urinary Zn- α -2 glycoprotein are possible new biomarkers of disease activity in Crohn's disease.

7. LIST OF PUBLICATIONS

Cumulative impact factor: full papers 32.159, abstracts: 43.311

Cumulative impact factor of publications used in this thesis: full papers: 4.766 abstracts: 3.154

This thesis is based on the following publications:

1. **Markó L**, Cseh J, Kőszegi T, Szabó Z, Molnár GA, Mohás M, Szigeti N, Wittmann I. Storage at -80 degrees C decreases the concentration of HPLC-detected urinary albumin: possible mechanisms and implications. *J Nephrol* 2009;22(3):397-402. **IF: 1.252**
2. **Markó L**, Molnár GA, Wagner Z, Böddi K, Koszegi T, Szabó Z, Matus Z, Szijártó I, Mérei A, Nagy G, Wittmann I. Measurement of the modification and interference rate of urinary albumin detected by size-exclusion HPLC. *Physiol Meas* 2009;30(10):1137-1150. **IF: 1.430**
3. **Markó L**, Szigeti N, Szabó Z, Böddi K, Takátsy A, Ludány A, Kőszegi T, Molnár GA, Wittmann I. Potential urinary biomarkers of disease activity in Crohn's disease. *Scand J Gastroenterol* 2010 Jul 26. [Epub ahead of print] **IF: 2.084**
4. **Markó L**, Mikolás E., Molnár G. A., Wagner Z., Kőszegi T., Szijártó I. A., Mohás M., Matus Z., Szabó Z., Böddi K., Mérei Á., Wittmann I. Normo- és microalbuminuriás cukorbetegekben a HLCP-vel mért vizeletalbumin-fluoreszcencia a vesefunkciós paraméterekkel függ össze, nem a glikémiás értékkel. *Diab Hung* 2009;17(3):229-238.
5. **Markó L.**, Szijártó I. A., Cseh J., Kőszegi T., Szabó Z., Molnár G. A., Matus Z., Mérei Á., Wittmann I. A HPLC-vel mérhető vizeletalbumin koncentrációja -80 °C-os tárolás során jelentősen csökken: lehetséges mechanizmusok és következmények. *Hypertonia és Nephrologia* 2009;13(2):88-93.

This thesis is based on the following congress presentations and abstracts:

1. **Markó L.**, Molnár G. A., Wagner Z., Wagner L., Kőszegi T., Nagy J., Wittmann I.: Determination of protein glycoxidation-products in the urine of diabetic patients. *Nephrol Dial Transplant* 2006;21(S5):v84-85. **IF: 3.154**
Place of presentation: XLIII ERA-EDTA (European Renal Association-European Dialysis and Transplant Association) Congress, July 15-18, 2006, Glasgow, United Kingdom
2. **Markó L.**, Molnár G.A., Wagner Z., Wagner L., Matus Z., Kőszegi T., Laczy B., Tamaskó M., Mohás M., Cseh J., Nagy J., Wittmann I.: Vizelet fehérje glikoxidációs termékek meghatározása diabeteses betegekben. *Magyar Belorvosi Archivum* 2006;59(S2):111-112.
Place of presentation: Magyar Belgyógyász Társaság Dunántúli Szekciójának LIII. Vándorgyűlése, Sopron, 2006. június: Legjobb fiatal előadók díja: 3. helyezés

3. **Markó L.**, Molnár G. A., Wagner Z., Wagner L., Matus Z., Kőszegi T., Laczy B., Tamaskó M., Mohás M., Cseh J., Nagy J., Wittmann I.: Determination of protein glycooxidation-products in the urine of diabetic patients. *Acta Physiologica Hungarica* 2006:93(2-3):210.
Place of presentation: Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006. június
4. **Markó L.**, Wagner Z., Cseh J., Kőszegi T., Matus Z., Wittmann I.: HPLC és nephelometria (NM) összehasonlítása a mikroalbuminuria diagnózisában. *Hypertonia és Nephrologia* 2006:10(S5):107.
Place of posterpresentation: Magyar Nephrológiai Társaság XXIII. Nagygyűlése, Eger, 2006. október
5. **Markó L.**, Molnár G. A., Kőszegi T., Cseh J., Mohás M., Matus Z., Wittmann I. Analysis of albuminuria with high performance liquid chromatography (HPLC) and immunonephelometry (IN) in diabetic and/or hypertonic patients. *Acta Physiologica Hungarica* 2009:96(1):101.
Place of posterpresentation: A Magyar Élettani Társaság LXXII. Vándorgyűlése és a Magyar Kísérletes és Klinikai Farmakológiai Társaság közös konferenciája, Debrecen, 2008. június
6. **Markó L.**, Cseh J., Kőszegi T., Szabó Z., Molnár G. A., Mohás M., Szigeti N., Szijártó I., Wittmann I. A HPLC-vel mérhető vizelet albumin mennyisége a -80°C-os tárolás során jelentősen csökken. Lehetséges mechanizmusok és következmények. *Hypertonia és Nephrologia* 2008:12(S5):222.
Place of posterpresentation: Magyar Nephrológiai Társaság XXIII. Nagygyűlése, Szeged, 2008. szeptember

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