MEASUREMENT OF ALBUMINURIA WITH SIZE-EXCLUSION CHROMATOGRAPHY

CHARACTERIZATION AND NEW PERSPECTIVES

PHD THESIS

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Measurement of albuminuria with size-exclusion chromatography. Characterization and new perspectives.

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CONTENTS

ABBREVIATIONS

ACN ..... acetonitrile
ACR ..... albumin-creatinine ratio
AGE ..... advanced glycation end products
AusDiab ..... Australian Diabetes, Obesity and Lifestyle
BMI ..... body mass index
CD ..... Crohn's disease
CDAI ..... Crohn's disease activity index
CV ..... coefficient of variation
DMR ..... dimeric to monomeric ratio of urinary albumin
DTNB ..... 5, 5'-dithio-bis (2-nitrobenzoic acid)
ESR ..... erythrocyte sedimentation rate
fcc ..... final concentration
FDA ..... Food and Drug Administration
GSA ..... glycated human serum albumin
GSH ..... reduced glutathione
Hb A_{1c} ..... hemoglobin A1c
HDL ..... high-density lipoprotein
Hgb ..... hemoglobin
HPLC ..... high-performance liquid chromatography
HSA ..... human serum albumin
hs-CRP ..... high-sensitive C-reactive protein
IBD ..... inflammatory bowel diseases
IN ..... immunonephelometry
ir-uAlb ..... immunoreactive urinary albumin
IT ..... immunoturbidimetry
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 ..... microalbuminuric using both IN and HPLC methods
MS ..... mass spectrometry
NM ..... normoalbuminuric by IN, microalbuminuric by HPLC method
NN ..... normoalbuminuric patients using both IN and HPLC methods

PA .....peak area

PMF .....peptide mass fingerprinting

RAAS .....renin-angiotensin-aldosterone system

RF .....relative fluorescence

RIA .....radio-immuno assay

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

u-Creat .....urinary creatinine

Wbc .....white blood cell count

SUMMARY

SUMMARY

Albumin, under physiological conditions, is excreted in the urine in very small amounts of less than 30 mg per day. Since the 1980s, it is known that the excretion of very small amounts of albumin in the urine (30-300mg/day), so called microalbuminuria that could not be detected by standard dipsticks, predicts the excretion of large amounts of proteins in the urine (proteinuria) in patients with diabetes mellitus. A few years later, microalbuminuria was also proven to be a powerful predictor of mortality in these patients and later that it is also an independent predictor of cardiovascular diseases and mortality in the general population. Cardiovascular diseases are the leading cause of death in the Western world therefore the screening for subjects with an increased risk for cardiovascular diseases has a great importance. In this respect, the detection and the exact measurement of albumin in the urine plays an important role. However, there is no consensus regarding the way of the measurement of albumin.

Conventional 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 (later referred as 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. The albumin measured by high performance liquid chromatography is referred as total urinary albumin. 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. In this study, albuminuria was measured by immunonephelometry in fresh urine at the time of the original collection (1999-2000) and by high performance liquid chromatography in stored urine at first thaw after storage at -80°C in 2007.

Based on some publications which showed that storage could strongly decrease the concentration of 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 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 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 recognize as a novel, non-invasive, easy-to-access activity biomarkers in Crohn's disease.

INTRODUCTION AND AIMS OF THE THESIS

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DEFINITION AND PROPERTIES OF ALBUMIN

Albumin is one of the longest known and probably the most studied of all proteins. The name albumin comes from the more general term, *albumen*, the early German word for protein which origin was the Latin, *albus* (white), the color of that part of an egg surrounding the yolk when it is cooked.[1] 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. It is synthesized in the liver as preproalbumin which has a 24 amino acid extension at the N terminus of which 18 amino acids are removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is further cleaved in the Golgi vesicles to produce the secreted albumin. After being produced in the liver, it circulates as a non-glycosylated, negatively charged polypeptide chain of 585 amino acids which is crosslinked by 17 disulphide bonds. Approximately 30%-40% of the albumin synthesised is retained within the plasma compartment. The remaining pool is located mostly in the muscles and in the skin. Albumin is practically not stored hepatically (<2 g) and therefore there is no reserve for release on demand. However, under physiological circumstances only 20–30% of hepatocytes produce albumin and so synthesis can be increased on request by 200–300%.[2] Synthesis of the albumin is a constant process, regulated at both transcriptional and posttranscriptional levels by specific stimuli, but a change in interstitial colloid oncotic pressure is thought to be the predominant regulatory influence. Albumin has a molecular mass of 66,439 Dalton, a molecular radius of approximately 36 Å, and a pI of 5.6.[3,4] However chemical and physical data support a heart shaped structure for the albumin,[4] the molecule can rapidly change conformation.[3] The mean half-life of the albumin is 14.8 days.

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.

Albumin, under physiological conditions, is excreted in the urine in very small amounts of less than 30 mg per day. It is assumed that albumin passes through the glomerular filtration barrier, and is reabsorbed by the proximal tubular cells by receptors such as megalin and cubulin, after which it is delivered to the lysosomal system and broken down to amino acids which are delivered back into the circulation.[6,7] Increased amounts of albumin can appear in the urine, resulting from increased glomerular filtration and/or altered tubular reabsorption. However, these processes are still debated.[8]

ALBUMINURIA AS A WELL-ESTABLISHED RISK MARKER

Persistent excretion of albumin in the urine (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.[9-14] 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.[15-22] The underlying mechanism of leakage of albumin into the urine is suggested to be endothelial dysfunction which is most probably limited not only to the kidneys, but might be an indication of generalized endothelial dysfunction.[23,24]

MEASUREMENT OF ALBUMINURIA

Given the fact that diabetes mellitus and cardiovascular disease are the leading causes of death in industrialized countries, accurate measurement of albuminuria is of great importance to be able to identify those at risk in order to be able to start preventive treatment. 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 radio-immuno assay[25] (RIA), using ^{125}I labeled albumin which is based on immune reaction. Unfortunately this method was time and money-consuming and too expensive for routine laboratory measurement. Therefore other immuno-based (immunonephelometry and immunoturbidimetry)[26-28] 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

immunonephelometry or immunoturbidimetry) 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 studies using this new method have shown that urinary albumin concentration detected by HPLC is significantly higher compared with conventional assays.[29,30] Moreover, one study suggested that with the HPLC method microalbuminuria can be detected earlier in both type 1 and 2 diabetes mellitus.[31] 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.

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

The exact nature of HPLC-measured albumin is not known. In diabetic patients – and also in general patients - the free radicals are generated e.g. by glucose oxidation or non-enzymatic glycation of proteins. During non-enzymatic glycation, carbonyl groups of reducing sugars react with free amino groups of proteins forming reversible Schiff bases, thereafter intermediate Amadori adducts and finally advanced glycation end products (AGEs) through a complex cascade.[32] Since this glycation process includes oxidative steps it is called glycoxidation. Some of the AGEs, as well as their oxidation adducts, are fluorophores, and these can usually be used for detection at wavelengths of 370 nm of excitation and 440 nm of emission.[33-36] Moreover previously we have shown that fluorescence measured at these wavelengths correlates with the non-fluorescent AGE product, N^{ε}-(carboxymethyl)lysine-levels measured by competitive enzyme-linked immunosorbent assay in patients with type 2 diabetes.[37] It has been suggested that AGEs have a significant role in the development of diabetic complications.[38,39] Especially modified albumins, such as glycated albumin could have a crucial role in the development of renal insufficiency.[40]

Some authors proposed that oxidative stress-induced modification of albumin could be one of the reasons for immuno-unreactivity[41], 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.[42]

First aim of the PhD thesis was to decide these questions. Therefore a HPLC-based method has been worked out and applied for studying the reason for immuno-unreactivity. The role of interference with other substances affecting the detection and the change of immunoreactivity by oxidative stress has been considered. Our aim has also been to reveal a connection between the rates of the glycoxidative modifications of total urinary albumin with clinical parameters of patients with diabetes mellitus.

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

In the past decade, several large epidemiological studies have investigated albuminuria as a predictor for renal and cardiovascular disease using immuno-based methods. Since the introduction of the new HPLC-based urinary albumin measurement, several studies proved that HPLC detects more albumin than the immuno-based methods.[21,22,43-45] However, the clinical significance of the measurement of the total albumin remained unclear. That is why it was not surprising that some of these big studies repeated their measurements - with their stored samples - using the new HPLC method to recalculate predictivity in order to answer the question if HPLC-measured albuminuria has a clinical significance. The first paper which aimed to address this question was just a few years ago published.[46] In this article Magliano et al investigated in the longitudinal Australian Diabetes, Obesity and Lifestyle (AusDiab) study 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. The only advantage of the HPLC method was that it provided more information on mortality risk than IN. In this study albuminuria was measured by IN in fresh urine at the time of the original collection (1999-2000) and by HPLC 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.[47] Moreover, it was not even known how HPLC-detected total albumin affected by long-term storage. Therefore the second aim of the PhD thesis was 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.

At the time of the publication of Magliano et al it was also known that the non-immunoreactive albumin, which is measured by HPLC, is sensitive to reducing

conditions, as it fragments into smaller parts. It was 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.[48] It has been also suggested that urinary pH is a determinant of urinary albumin decrease. However, there are scant data on how pH affects urinary albumin concentration.[49] Because of that further aims of this study were also to find factors which can be responsible for the loss of albumin in prolonged stored urine samples, especially regarding to HPLC-measured total urinary albumin. That is why we aimed to examine possible pH-dependency of decline of albumin concentration and to assess the reducing capacity of stored and fresh urines by measuring the total sulfhydryl groups of the urine samples.

NEW POTENTIAL BIOMARKERS DISCOVERED BY MEASURING ALBUMINURIA WITH HPLC (PART III OF THIS THESIS)

Crohn's disease (CD) represents one of the two main forms of inflammatory bowel diseases (IBD). It is characterized by a chronic course with phases of remissions and acute episodes.[50] Assessment of the activity and the severity of CD is sometimes still a challenge for physicians despite the hallmark of diagnostic tools, laboratory markers and clinical indices.[51] Laboratory markers in CD are measured mostly in blood or stool which sampling procedure is cumbersome. However, urine as a non-invasive, easy-to-access source is incomprehensibly ignored.

Previously, it has been demonstrated that measurement of albuminuria has the potential to be an effective objective method in the monitoring of disease activity and response to treatment in IBD.[52] Moreover, it has been shown that its concentration correlates with the intestinal histopathological grading[53] and with serum concentration of tumor necrosis factor-alpha, an important pathophysiological factor of CD, which suggests that it may be important in the pathogenesis of microalbuminuria.[54] Recently, we encountered a young CD patient with frequent exacerbation phases as part of a cross-sectional study, published lately by our workgroup.[55] Since no data existed about the measurement of total urinary albumin in CD, as a pilot study we followed up this patient in the course of his disease and measured the concentration of urinary immunoreactive and toal albumin. The surprising results led us to further analyze the total albumin of our CD patient using techniques that allow the identification of possible biomarkers.

MATERIALS AND RESULTS

PART I

MEASUREMENT OF MODIFICATION AND INTERFERENCE RATE OF URINARY ALBUMIN DETECTED BY SIZE-EXCLUSION HPLC.

Physiological Measurements 2009;30(10):1137-50

MATERIALS

PREPARATION OF THE DIFFERENT FORMS OF ALBUMIN IN VITRO

In order to decide whether oxidative 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 AGEs forming agent.[56] Preparation of MGO-HSA was performed by the method of Westwood et al.[57] Briefly, 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.

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 immunonephelometry (IN) diagnosed normoalbuminuria (n=59) and microalbuminuria (n=20) 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.

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 Cockroft-Gault formula.[58]

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.

MEASUREMENT OF THE CONCENTRATION OF ALBUMIN

The in vitro prepared different forms of albumin as well as urinary albumin concentrations were measured in triplicate 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 FDA approved Accumin^{TM} 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 Accumin^{TM} 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).

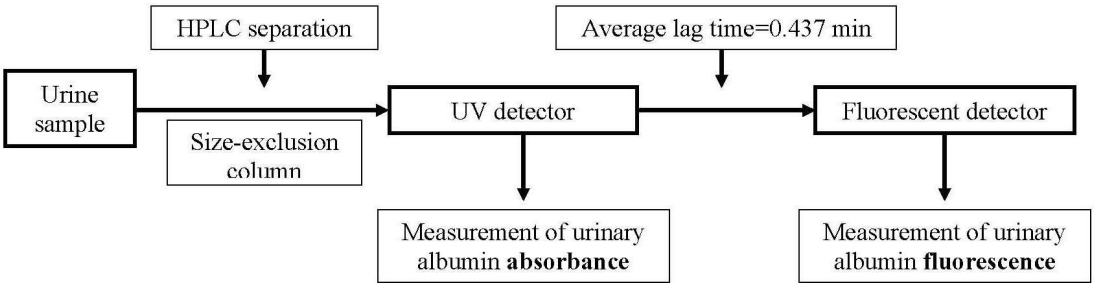


Figure 1. Schematic structure of the HPLC system.

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).

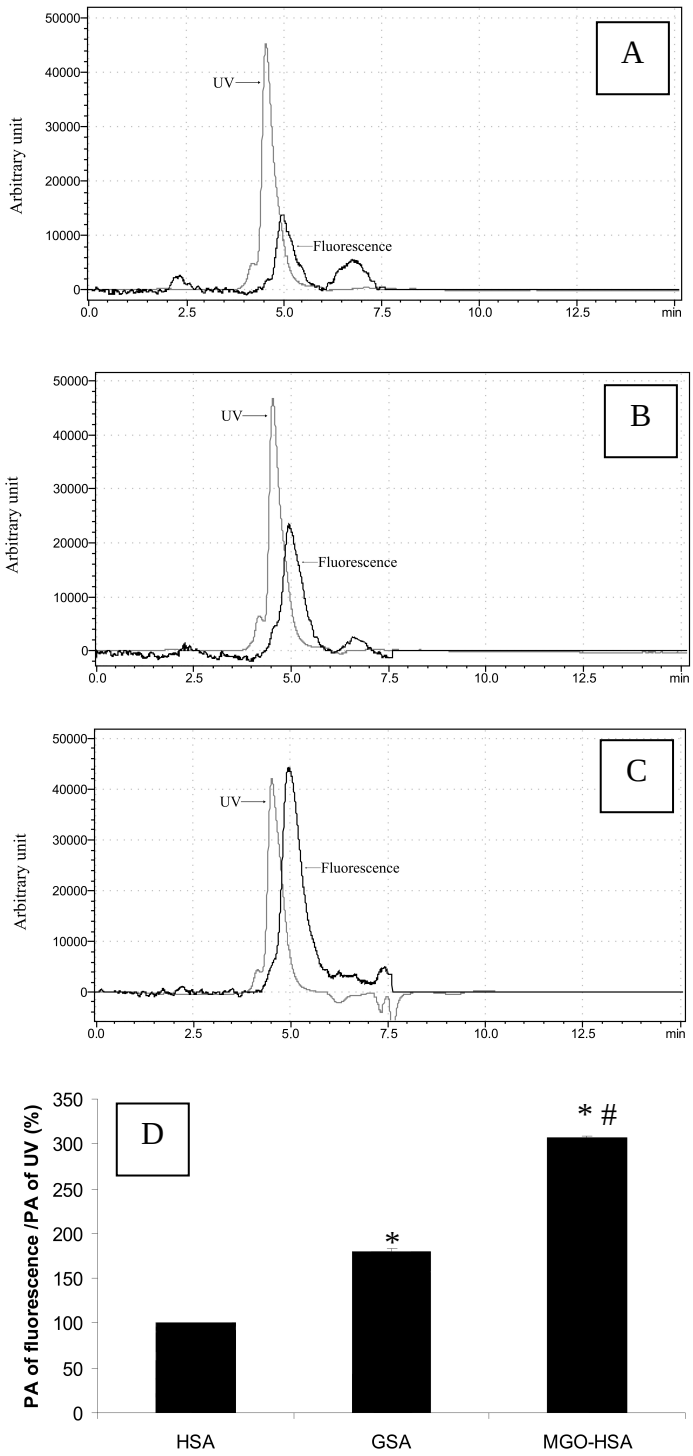
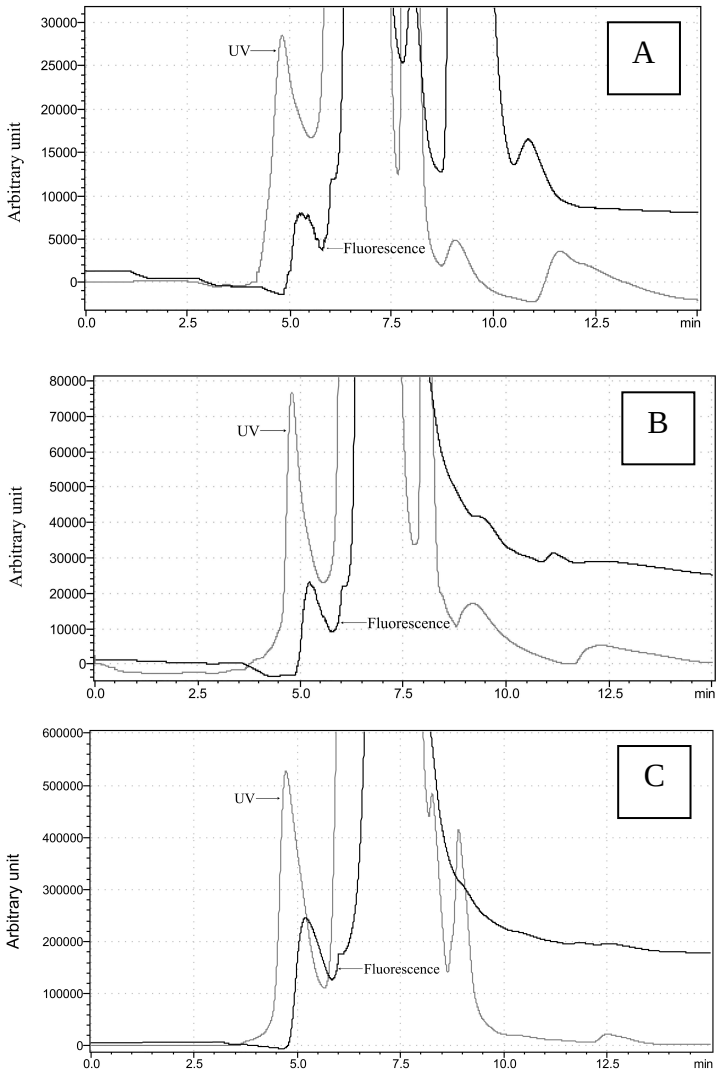


Figure 2. Detection of relative fluorescence of different forms of albumin in the in vitro experiment. Panel A shows the chromatograms of human serum albumin (HSA), panel B of glycated human serum albumin (GSA) and panel C of human serum albumin modified with 1 mM methylglyoxal (MGO-HSA) of the same concentration (33 mg/ml). The gray line shows the chromatogram of UV detection (λ=214 nm) and the black line shows the chromatogram of fluorescence detection (excitation at 370 nm and emission at 440 nm). Average lag time between UV and fluorescence detection was 0.437 min. Panel D shows the ratio of the peak area (PA) of fluorescence and PA of UV of different forms of albumin (n=5 in each case) where the average of the ratio of HSA was considered to be 100%. Results are expressed as mean±SEM; $p<0.001$ Kruskall–Wallis test; *=versus HSA, $p<0.001$; #=versus GSA, $p<0.001$ Mann–Whitney U-test.

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) (Figure 1). 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. On average there was a 0.437 min delay (the time of the albumin to get from the UV detector to the fluorescent detector) between the UV and the fluorescent signal of the albumin.



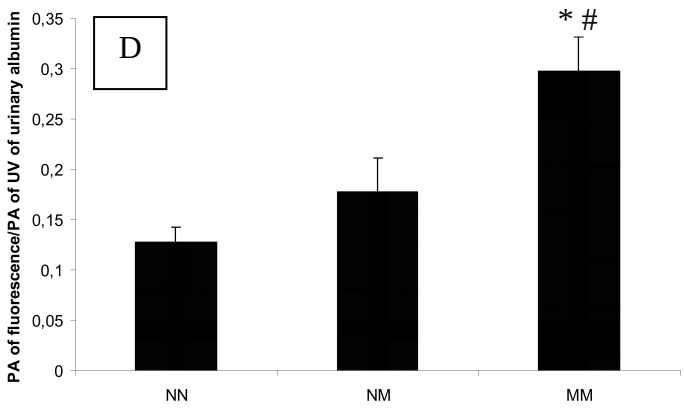


Figure 3. Detection of glycoxidation of urinary albumin in diabetic patients. Panel A shows the chromatograms of a patient normoalbuminuric using both immunonephelometry and HPLC (NN). Panel B shows chromatograms of a patient normoalbuminuric by immunonephelometry but microalbuminuric by HPLC (NM) and panel C of a patient microalbuminuric by both methods (MM). The gray line shows the chromatogram of UV detection (λ=214 nm) and the black line shows the chromatogram of fluorescence detection (excitation at 370 nm and emission at 440 nm). Panel D shows the ratio of the peak area (PA) of fluorescence and the PA of UV of the patient groups. Results are expressed as mean±SEM. $p<0.001$ Kruskall–Wallis test; NN versus NM, $p=0.852$; *= versus NN, $p<0.001$; #=versus NM, $p=0.001$ Mann–Whitney U-test.

Chromatograms obtained in the case of sample running of HSA, GSA and MGO-HSA can be seen in Figures 2A, B and C. In each figure two chromatograms can be seen. The first was obtained through UV detection while the second one provided fluorescent detection. In each case the first peak immediately preceding the albumin peak is that of albumin dimer and was not taken into consideration during albumin measurements. A peak following the albumin peak can be noticed on the chromatograms of fluorescent detection. It was proved to be an artefact by injecting a phosphate buffer dose (blind sample) and was most likely caused by the flow rate change imposed after the elution of the albumin (see time program of HPLC measurements).

Chromatograms obtained in the case of samples obtained from diabetic patients can be seen in Figures 3A, B and C. The chromatograms are similar to those chromatograms obtained in the case of in vitro prepared albumins. It must be noticed that during the running of urine samples the peak of albumin dimer was not always present. The peak of albumin was followed by other components of urine which were not identified.

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:

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

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 μl. These samples were analysed further using a RP-HPLC method.

For the separation a lately developed non-porous Kovasil MS C18 column (particle size: 1.5 μm, 33×4,6 mm, Zeochem AG, Uetikon, Switzerland) was used, which enables a short analysis time and sensitive separation of complex samples.[59] 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 (Figure not shown). 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 (PA) to the total PA.

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 Kruskall-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±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.

RESULTS

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±9%, $p<0.05$) and a non-significant increase in the fluorescent signal (11±20%, mean±SD, $p=0.093$).

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±10%, GSA: 120±8% and MGO-HSA: 142±8%).

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 %. Figure 2D shows that RF of GSA and of MGO-HSA is higher ($p<0.001$ for both) compared with HSA, which indicates extensive changes in the albumin structure of both GSA and MGO-HSA.

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)[23] 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. Clinical characteristics of the groups of patients are summarized in Table 1. In the NM and the MM groups serum creatinine levels were significantly higher and eGFR levels were significantly lower compared to the NN group; 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.

Figure 4 shows a Bland-Altman bias plot for both assays. ACR values of both methods were log-transformed before plotting because data showed non-normal distribution. It is apparent 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. This phenomenon, which was observed in our in vitro measurements as well, is already reported.[43]

Table 1. Clinical characteristics of the groups.

	NN	NM	MM	P		
	(n=47)	(n=12)	(n=20)	NN vs. NM	NN vs. MM	NM vs. MM
Age (years)	52±2	60±4	59±2	NS	NS	NS
Gender (male/female)	22/25	7/5	11/9	NS	NS	NS
Type of diabetes mellitus (type 1/type 2)	13/34	2/10	5/15	NS	NS	NS
Smokers/non-smokers	5/42	1/11	5/15	NS	NS	NS
Angiotensin converting enzyme inhibitor (yes/no)[b]	37/10	11/1	20/0	NS	<0.05	NS
Angiotensin II receptor blockers (yes/no)[b]	11/36	2/10	7/13	NS	NS	NS
ACR IN (mg/mmol)[a]	0.42 (0.21-0.77)	1.56 (0.89-2.16)	6.92 (5.06-10.48)	<0.001	<0.001	<0.001
ACR HPLC (mg/mmol) [a]	0.75 (0.36-1.26)	4.43 (3.05-7.35)	8.69 (6.75-13.84)	<0.001	<0.001	<0.001
Random plasma glucose (mmol/l)	8.72±0.51	10.12±1.60	7.57±0.70	NS	NS	NS
Fructosamine (µmol/l)	317±11	333±30	303±16	NS	NS	NS
Hemoglobin A_{1c} (%)	7.61±0.27	7.15±0.35	6.84±0.37	NS	NS	NS
Systolic blood pressure (mmHg)[a]	120 (110-130)	120 (112-130)	130 (120-150)	NS	NS	NS
Diastolic blood pressure (mmHg)[a]	70 (70-80)	70 (61-70)	80 (60-80)	NS	NS	NS
BMI (kg/m^2)	30.51±0.98	32.60±3.57	32.68±1.13	NS	NS	NS
Cholesterol (mmol/l)	4.44±0.14	4.27±0.26	4.77±0.41	NS	NS	NS
Triglycerides (mmol/l)	2.07±0.23	2.10±0.33	2.50±0.40	NS	NS	NS
LDL cholesterol (mmol/l)	2.50±0.13	2.35±0.18	2.53±0.30	NS	NS	NS
HDL cholesterol (mmol/l)[a]	1.18 (0.98-1.45)	1.10 (0.88-1.22)	1.07 (0.94-1.16)	NS	NS	NS
Hemoglobin (g/l)	132.0±2.4	134.8±4.4	131.3±4.7	NS	NS	NS
Hematocrit (%)	40.0±0.6	40.5±1.1	39.5±1.4	NS	NS	NS
Serum creatinine (µmol/l)[a]	78 (66-92)	96 (77-154)	101 (66-157)	<0.05	<0.05	NS
Estimated glomerular filtration rate (Cockroft-Gault) (ml/min)	102±6	73±9	79.3±9	<0.05	<0.05	NS
Urine pH[a]	5.90 (5.30-6.20)	6.2 (5.00-6.50)	5.45 (5.00-5.90)	NS	NS	NS

[a]Data of non-normal distribution are presented as median (25-75 percentiles). All other parameters are normally distributed and are expressed as mean±SEM. [b]Please note that some patients take both angiotensin converting enzyme inhibitor and angiotensin II receptor blockers and 5 patients of group NN did not take any of these medications. NN=normoalbuminuric patients using both immunonephelometry and HPLC, NM=normoalbuminuric by immunonephelometry but microalbuminuric by HPLC, MM=microalbuminuric using both methods. ACR=albumin-creatinine ratio, IN=immunonephelometry, HPLC=high performance liquid chromatography, BMI=body mass index.

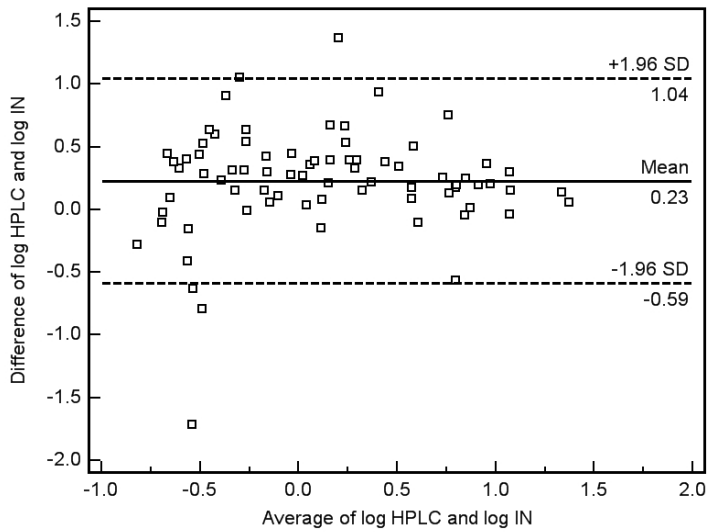


Figure 4. Bland-Altman bias plot of albumin-creatinine ratio calculated by albumin concentration ascertained by size-exclusion high performance liquid chromatography (HPLC) and by immunonephelometry (IN). The continual line represents the mean; the dashed lines represent the ± 1.96 SD limits.

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 ($p=0.201$) (Figure 3D). 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.

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 PA to total PA) was present in 12.7±1.9% in the albumin peak of size-exclusion HPLC.

PART II

STORAGE AT -80°C DECREASES THE CONCENTRATION OF HPLC-DETECTED URINARY ALBUMIN: POSSIBLE MECHANISMS AND IMPLICATIONS

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METHODS

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. 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 (see Table 2). Both studies were approved by the Ethical Committee of the Medical Faculty of the University of Pécs, Hungary. Written informed consent was obtained from all participants.

Table 2. Clinical characteristics.

	Stored urine group (n=30)	**Fresh urine group (n=30)**	**p value**
Age (years)	61±14	56±12	0.109
Gender (male/female)	15/15	19/11	0.436
Medication on RAAS (yes/no)	27/3	23/7	0.166
Random plasma glucose (mmol/l)	7.3±2.7	9.5±5.4	0.065
Fructosamine (µmol/l)	285±74	304±79	0.366
Hb A_{1c} (%)	7.2±1.5	7.7±1.6	0.259
Systolic blood pressure (mmHg)	128±17	127±12	0.777
Diastolic blood pressure (mmHg)	73±11	75±9	0.424
BMI (kg/m^2)	30±5	29±5	0.924
Cholesterol (mmol/l)	4.61±1.15	4.59±1.06	0.923
Triglycerides (mmol/l)	2.39±1.58	2.49±2.92	0.169
LDL cholesterol (mmol/l)	2.62±1.02	2.33±0.84	0.281
HDL cholesterol (mmol/l)	1.19±0.36	1.42±0.50	0.053
Blood urea nitrogen (mmol/l)	8.68±5.52	7.79±5.07	0.673
Serum creatinine (µmol/l)	107±55	101±67	0.355
Estimated glomerular filtration rate (Cockroft-Gault) (ml/min)	72±33	84±28	0.144
Urine pH	6.0±0.7	5.8±0.8	0.274

Continuous data are expressed as mean±SD. RAAS=renin-angiotensin-aldosterone system, Hb A_{1c}=hemoglobin A_{1c}, BMI=body mass index, LDL=low-density lipoprotein, HDL=high-density lipoprotein.

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 as previously described in detail with the FDA-approved HPLC Accumin^{TM} 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) 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 AccuminTM 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.

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 (fcc) of 100 μM) 10 μl of colorimetric Ellman's reagent, 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, Schnelldorf, 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 (fcc of 10 μM) of freshly prepared reduced glutathione (GSH) (Sigma-Aldrich, Schnelldorf, 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.

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±SD. P values <0.05 were considered as statistically significant.

RESULTS

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, data not shown).

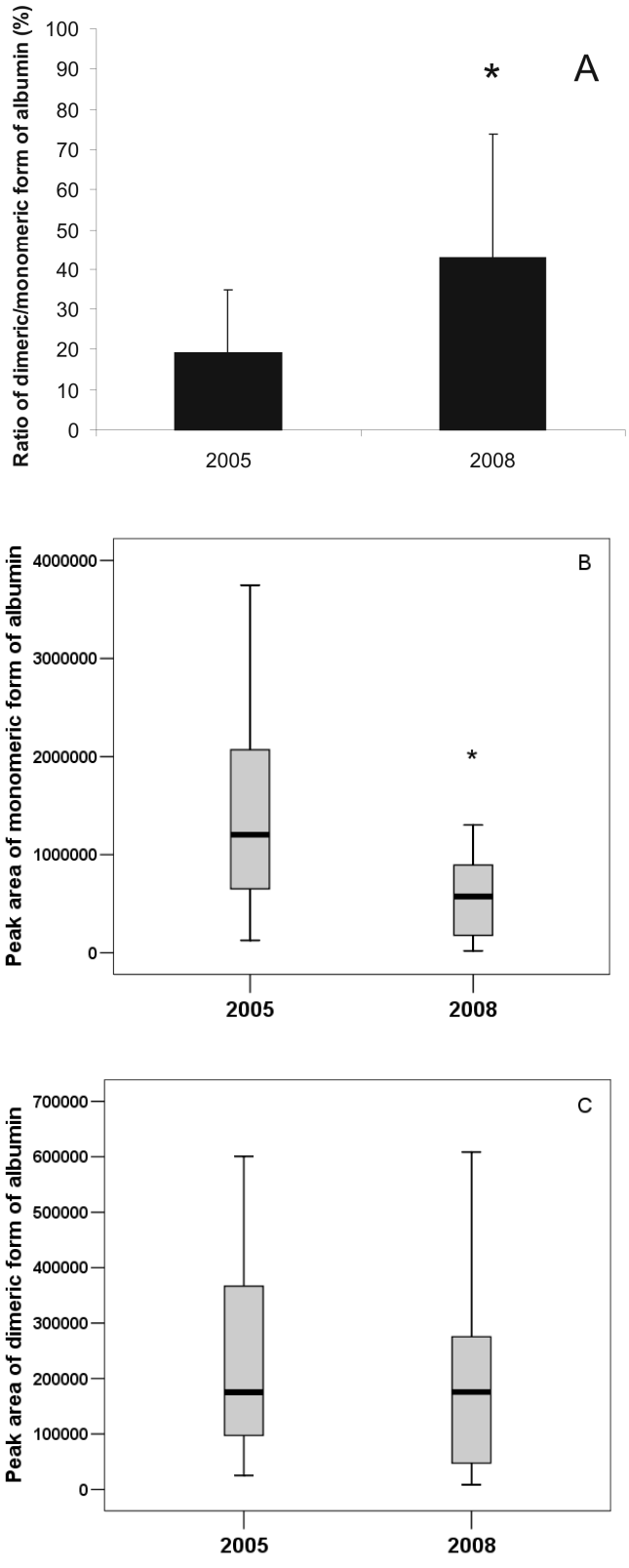

Figure 5. Panel A shows the increase ($p<0.001$) of the dimeric to monomeric urinary albumin ratio (DMR) after 2.5 years of storage. Panel B shows the changes ($p<0.001$) of peak areas of monomeric form and Panel C the non-significant changes ($p=0.275$) of peak areas of dimeric form of albumin after 2.5 years. Since the peak area data of both monomeric and dimeric forms of albumin showed a non-normal distribution data are presented in box plot. The thick line represents the median; the end of the rectangles shows the 25 and 75 percentile and the end of the box plots shows the minimum and maximum.

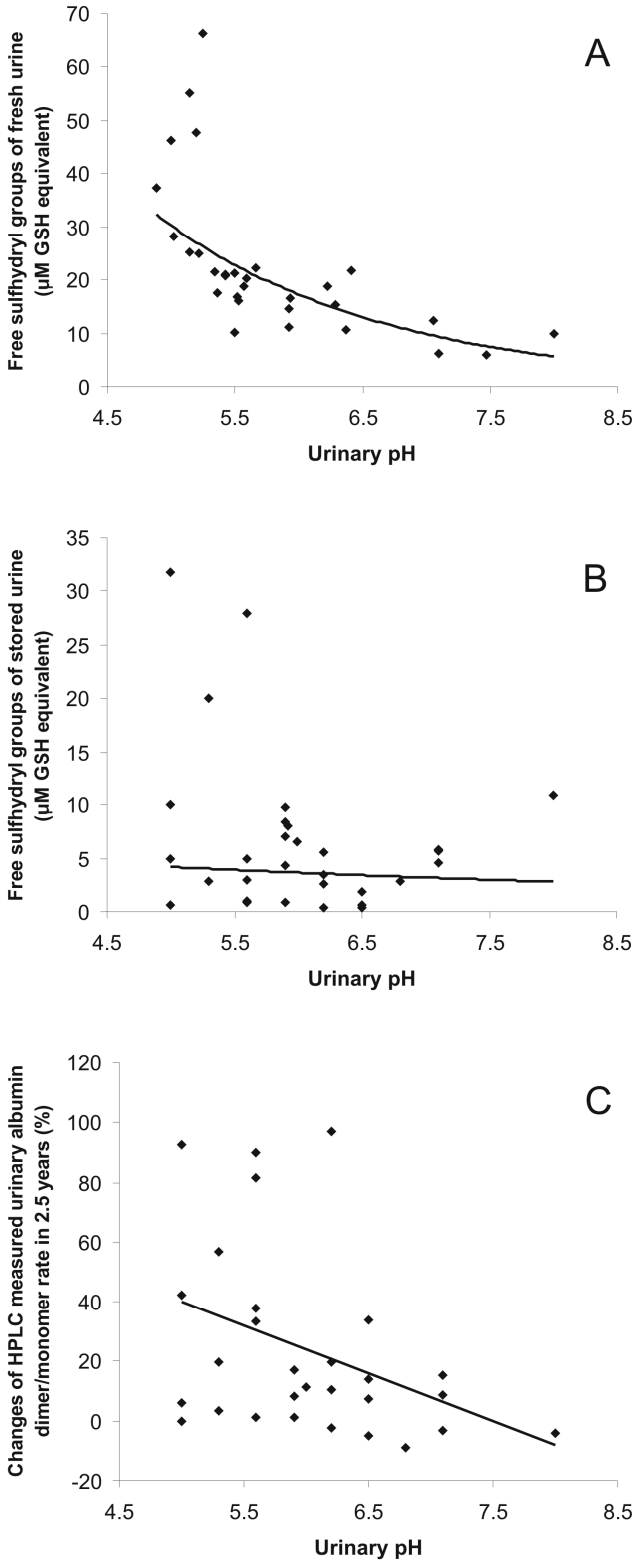

Figure 6. Panel A shows the correlation (r=-0.795; p<0.001 for linear correlation) between urinary pH and free sulfhydryl groups of fresh urine (in µM reduced glutathione (GSH) equivalent). Panel B shows the loss of this correlation (r=-0.216; p=0.261 for linear correlation) in 2.5 year stored urine. Please note that at pH 7.1 and 5.6 2-2 samples had very similar data (2.86, 2.89 and 0.41, 0.46). Panel C shows the correlation (r=-0.382, p=0.041) between urinary pH and high performance liquid chromatography (HPLC) measured alteration of urinary albumin dimer/monomer ratio in the 2.5 year period.

On the other hand, a significant increase could be observed in the DMR (Figure 5, Panel A; $p<0.001$). However, only peak areas of the monomeric form of albumin changed significantly (Figure 5, Panel B; $p<0.001$), while peak areas of the dimeric form of albumin did not change significantly (Figure 5, Panel C; $p=0.275$). We found an exponential correlation between urinary pH and the TFSG of fresh urine samples (Figure 6, Panel A) ($r=-0.795$; $p<0.001$ for linear correlation), but not in 2.5 year stored urine samples (Figure 6, Panel B) ($r=-0.216$; $p=0.261$ 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 (Figure 6, Panel C) ($r=-0.382$, $p=0.041$).

Part III

POTENTIAL URINARY BIOMARKERS OF DISEASE ACTIVITY IN CROHN'S DISEASE

Scand J Gastroenterol 2010 Jul 26. [Epub ahead of print]

METHODS

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)[60] 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 (CDAI) was used. Scores ≥150 are defined as active.[61]

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.

URINARY ALBUMIN ASSAYS

Concentrations of immunoreactive (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 SE-HPLC protocol.

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

Central fractions of albumin peaks of the analyzed urines were collected and concentrated ~15 to 20-fold by use of a 3 kDa cutoff membrane (Millipore Corp., Bedford, USA). The HPLC instrument used for the analysis of the concentrated albumin peaks consisted of a Dionex P680 gradient pump, a Rheodyne 8125 injection valve and

a Dionex UVD 170U UV-VIS detector (all parts purchased from Germering, Germany). Data acquisition was carried out on Chromeleon software (version: 6.6). Proteins being present in the size-exclusion peak were separated on a Kovasil MS-C18 non-porous column (Zeochem AG, Uetikon, Switzerland). Eluent "A" consisted of 5 % (v/v) ACN in water and 0.1 % TFA and eluent "B" contained 95 % (v/v) ACN and 0.1 % TFA. The gradient applied was: 0-20 min: 0 % „B" → 60 % „B", 20-25 min: 60 % „B" → 100 % „B". The flow rate was: 0.7 ml/min. Chromatograms were acquired at 214 nm. 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 as described.[62]

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 needed and was performed as described.[62] 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.[63] 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[64] following the traditional Coomassie brilliant blue R-250 staining. Proteins identified were excised from gel and after in-gel digestion as described[65] were analyzed by MALDI-TOF/MS.

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 µl of the matrix was deposited on a stainless steel target together with 1 µl of the sample. All mass spectra were monitored in positive mode with pulsed ionization (λ = 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.

RESULTS

ALBUMIN ASSAYS

Clinical characteristics of the patient during the time of the study are summarized in Table 3. 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. A representative chromatogram of urine obtained during active phase (black line) with albumin standard (grey line; 306 mg/l) is presented on Figure 7. This unexpectedly high difference between the t-uAlb and ir-uAlb led us to analyze further our results.

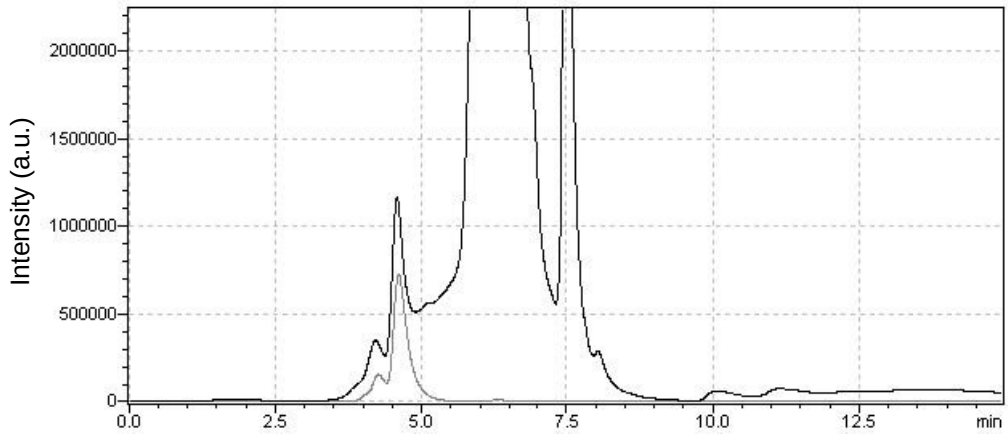

Figure 7. Representative chromatogram of a urine sample obtained during active phase of the Crohn's disease patient (black line), shown with albumin standard (grey line; 306 mg/l).

Table 3. Patient characteristics.

		CDAI (a.u.)	ir-uAlb (mg/L)	t-uAlb (mg/L)	u-Creat (mmol/L)	ir-uAlb/ u-Creat (mg/mmol)	t-uAlb/ u-Creat (mg/mmol)	t-uAlb/ ir-uAlb ratio	hs-CRP (mg/L)	ESR (mm/h)	Ferritin (ng/mL)	Wbc (G/L)	Hgb (g/L)	Platelet count (G/L)
Study entry	**16.08.2007**	81	7	72.7	33.0	0.2	2.2	10.4	56.3	66	162	11.5	131	467
Acute phase	**25.10.2007**	269	37	548.2	61.2	0.6	9.0	14.8	247.7	85	412	13.0	125	737
Remission	**01.11.2007**	62	13	81.8	41.3	0.3	2.0	6.3	25.3	40	363	16.9	122	491
Acute phase	**05.02.2008**	232	35	518.1	62.8	0.6	8.2	14.8	182.1	86	n.m.	12.5	131	389
Remission	**14.02.2008**	108	4	22.9	16.8	0.2	1.4	5.7	21.1	30	128	12.7	129	448

CDAI: Crohn's disease activity index; ir-uAlb: immunoreactive urinary albumin; t-uAlb: total urinary albumin; u-Creat: urinary creatinine; hs-CRP: high-sensitive C-reactive protein; ESR: erythrocyte sedimentation rate; Wbc: white blood cell count; Hgb: hemoglobin; n.m: not measured.

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 shown by Figure 8 (black line) clearly indicates the presence of co-eluted proteins. Two fractions were collected from the RP-separation. Fraction 1 included those proteins eluted at 12.40 min and 12.69 min, being recognized as two partially resolved constituents, while Fraction 2 contained actually uAlb that was verified by injecting albumin standard solution at a concentration of 306 mg/l (Figure 8, grey line) and later by MALDI-TOF/MS. Considerable decrease of first-fraction-proteins but not albumin could be observed in the urine obtained in remission (Figure 9 and 10). Presence of two co-eluting proteins was proven by SDS-PAGE, as well (Figure 11).

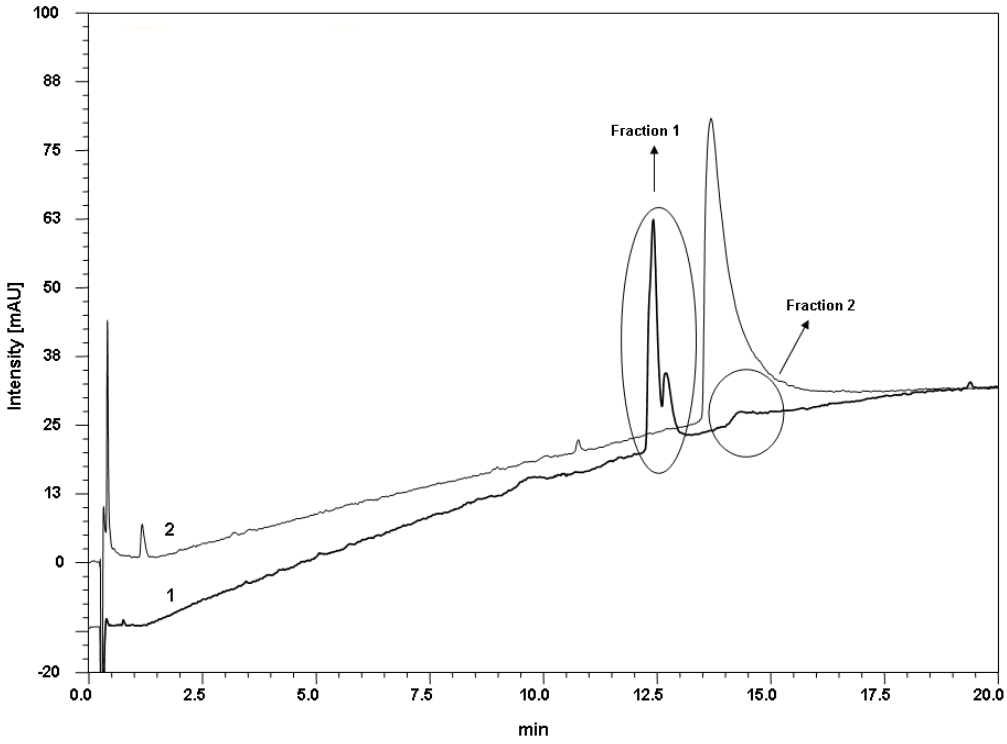

Figure 8. Chromatogram obtained by reverse-phase high performance liquid chromatography by running the albumin fraction of size-exclusion high performance liquid chromatography obtained during acute phase (black line) shown with albumin standard (grey line; 306 mg/l).

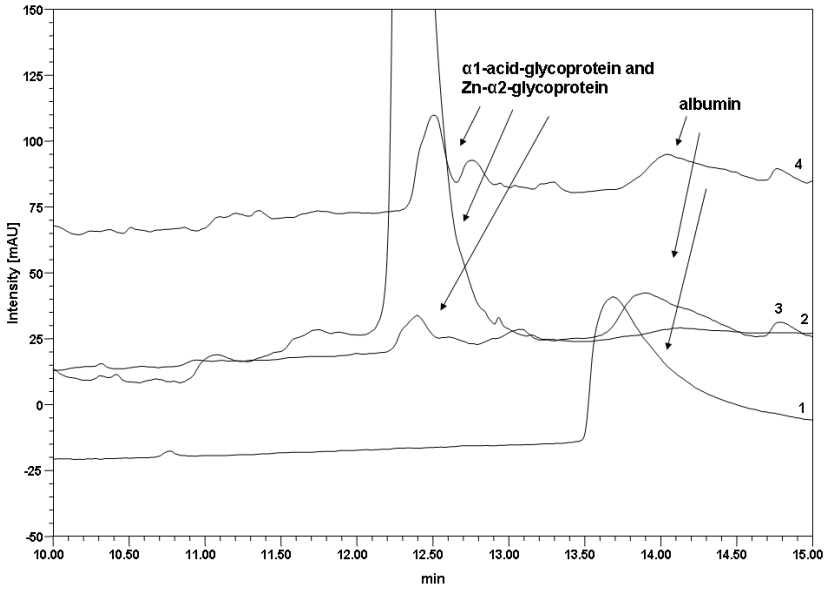

Figure 9. Changes of the levels of the proteins in the size-exclusion central peak of albumin by reversed-phase HPLC. First relapse. Chromatogram number 1 is albumin standard of 153 mg/L, whereas number 2 is study entry, number 3 is acute episode and chromatogram number 4 is remission.

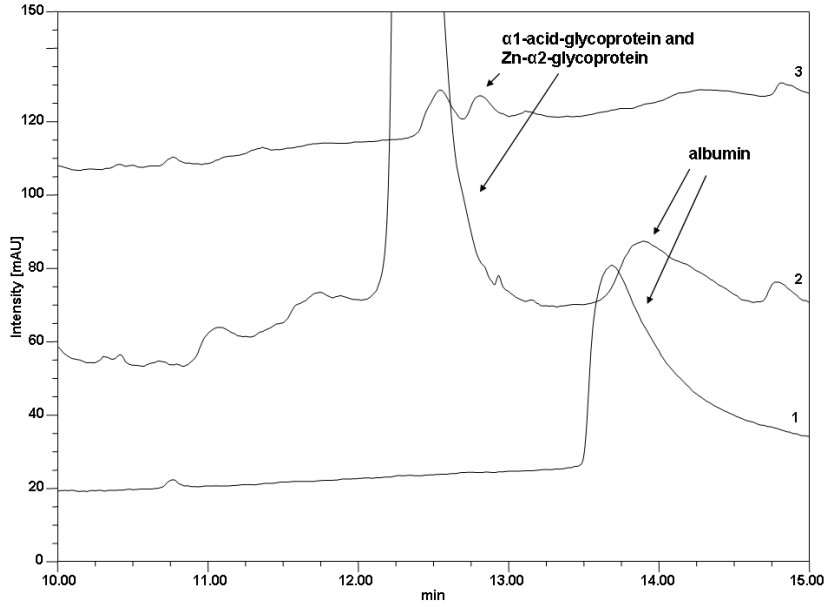

Figure 10. Changes of the levels of the proteins in the size-exclusion central peak of albumin by reversed-phase HPLC. Second relapse. Chromatogram number 1 is albumin standard of 153 mg/L, whereas number 2 is acute episode and number 3 is remission.

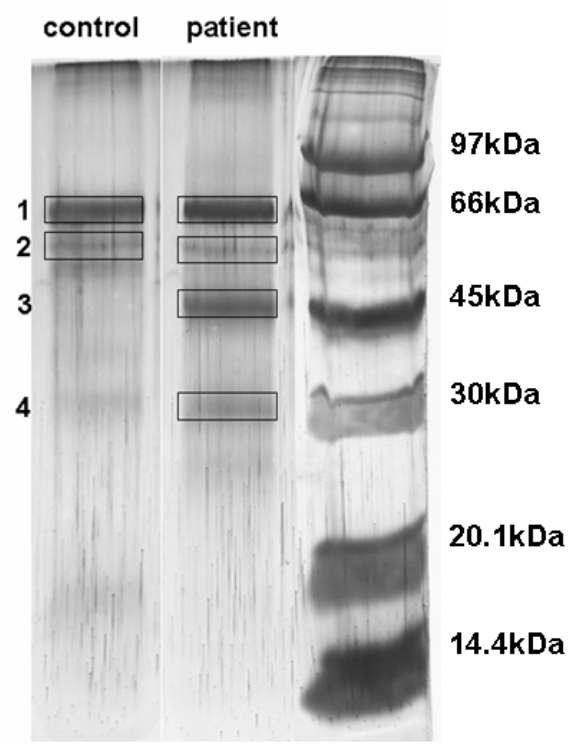

Figure 11. Sodiumdodecylsulphate polyacrylamide gel-electrophoresis of Crohn's disease patient urine obtained during acute phase compared with control urine from a healthy subject, prepared the same as the urine of Crohn's disease patient's.

MALDI-TOF/MS MEASUREMENTS

Mass spectrum measured from the first fraction of RP-HPLC is shown by Figure 12A. The peak appearing at 70.3 kDa can be considered to be the dimer of the protein with a mass of 34.7 kDa. Investigating this mass spectrum suggests the presence of two proteins in the first fraction, which observation is also supported by the RP-chromatogram.

Figure 12B exhibits the resulted peptide mass fingerprinting (PMF) and in Table 4 all the peptides of the PMF recognized by Mascot data base search engine are listed. Three proteins, α1-acid-glycoprotein-1, α1-acid-glycoprotein-2 and Zn-α2-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.

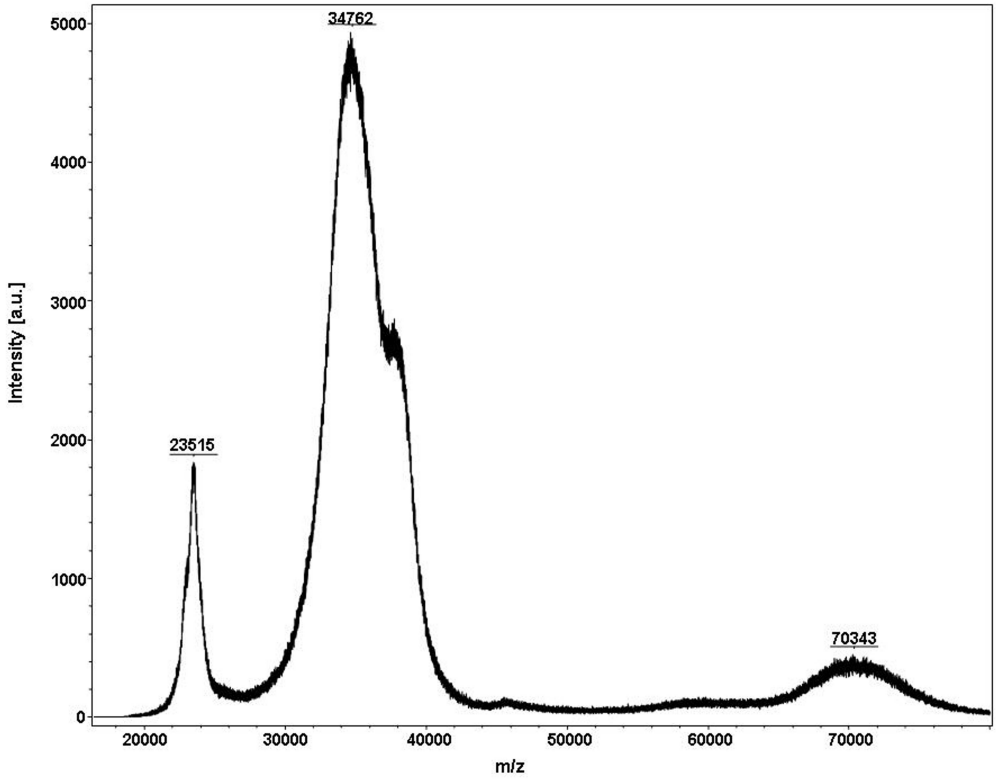

Figure 12A. Mass spectrum measured from the first fraction of reverse-phase high performance liquid chromatography.

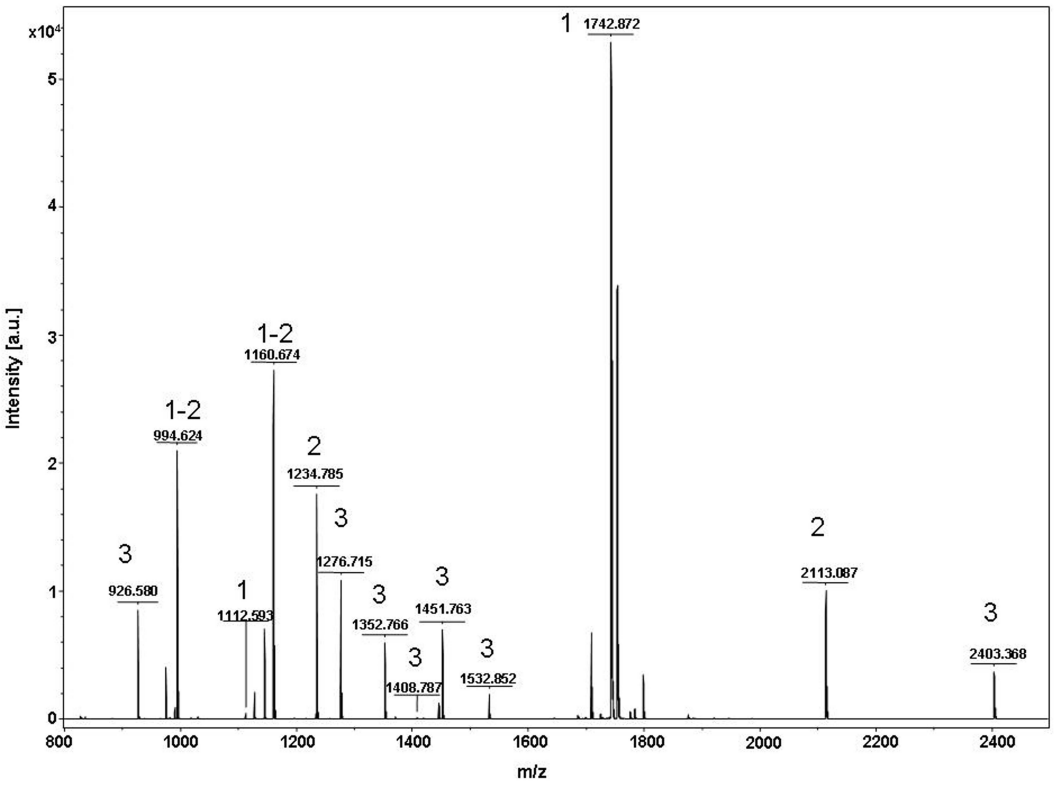

Figure 12B. Peptide mass fingerprint of the digested first fraction of reverse-phase high performance liquid chromatography. The numbers 1-2 refer to α-1 acid glycoprotein 1 and 2 and number 3 refers to Zn-α-2 glycoprotein. Results summarized in Table 4.

Table 4. Peptides of the peptide mass fingerprinting recognized by Mascot database from Fraction 2 of reversed-phase HPLC.

observed mass [Da]	theoretical mass [Da]	mass error [ppm]	data-base	sequence	access key	protein
808.389	808.3512	46	NCBI	YYYDGK	CAA49574	zn-α-2-glycoprotein
926.58	926.4843	97	NCBI	IDVHWTR	CAA49574	zn-α-2-glycoprotein
974.583	974.4876	98	NCBI	SQPMGLWR	CAA49574	zn-α-2-glycoprotein
994.624	994.5204	91	NCBI	TEDTIFLR	P02763/P19652	α-1-acid-glycoprotein-1-2
1018.604	1018.4874	94	NCBI	DKCEPLEK	P02763/P19652	α-1-acid-glycoprotein-1-2
1112.593	1112.5259	60	NCBI	SDVVYTDWK	P02763	α-1-acid-glycoprotein-1
1127.64	1127.5691	63	NCBI	AGEVQEPELR	CAA49574	zn-α-2-glycoprotein
1144.589	1144.4979	80	NCBI	SDVMYTDWK	P19652	α-1-acid-glycoprotein-2
1160.674	1160.5887	73	NCBI	WFYIASAFR	P02763	α-1-acid-glycoprotein-1-2
1233.661	1233.5609	81	NCBI	CLAYDFYPGK	CAA49574	zn-α-2-glycoprotein
1234.785	1234.7055	65	NCBI	EHVAHLLFLR	P19652	α-1-acid-glycoprotein-2
1276.715	1276.6321	65	NCBI	WEAEPVYVQR	CAA49574	zn-α-2-glycoprotein
1352.766	1352.6879	58	NCBI	AREDIFMETLK	CAA49574	zn-α-2-glycoprotein
1408.787	1408.7359	36	NCBI	YSLTYIYTGLSK	CAA49574	zn-α-2-glycoprotein
1418.711	1418.6508	36	NCBI	TLMFGSYLDDEK	P19652	α-1-acid-glycoprotein-2
1445.724	1445.6617	43	NCBI	TYMLAFDVNDEK	P02763	α-1-acid-glycoprotein-1
1451.763	1451.6835	55	NCBI	AYLEEECPATLR	CAA49574	zn-α-2-glycoprotein
1515.844	1515.7148	85	NCBI	SDVMYTDWKKDK	P19652	α-1-acid-glycoprotein-2
1532.852	1532.7856	43	NCBI	QKWEAEPVYVQR	CAA49574	zn-α-2-glycoprotein
1742.872	1742.8054	38	NCBI	EQLGEFYEALDCLR	P02763	α-1-acid-glycoprotein-1
1778.987	1778.9105	43	NCBI	AKAYLEEECPATLRK	CAA49574	zn-α-2-glycoprotein
1790.049	1789.8313	94	NCBI	DTKTYMLAFDVNDEK	P02763	α-1-acid-glycoprotein-1
1920.081	1919.9538	66	NCBI	SVQEIQATFFYFTPNK	P02763/P19652	α-1-acid-glycoprotein-1-2
2113.087	2112.9729	54	NCBI	EQLGEFYEALDCLCIPR	P19652	α-1-acid-glycoprotein-2
2403.368	2403.2092	66	NCBI	HVEDVPAFQALGSLNDLQFFR	CAA49574	zn-α-2-glycoprotein

Proteins identified from the excised gel slabs also confirmed these results whereas albumin is band 1 and 2 (66 kDa and albumin fragment of 55 kDa[66]), Zn-α2-glycoprotein is band 3, α-1-acid-glycoprotein is band 4. Investigating control urine from healthy individual allowed only the identification of albumin. Results of MALDI-TOF/MS measurements are summarized in Table 5.

Table 5. Proteins identified from the excised gel slabs by MALDI-TOF/MS.

	protein	mass [Da]	sequence coverage	score	error [ppm]	access key	database
control							
band 1	human serum albumin	66 kDa	27%	142.15	49	CAA01217	NCBI
band 2	human serum albumin	55 kDa	20%	83.09	58	(see reference 15)	NCBI
patient							
band 1	human serum albumin	66 kDa	29%	186.25	30	CAA01217	NCBI
band 2	human serum albumin	55 kDa	20%	73.44	65	(see reference 15)	NCBI
band 3	zn-α-2-glycoprotein	41 kDa	26%	122.06	45	CAA49574	NCBI
band 4	α-1-acid-glycoprotein-1-2	45 kDa	23%	57.33	75	P02763/P19652	NCBI

DISCUSSION

DISCUSSION

While there are an increasing number of publications on the significance of HPLC-measured albuminuria in diabetic patients[29-31], its properties and possible connection to clinical parameters are hardly addressed. The results of these studies proved that HPLC measures total urinary albumin, which includes a fraction of albumin which is not detectable by conventional immunochemical methods. The exact nature of this form is not fully understood. Agarwal[41] proposed that a fraction of albumin is modified by oxidative stress, which modification could be the reason of immuno-unreactivity. Sviridov et al[42] proposed that the size-exclusion HPLC method does not have sufficient resolution to separate albumin from other similar molecular size molecules, so the higher concentration by HPLC is an artefact caused by unresolved interferences.

To investigate the hypothesis above, that modification of albumin may alter immunoreactivity, **as the first part of this PhD thesis** we have measured known concentrations of in vitro differently modified forms of albumin with IN and with size-exclusion HPLC. To be able to verify the modification rate of the different forms of albumin we have established an HPLC method equipped with tandem UV and fluorescent detection. Results based on IN and HPLC measurements of in vitro differently modified forms of albumin showed no significant difference, which allows us to assume that modification (specifically glycation and/or oxidation) has a very low or no effect on immunoreactivity. As a matter of fact there is already one study which proposes that immuno-unreactive albumin is a form of albumin which has a limited number of polypeptide chain scissions but is held together by non-covalent intra-chain bonding disulfide bridges.[48]

In our study we have also used our tandem UV-fluorescent detector HPLC system for clinical purposes. For the first time, we have assessed the glycoxidation rate (in terms of relative fluorescence, RF) of HPLC-measured total urinary albumin and its connection to clinical parameters on diabetic patients. Our study showed that the glycoxidation rate of total urinary albumin reflects kidney pathophysiology by showing correlations with serum creatinine and eGFR levels but not with the parameters of glycaemia (concentration of plasma glucose, concentration of fructosamine and levels of hemoglobin A_{1c}). Moreover, in linear regression models serum creatinine and eGFR levels proved to be the independent predictors of glycoxidation rate of urinary albumin. Regarding the handling of modified forms of albumin by the kidney (mainly glycated albumin was studied), inconsistent conclusions can be found in the literature. An earlier

work suggested that excretion of modified (glycated) albumin is facilitated in diabetic patients with normo- and microalbuminuria in contrast to non-glycated albumin.[67] It was also assumed that glycated proteins have a preferential transport to the mesangial space and they arise in a greater amount in the urinary space.[68,69] Others could not verify preferential urinary excretion of glycated albumin.[70] Our findings with the tandem UV-fluorescent detector HPLC method showed that urinary albumin is modified to a higher rate in patients with microalbuminuria than in patients with normoalbuminuria, which finding agrees with the results of earlier works. Hyperglycaemia can induce oxidative stress in loco in the kidney of diabetic patients[71] which may contribute to the formation of glycated (fluorescent) albumin. We assume that this oxidative milieu could cause rapid modifications in molecules present in the urinary tracts, just like the short treatment of HSA with 1mM methylglyoxal in our in vitro experiment. This would mean that a more severe renal function decline - which means a higher oxidative stress level in the kidney – would produce more modified molecules (including albumin) in loco. Therefore the measurement of modified albumin would reflect the status of the kidney instead of generalized oxidative stress.

From a recent study it is known that urinary albumin concentration measured by HPLC significantly decreases during long-term freezing.[72] In our study as a part of characterization of the UV-fluorescent HPLC system we have measured reproducibility after one year of -80°C storage and we have found a significant -25%, decrease in the PA of albumin. This finding agrees with the finding of Brinkman et al (29%). A new result of our study is that we found that in vitro prepared albumin forms are not affected by long-term freezing. Moreover, it can also be ascertained that RF of urinary albumin is less and inversely affected by freezing, which is presumably because of albumin still being exposed to the oxidative agents of urine during freezing.

Some authors proposed that other proteins may coeluate during size-exclusion HPLC measurements with albumin in such quantities that could cause higher albumin concentrations than that one obtained with immunochemical methods. Based on the rough results of our RP-HPLC examinations this interference is only 12.7% on average, which is not more than has been assessed by others.[73] We propose that this level of interference could only have a small significance in contrast to the 2-3 fold concentration-differences between HPLC and IN.

A limitation of our study is that our method detects non-specific fluorescence of AGEs, fluorescent oxidation and lipoxidation adducts only and does not detect non-

fluorescent AGEs. However, concentration of some of these non-fluorescent products correlates with the concentration of fluorescent products.[37]

Since the introduction of HPLC-based albuminuria measurement, several studies have proven that HPLC detects more albumin than the immuno-based methods.[30,43-45] Recently, Magliano et al published their results of the impact of HPLC-detected albuminuria on mortality by measuring albuminuria with HPLC after 7 years of -80°C sample storage.[46] However, the studies to date did not pay enough attention to the reproducibility of the measurements after long-term storage. Brinkman et al published that loss of albumin after freezing urine depends not only on freezing temperature but also on the detection method, as they found a non-significant 5% change in albuminuria by IN and a significant 29% decrease by HPLC after 12 months of storage at -80°C.[72] As **the second part of this PhD thesis** we have confirmed this observation by measuring our samples after 2.5 years of storage at -80°C and found a 24% decrease by HPLC. This decrease in long-term frozen urine may reduce the value of albuminuria for the prediction of mortality as was proven by IN-detected albuminuria in prolonged frozen storage of urine samples at -20 °C.[74] However, a prospective study needs to elucidate this question.

In accordance with Brinkman et al we have also found a connection between urinary pH and a decrease of albuminuria as measured by HPLC.[72] As a possible explanation increased aggregation of urinary albumin at low pH was assumed because the isoelectric point of albumin is at pH 4.7.[72] However, only dimerization of bovine serum albumin under mildly acidic conditions was investigated in the literature[75] and we did not find any data on human urine analyses, especially on HPLC-detected albuminuria. Our study provided the opportunity to assess the change of monomeric and dimeric forms of urinary albumin in 2.5 years and we have found that only the monomeric form decreased significantly, whereas the dimeric form did not change significantly - as was previously proposed. The significant change in 2.5 years of the derived parameter of DMR, which represents the percentage of the dimeric form to the monomeric form of albumin, was found to correlate with the pH. However, this change and correlation with pH is a consequence of the change of the monomeric form only. This finding confirms that a decrease of urinary albumin determined by HPLC is pH-dependent and not due to an aggregation to the dimeric form. In this way other mechanisms such as degradation could be the reason for the observed decrease. In fact,

our hypothesis was just recently confirmed by Kania et al who showed that proteases are present and active in stored human urine samples which rapidly degraded urinary albumin into large fragments within minutes after adjustment to low pH. They proposed that adjustment to neutral pH or addition of protease inhibitors may be useful techniques for sample preservation.[76]

It is also known that the non-immunoreactive form of urinary albumin is a partially cleaved form which is maintained with an intact relative molecular mass (66 kDa) by the help of the disulfide bonds and which form fragments into smaller parts to reducing agents.[48] Therefore we measured the total sulfhydryl groups of both stored and newly collected fresh urine in an attempt to assess whether this free sulfhydryl group capacity could play a role in the decrease of HPLC-detected albuminuria by reducing disulfide bonds of albumin. We also aimed to determine which factors influence the concentration of these groups. 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. This means that urine has a potentially high level of reducing activity which is pH-dependent and it may play a role in changes of HPLC-detected albuminuria by breaking up the cleaved non-immunoreactive form of urinary albumin.

Crohn's disease is a lifelong illness with unpredictable disease course. The main goal in treating CD is to control acute flares of the disease and to maintain remission. To control relapses early recognition is essential. Despite the hallmark of available methods for recognizing disease activity, a perfect biomarker is still lacking.

Changes in the levels of ir-uAlb have been already shown to be a useful marker in CD.[52,53] Recently, it was proven that albumin in urine exists in non-immunoreactive form, as well[29] and so the concentration of albumin in urine is at least 3-times higher, as earlier believed.[43] Moreover, it was also suggested by one study that SE-HPLC-measured t-uAlb predicts diabetic nephropathy earlier than ir-uAlb, measured by conventional immunoassay, in both type 1 and 2 diabetes mellitus.[31] **In the third part of this PhD thesis** we investigated the hypothesis that measurement of t-uAlb may provide also some advantages in CD, as well. However, the huge difference between the concentrations measured by the two methods, especially in acute episode (almost 15-fold), led us to analyze the peak of the albumin of SE-HPLC further. As a result of further RP-HPLC, SDS-PAGE and MALDI-TOF/MS measurements we have identified

two other major proteins in the peak of the albumin of SE-HPLC which were practically absent in the urine samples obtained in remission.

Alpha-1-acid glycoprotein in serum, an acute phase reactant and a potential factor in the development of IBD,[77] is already known as an activity marker in CD; however its half-life of five days made it less useful.[51] Nevertheless, urinary α-1-acid-glycoprotein was only examined thoroughly in diabetic patients, but never in CD patients, and its concentration was found to be unaffected by the serum concentration.[78] Besides, it was found to be a powerful predictor of cardiovascular mortality in normoalbuminuric diabetic patients.[79] The fact that serum and urinary concentrations are independent from each other means that measurement of α-1-acid-glycoprotein in urine can be a new activity marker in CD. Moreover, it can also explain the phenomena we found that the molecular weight of native α-1-acid-glycoprotein was only 23.5 kDa (mass of peptide backbone without glycans) was found in the urine samples instead of 41-43 kDa.

The same phenomena could be observed with Zn-α-2-glycoprotein; only 34.7 kDa was detected by MALDI-TOF/MS, instead of the carbohydrate-containing protein with a mass of 41 kDa. However, until nowadays exact function of this protein is unknown. Structurally it shows high similarities with major histocompatibility complex class I proteins.[80] It is thought to be involved in depletion of fatty acids from adipose tissues and it is already a potential serum marker of several cancers.[81] We speculate that the elevated expression and excretion of this protein in acute phase of CD is also related with fatty acid mobilization. However, to the best of our knowledge this protein was never investigated in CD or in any IBD.

Undoubted weakness of this study is that deductions are based on one single patient, however examination of stored urine of other CD patients in relapse from our lately published cross-sectional study[55] show also high excretion of α-1 acid glycoprotein and the Zn-α-2 glycoprotein (Figure 13).

Summarizing our findings we have shown that uAlb measured by the new SE-HPLC method in CD is not reliable since it measures a high amount of other proteins. As a matter of fact the resolution of SE-HPLC has been addressed by others[42] and by us[82] however, coeluting proteins was expected to be ~20-30%. On the other side these coeluting urinary proteins, the α-1 acid glycoprotein and the Zn-α-2 glycoprotein, in the peak of the albumin showed an almost perfect association with the clinical status; they were highly excreted in relapse and were almost absent in remission (as shown in

Figure 9 and 10). Furthermore, as well as urinary α-1 acid glycoprotein, urinary Zn-α-2 glycoprotein has also never been investigated, moreover, not even associated with CD.

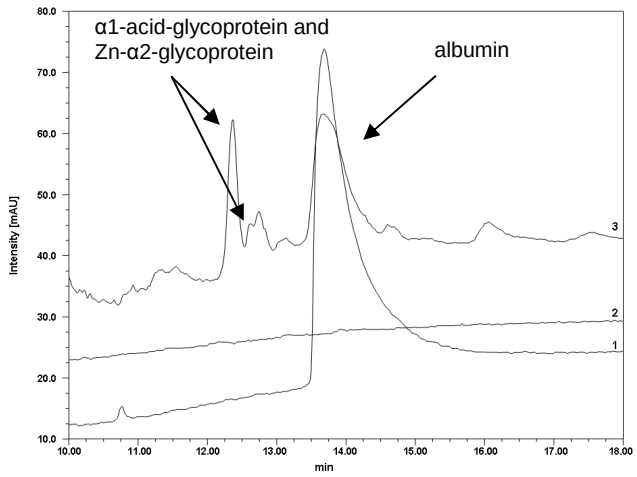

Figure 13. Levels of the different proteins in the size-exclusion peak of albumin by reversed-phase high performance liquid chromatography. Chromatogram number 1 is albumin standard of 153 mg/l, whereas number 2 is chromatogram of a healthy subject and number 3 is chromatogram of another Crohn's disease patient with relapse.

CONCLUSIONS

CONCLUSIONS

To answer the questions raised in **the first part of the thesis** firstly, we have established a high performance liquid chromatography method coupled with UV-fluorescent detection to be able to detect relative fluorescence of total urinary albumin, and a reverse-phase high performance liquid chromatography to assess interference rate of non-albumin proteins in the size-exclusion peak of albumin. Based on our in vitro studies with differently modified forms of albumin we have shown that relative fluorescence reflects the glycoxidative modification rate of the albumin and that modification of albumin potentially did not cause the loss of immunoreactivity. Measurements of urine samples of diabetic patients with our coupled UV-flourescent high performance liquid chromatography revealed the glycoxidation rate of total urinary albumin reflects kidney pathophysiology by showing correlations with serum creatinine and estimated glomerular filtration rate but not with the parameters of glycaemia. Performing a reverse-phase high performance liquid chromatography separation of the size-exclusion peak of albumin of urine of diabetic patients we were able to provide an estimation of an average interference rate of 12.7% non-albumin material being present in the albumin peak.

In the **second part of the thesis** we have investigated the possibility of the decrease of the concentration of albumin measured by high performance liquid chromatography in stored urine samples. We concluded 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 in accordance with some others, performed similar measurements by immuno-based methods. That is why we concluded that for the measurement of urinary albumin by high performance liquid chromatography only fresh urine samples should be used and we think that conclusions based on stored urine measurements should be handled with caution. We have found also a strong correlation between the amount of the free sulfhydryl groups and urinary pH in fresh urine samples which correlation could not be observed, and concentration of free sulfhydryl groups significantly decreased in stored urine. Since it was suggested by one study that 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 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.

The serendipitous findings of the **third aim of this thesis** are more than interesting in the era of the biomarkers. Our aim of comparing the changes of the concentration of total albumin by high performance liquid chromatography, and the immunoreactive albumin by immunoturbidimetry in pilot study in a single Crohn's disease patient with frequent exacerbation phases has exceeded our expectation. The surprisingly high difference between the two methods led us to further analyze the peak of the total albumin using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, a technique that allowed us the identification of two possible biomarkers: the α-1 acid glycoprotein and the Zn-α-2 glycoprotein. However, from this study we had to conclude also the fact that urinary albumin measured by size-exclusion chromatography method in the acute phase of Crohn's disease is not reliable, since it measures a high amount of other proteins.

LIST OF PHD THESES

LIST OF PHD THESES

1) Glycoxidative modification of the albumin does not affect immunoreactivity.

2) Glycoxidative modification rate of total urinary albumin in patients with diabetes mellitus reflects renal pathophysiology.

3) Coeluating 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.

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LIST OF PUBLICATIONS

LIST OF PUBLICATIONS
Cumlative impact factor: publications 32.159, abstracts: 43.311
Cumulative impact factor of publications used in this thesis: publications: 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, WittmannI. 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.

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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 (Eurpoean 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.
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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 glycoxidation-products in the urine of diabetic patients. *Acta Physiologia Hungarica* 2006:93(2-3):210.
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1. Molnár G. A., Wagner Z., **Markó L.**, Kőszegi T., Mohás M., Kocsis B., Matus Z., Wagner L., Tamaskó M., Mazák I., Laczy B., Nagy J., Wittmann I**.:** Urinary ortho-tyrosine excretion in diabetes mellitus and renal failure: evidence for hydroxyl radical production. *Kidney Int* 2005:68(5):2281-2287. **IF: 4.927, No. of citation: 5**

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