**Univerzita Karlova v Praze**

**1. lékařská fakulta**

PhD Thesis Summary

# STUDY OF PROTEIN CHANGES IN PATIENTS WITH NEPHROTIC SYNDROME AND ANDERSON-FABRY DISEASE

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## 1. Content

## 2. Abstrakt

**Úvod:** Závažná proteinurie může být způsobena jednak zvýšenou permeabilitou glomerulární bazální membrány, porušením struktury membrány či podocytů, tak také porušením sekrečně-reabsorpčních tubulárních procesů. Metodou 2D elektroforézy jsme analyzovali 60 pacientů s nefrotickou proteinurií a jinými diagnózami (lupusová nefritida, membranózní nefropatie, IgA nefropatie, Wegenerova granulomatóza) a 20 pacientů s Andersonovo-Fabryho chorobou (AFD), jenž je X-vázané genetické onemocnění postrádající aktivitu α-galaktozidázy A. Hlavním cílem této práce bylo nalézt možné rozdíly močových proteinů u nefropatií, mezi zdravými kontrolami a AFD pacienty a identifikovat abnormální proteiny jako potenciální biomarkery nemoci.

**Metodika:** Močové proteiny byly děleny metodou izoelektrické fokusace s použitím polyakrylamidových stripů (pH 3-10 lineární). SDS elektroforéza byla provedena v 12% polyakrylamidovém gelu. Proteiny byly vizualizovány stříbrem a identifikovány MALDI-TOF MS. Gely byly hodnoceny softwarem Phoretix 2D expression verze 2005.

**Výsledky:** Zjistili jsme, že bez přídavku inhibitorů proteáz můžeme detekovat proteolýzu se zvýšeným množstvím proteinů nacházejících se v oblasti kolem 10 kDa a sníženým množstvím proteinů vyskytujících se v oblasti kolem 50 kDa. Odstranění albuminu zlepšilo přehlednost močových proteomů. Srovnání močových map ukázalo významné změny proteinů, které jsou typické pro Andersonovu-Fabryho chorobu a nefropatie. U AFD pacientů byla nalezena glykosylace prostaglandin H2 D-izomerázy v místě Asn51 a Asn78. Pomocí MALDI-TOF MS byl identifikován albumin, transferin, alfa-1 antitrypsin a transthyretin prekurzor.

**Závěr:** Změny močových proteinů mohou být důležité pro diagnózu a vývoj renálního onemocnění. Močová proteomika u AFD pacientů odhalila zvýšenou sekreci některých proteinů. Předpokládáme, že pozorované rozdíly v množství a pozici prostaglandin H2 D-izomerázy na 2D gelu, mohou být spojeny s odlišnou glykosylací u AFD jedinců.

**Klíčová slova:** Andersonova-Fabryho choroba/ MALDI-TOF MS/ dvou rozměrná elektroforéza/ močové proteomy / nefrotický syndrom

## 3. Summary

**Background:** Heavy proteinuria may be caused by either increased glomerulal basement membrane permeability or membrane or podocyte structural damage, and also by impairment of secretion-reabsorption tubular processes. In this study, 60 patients with nephrotic proteinuria and other diagnoses (lupus nephritis, membranous nephropathy, IgA nephropathy, Wegener´s granulomatosis) and 20 patients with Anderson-Fabry disease (AFD),which is an X-linked genetic disorder with deficient *a*-galactosidase A activity, were analysed by the 2D electrophoresis method. The main aim of this work was to investigate possible differences in urine proteins in nephropaties, between healthy controls and AFD patients and to identify abnormal proteins as potential biomarkers of disease.

**Methods:** The urine proteins were devided by isoelectric focusing method using polyacrylamide strips (pH 3-10 linear). The second dimensional SDS electrophoresis was performed in 12 % polyacrylamide gel. The proteins were visualized by silver method and selected proteins were identified by MALDI-TOF MS. The gels were evaluated by Phoretix 2D expression software 2005.

**Results:** We found out that without adding protease inhibitors we can detect proteolysis, with increased quantity of proteins manifested in the area about 10 kDa and decreased quantity of proteins detectable in the area with molecular weights about 50 kDa. The separation of albumin caused higher lucidity of the urinary proteomes. The urinary maps comparison brought out that there are significant proteins´ changes, which are typical for Anderson-Fabry´s disease and other nephropathies and possible glycosylation at Asn51 and Asn78 sites of the prostaglandin H2 D-isomerase was detected in AFD patients. Also albumin, transferrin, alpha-1 antitrypsin and transthyretin precursor were identified by MALDI-TOF MS.

**Conclusion:** Changes of urinary proteins should be important for renal diagnosis and progression. AFD urinary proteomics revealed increased secretion of several proteins. We postulate that the observed difference in the amount of prostaglandin H2 D-isomerase and its position on two dimensional gels might be related to different glycosylation in AFD subjects.

**Keywords:** Anderson-Fabry disease/ MALDI-TOF MS/ two-dimensional electrophoresis/ urinary proteomes / nephrotic syndrome

## 4. Abbreviation

| | |
|---|---|
| AAV | ANCA-associated vasculitis |
| ACN | acetonitrile |
| AFD | Anderson-Fabry disease |
| CHCA | *a*-cyano-4-hydroxycinnamic acid |
| ERT | enzyme replacement theraphy |
| FSGS | focal segmental glomerulosclerosis |
| $Gb_3$ | globotriaosylceramide |
| HPLC | high preasure liquid chromatography |
| IEF | isoelectric focusing method |
| IgAN | IgA nefropathy |
| LC | liquid chromatography |
| Lyso- $Gb_3$ | globotriaosylsphingosine |
| MALDI-TOF | matrix associated laser desorption/ionization time of flight |
| MN | membranous nefropathy |
| MS | mass spectrophotometry |
| PAGE | electrophoresis in polyacrylamid gel |
| pI | isoelectric point |
| SELDI-TOF | surface-enhanced laser desorption/ionization TOF |
| SLE | lupus nephritis |
| TFA | trifluoroacetic acid |
| WG | Wegener´s granulomatosis |

## 5. Introduction

Proteinuria is a well-recognized negative prognostic factor for patients with kidney disease. The possible presence of proteases among various urinary proteins should be stressed, as they may play an important role in degradation and changing structure and features of the filtered proteins. Ultimately the reabsorption by tubular cells may be affected. Common laboratory methods do not allow detection of changes in these urinary protein molecules. However, these changes of proteins may contribute to renal damage and its progression. Proteomic analysis (2D, LC-MS/MS, HPLC-MS/MS) provides many advantages compared with conventional methods such as Western blot analysis, because a large complement of expected and unexpected proteins can be examined simultaneously. Western blot analysis is limited by the relatively small number of proteins that can be studied in a single experiment and the availability of a specific antibody to the targeted protein is also necessary. However, HPLC coupled with tandem MS (HPLCMS/MS) requires complicated instrumentation and MS analysis is required for all experiments. Construction of a urinary proteome map on 2D gel may therefore be a useful technique, because the recent methodology of 2D PAGE provides a consistent protein spot pattern (Thongboonkerd, 2004).

The presented data are focused on the study of urinary protein changes in glomerular renal diseases (predominantly with the nephrotic syndrome) and Anderson-Fabry disease (AFD) using modern analytical techniques such as isoelectric focusing and two-dimensional (2D) electrophoresis in polyacrylamide gels. A similar technique of protein determination by 2D electrophoresis is being studied in other laboratories, which are focused on different types of diseases such as tubular renal disease (Fanconi syndrome) or various carcinomas (Cutillas et al., 2004; Pieper et al., 2004; Rossing et al., 2005). Protein analysis using 2D electrophoresis gives a very clear picture about proteins contained in the sample. Earlier studies have shown that this technique is comfortable for protein analysis from animal sources (Gygi et al., 2000; Washburn et al., 2001; Oh et al., 2004).

We have focused on detailed urinary protein analysis (technique optimization, sample preparation, and protein stability) in patients with glomerulopathies accompanied mostly with nephrotic syndrome and Anderson-Fabry disease. The urinary protein in healthy people does not exceed 150 mg/day and its increase above 250 mg/day indicates kidney damage. Proteinuria higher than 3,5 g/ 24h is typical for the nephrotic syndrome, which is a clinical and laboratory syndrome characterized by proteinuria, hypoproteinemia, edema and

hypercholesterolemia. Heavy proteinuria is caused by increased glomerulal basement membrane permeability and membrane or podocytes damage. The most common primary glomerulopathies leading to progress of nephrotic syndrome are focal segmental glomerulosclerosis, idiopatic membranous nephropathy and nephrotic syndrome with minimal change disease which is the main reason of nephrotic syndrome in childhood. These three diseases are the cause of the formation of nephrotic syndrome in 60-95 per cent of the cases and its distribution depends on age. Anderson-Fabry disease (AFD) is an X-linked glycolipid storage disorder that is caused by deficient activity of the lysosomal enzyme *a*-galactosidase A (*a*-gal A). (Beck et al., 2004; Mehta et al., 2004) Globotriaosylceramide (Gb3), the glycolipid substrate for this enzyme, progressively accumulates within vulnerable cells and tissues of affected patients, which leads to a wide clinical spectrum of clinical manifestations. The diagnosis of AFD is usually established by detection of low *a*-gal A activity in plasma, leukocytes, or fibroblasts, or by detection of increased $Gb_3$ levels in urine kidney, skin, and heart, or by detection of gene mutations.(Tesar et al., 2003) The incidence of AFD is estimated to be 1:40,000, (Schiffmann et al., 2000; Spada et al., 2006) in some studies up to 1:3500. (Spada et al., 2006) Enzyme replacement therapy (ERT) with human recombinant *a*-gal A has become available since 2001.(Eng et al., 2001; Schiffmann et al., 2001) Recent evidence suggests that the ERT is more efficacious if started early, before the development of advanced kidney involvement with overt proteinuria.(Schiffmann et al.,2000; Banikazemi et al., 2007) Until now, assessment of $Gb_3$ excretion and its concentration in plasma and urine has been used as a biomarker to monitor the disease response to the treatment. Recently, lyso-$Gb_3$ has been suggested as a more promising tool.

However, there is a critical need to find new biomarkers for assessment of the disease severity in nephrotic patients and the ERT efficacy (Schiffmann et al., 2010) in AFD patients. Our study was designed to analyze potential differences in urinary proteomes in nephrotic patients and differencies between AFD patients and healthy controls, using modern analytical techniques, such as isoelectric focusing and two-dimensional (2D) electrophoresis in polyacrylamide gels.

The main aim of this work was to identify potential disease-specific proteins using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We expect this finding to facilitate better understanding of nephropathies.

## 6. Aims

The main aim of this work was to suggest 2D method which would provide clear spectrum of urine proteins in nephrotic patients and which would show possible protein changes caused by presence of proteases or due to illness and therapy.

1. Development of method providing a lucid spectrum of urine proteins in nephrotic patients with detectable protein´s changes
    - optimization of two-dimensional electrophoresis technique for urine samples of nephrotic patients
    - effect of albumin separation from the sample
    - study of stability of urine samples
    - detection of protein changes due to presence of proteases

2. Study of urine proteins in patients with nephrotic proteinuria
    - comparison of 2D spectrum of proteins between individual groups
    - characterization of selected proteins using MALDI-TOF mass spectrophotometry

3. Study of urine proteomes in patients with Anderson-Fabry disease
    - detection of differences in proteomes between AFD patients and healthy controls
    - identification of potential specific proteins of the disease using MALDI-TOF MS

## 7. Material and Methods

### 7.1 Studied groups

Urinary samples from 60 patients with nephrotic proteinuria and other diagnoses (systemic lupus erythematodes with renal involvement (lupus nephritis - SLE) and ANCA-associated vasculitis (AAV), amyloidosis AL, membranous nephropathy, IgA nephropathy, primary focal segmental glomerulosclerosis (FSGS), Wegener´s granulomatosis (45 mens, 15 women, age range 18-78 years) were analysed.Their proteinuria varied between 0.23 and 19 g per day. Urinary samples from 20 patients with AFD (11 males, 9 females, age range 18–69 years) were collected. Their proteinuria varied between 0.1 and 1 g/24 h; 13 patients were on ERT (nine of them were given agalsidase beta (Fabrazyme®) 1 mg/kg every other week, four of them were given agalsidase alfa (Replagal®) 0.2 mg/kg every other week) and 7 subjects received no therapy. Physiologic proteinuria is usually estimated to be between 30 and 50 mg/day and is mostly composed of tubular proteins, namely uromodulin (Tamm–Horsfall protein). Proteinuria of 150 mg/day may be accepted as an upper limit of "normal" proteinuria. The control group consisted of 10 healthy individuals (five males and five females aged between 27 and 42 years of age).

### 7.2 Preparation of urine sample

#### 7.2.1 Concentration and storage of urine samples

Samples (30 mL) from their 24-h urine collections were used for the analysis. Total protein concentration was determined using pyrogallol red. Proteinuria is usually very low (up to 1 g/24 h) in patients with Anderson - Fabry disease; therefore, most of the urine samples (proteinuria of less than 1 g/24 h) had to be concentrated (10 kDa, 5 min, 3000 × *g*, 4°C) using Amicon Ultra (Millipore, Massachusetts) before their 2D analysis. (Joo et al., 2003). The Bradford spectrophotometric assay (Bradford, 1976) was used to determine protein concentration in urine in concentrated samples. The samples were analysed immediately after collection or stored in small aliquots at -80°C.

### 7.3 Determination of urine proteins using 2D electrophoresis

The proteins were divided by the isoelectric focusing method (IEF) using 7-cm polyacrylamide strips with immobilized linear gradient pH 3–10 (IPG strip pH 3–10L, Amersham Biosciences). The total amount of protein placed on each polyacrylamide strip was 20 μg. Each strip was rehydrated overnight with a rehydration solution. The isoelectric focusing was performed in three steps to reach 3500 V. After completion of the IEF, the proteins on the strip were equilibrated with an equilibration buffer or the strip was frozen at –80°C for later use. The IPG strip was then transferred onto 12% polyacrylamide gel and the second dimensional separation was performed in SE260 Mini-Vertical Electrophoresis Unit (Amersham Biosciences). The proteins were then visualized by the Silver Method (Silver Bullit Kit, Amresco, St. Louis, MO).

The software Phoretix 2D expression 2005 was used for protein analysis. The patient's urine sample that was analysed immediately after collection was used as a reference gel. This reference gel was then used to compare proteins between investigated gels with or without added protease inhibitors. The matched protein spots by an automatic matching were then confirmed and edited manually. Background subtraction was performed, and the intensity volume of each spot was normalized with total intensity volume, next named as normalized quantity of protein. The protein content (in %) was calculated using the following formula:
% protein: [total protein in the sample (with or without inhibitors)/total protein in the sample analysed immediately after collection] x 100

### 7.4 Application of optimized technique

#### 7.4.1 Stability of urine samples

Samples that were not analysed immediately were stored at –80 °C. Their stability was validated by repeated analysis after 30 and 180 days, respectively. Again, we used the centrifugation method described above where the quantity of proteins in the samples was lower than 1 g/24 h. To test the stability of proteins, we studied five different samples of patients with the nephrotic syndrome. Protein spectra of samples analysed immediately after collection, 30 and 180 days after storage at –80 °C, were compared using software Phoretix 2D expression 2005.

### 7.4.2 Albumin separation from samples

Albumin in urine samples was determined by nephelometry technique using immunochemistry system IMMAGE. Albumin was separated from samples containing albumin concentrations higher than 3 g/ 24 h using ammonium sulphate (Harlow and Lane, 1999). The amount of ammonium sulphate was added to the given volume of each of the five samples studied, so as to get 50% saturated solution. The samples were incubated and mixed at 4°C for 16 h. Exactly 50% saturation of the sample by ammonium sulphate causes protein agglutination and only albumin remains diluted in the solution. The mixture was next centrifuged (Amicon Ultra 10 kDa, 10 min, 10 000 x *g*, 4°C). The pellet containing all urine proteins except albumin was diluted in distilled water and transferred to the dialysis membrane (molecular mass cut-off at 10 kDa). The following dialysis against 1.5 M Tris buffer, pH = 7.5, removed the ammonium sulphate, which might disturb the subsequent detection of proteins. The protein concentration after removing albumin was determined by Bradford's assay. The samples prepared by this method were analysed immediately.

### 7.4.3 Effect of proteases

The presence of proteases was studied in samples from five patients with primary glomerulopathy after 48 h incubation at 37 °C. Three aliqouts from each patient were prepared in this part of the study. Two identical 5-ml aliquots from each urine sample were separated immediately after collection. Forty µl of a cocktail containing protease inhibitors (serine, cysteine, aminopeptidase) and sodium azide were added to one aliquot of each sample. The second aliquot contained only sodium azide. These two aliquots were incubated 48 h at 37°C. Another (third) aliquot obtained from each sample was frozen at –80°C immediately after the collection without adding any other substances. All aliquots were analysed together at the same time.

### 7.4.4 In gel protein digestion

The silver-stained protein spots were excised from the 2D gels, cut into small pieces, and washed several times with fresh 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. Upon complete destaining, the gel pieces were washed with water, dehydrated in acetonitrile (ACN), and again rehydrated in water. The supernatant was removed and the gel was partly dried in a vacuum concentrator. The gel pieces were then

reconstituted in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 1 mM $CaCl_2$, 10% ACN, and sequencing grade modified trypsin (50 ng/ mL, Promega, Wisconsin). After overnight digestion, the resulting peptides were extracted with 40% ACN/0.5% trifluoroacetic acid (TFA). For the low-abundance proteins, the peptides were purified and concentrated prior to mass spectrometric analysis according to manufacturer's user guide using ZipTip tips with 0.6 μL C18 resin (Millipore,Massachusetts).

### 7.4.5 MALDI-TOF MS a protein identification

Ten milligrams per milliliter solution of *a*-cyano-4-hydroxycinnamic acid (Bruker Daltonik, Germany) in 30% ACN/30% MeOH water solution containing 0.2% TFA was used as a MALDI matrix. A sample (1 mL) was placed on the polished steel MALDI target plate, allowed to air-dry at room temperature, and then 1 mL of the matrix solution was added. Positive ion mass spectra were obtained using a matrix-assisted laser desorption/ionization time-offlight/ time-of-flight (MALDI-TOF/TOF) mass spectrometer (Autoflex II, Bruker Daltonik) in reflectron mode, equipped with a 337 nm nitrogen laser. The generated spectra were within the mass range of 700–4500 Da. The data were calibrated against the Peptide calibration standard I (Bruker Daltonik) as an external calibration standard, using a seven-point calibration. Mass spectrometric sequencing of selected peptides was done by MALDI LIFT-TOF/TOF MS of the same prepared samples with the above instrument operated in MS/MS mode. Using the FlexAnalysis 2.4 program with the SNAP peak detection algorithm, peak lists in the XML data format were created. Statistical calibration was included in the program and no further adjustments were applied; the maximum number of assigned peaks was set to 50. After the peak labeling, all known contaminant signals were manually removed. The peak lists were examined against Swiss-Prot or NCBInr database subsets of human proteins, using the MASCOT search engine with the following search criteria settings: peptide tolerance of 100 ppm, the number of allowed missed cleavage sites was set to 1, fixed modifications of cysteine carbamido-methylation, and variable methionine oxidation. No restriction criteria were set with respect to protein molecular weight and p*I* values. Identity of a particular candidate protein was confirmed by MS/MS peptide sequencing, when either the probability-based Mowse score was only slightly higher than the threshold value calculated for the parameters used or the sequence coverage was too low.

### 7.4.6 Statistical evaluation

The statistical analysis was performed using Statistica CZ 9 (StatSoft, Prague 6, and Czech Republic). All values are reported as mean ± standard deviation or as shift percentages. Repeated measures ANOVA was used for data analysis and post-hoc p-levels for the Tukey honest significant difference (HSD) were considered to be statistically significant at the $p<0.05$ level.

## 8. Results

### 8.1 Optimization of 2D technique

#### 8.1.1 Separation of albumin

Albumin, which in samples with higher proteinuria (above 5 g/ 24h) deteriorated the lucidity of urinary proteomes, was removed from samples by a method based on different protein solubility at a given concentration of salts. Albumin concentration in these samples was about 3 g/ 24h. Two protein maps of patient´s sample with proteinuria are shown in Fig. 1

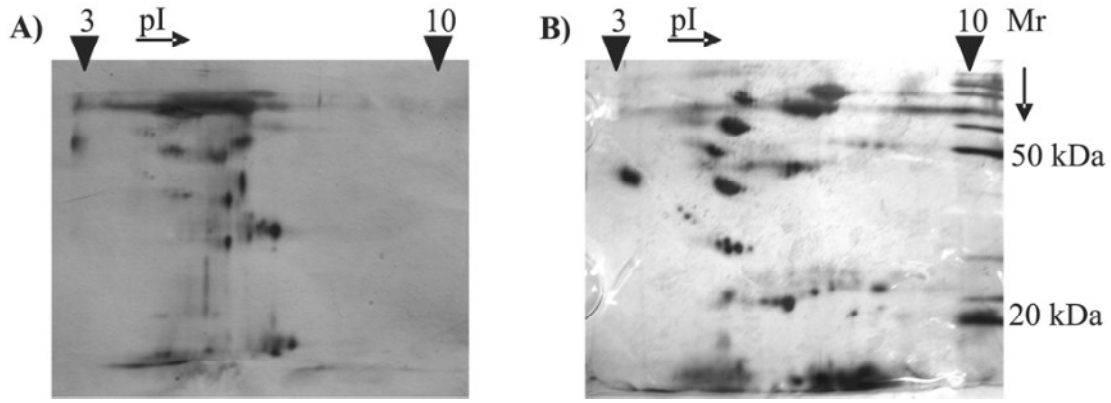


Figure 1. Comparison of protein map before and after removal of albumin. The urine samples were analysed immediately after collection using 7 cm polyacrylamide strips pH 3–10L by the IEF method, where 20 µg of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver. The protein spectrum of a patient with nephrotic syndrome before (A) and after (B) removal of albumin.

The separation of albumin from sample resulted in higher lucidity of the urinary proteomes and higher protein resolution in an area with molecular weight > 30 kDa. We obtained normalized levels of albumin quantity using software 2D expression 2005 and found out lower amount of albumin (75 per cent decrease) in the sample after separation. (Table I.)

Table I. Albumin with significant changes of intensity in quantity before and after separation from the sample.

| Normalized level of quantity (mean ± SD)[a] | | | |
|---|---|---|---|
| | albumin before extraction | albumin after extraction | p- level |
| albumin | 26,53 ± 1,66* | 6,63 ± 0, 63* | 0, 000292* |

[a] N=5 electrophoreograms from five independent urine aliquots. * Data were analysed by ANOVA test at the $p < 0, 05$ level and compared by Tukey HSD test.

The lower amount of albumin in the sample is visible at a 1D electrophoreogram of three fractions, namely proteins before extraction of the albumin, supernatant after centrifugation, and urine proteins after separation of the albumin (see Fig. 2).


Figure 2. SDS-PAGE in 12% polyacrylamide gel detected by silver: 1. standard; 2. urine proteins before albumin extraction; 3. supernatant containing albumin after centrifugation; 4. urine proteins after albumin extraction.

Repeat Measures ANOVA test at the $p < 0, 05$ level followed by the Tukey test analysis was statistically significant for differences between quantity of albumin before and after separation from the sample.

### 8.1.2 Stability of samples

The protein content of urine in proteinuria is higher than 1 g/ 24h. It was therefore not necessary to concentrate urine samples before 2D analysis when silver detection is used for protein visualization. Two different urine samples from patients with the nephrotic syndrome analysed on the first and 30th day after collection of samples are shown in Fig. 3. The study of the protein spectrum using software Phoretix 2D expression 2005 before and after 30 days of storage at –80 °C did not show protein changes in the sample. Even after 6-month storage of the sample, its reanalysis produced identical results. Normalized quantity of five selected proteins is shown in table II.

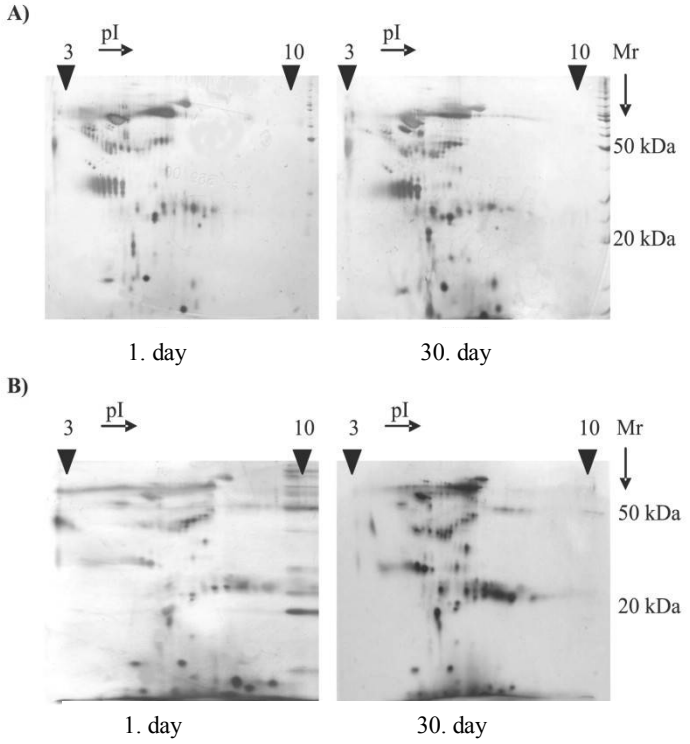


Figure. 3. Comparison of 2D electrophoreograms analysed in different intervals after sample collection. The urine samples were analysed immediately after collection using 7-cm polyacrylamide strips pH 3–10L by the IEF method, where 20 μg of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver.

Repeat Measures ANOVA test at the $p < 0, 05$ level followed by the Tukey test analysis was not statistically significant for differences between quantity of selected proteins in samples analysed first day and 180 day after sample collection and stored at -80˚C.

Table II. Data of proteins – 1st and 180th day after urine collection

| **Analyzed data (intensity of proteins quantity) using Phoretix 2D expression v2005 (mean ± SD)[a]** | | | |
|---|---|---|---|
| Number of protein | 1. day after collection | 180. day after collection | p-level |
| protein 1 | 10,11 ± 1,51* | 10,06 ± 1, 57* | 1,000000* |
| protein 2 | 7,84 ± 0,76* | 7, 95 ± 0,77* | 0,999998* |
| protein 3 | 5,14 ± 0,52* | 5,01 ± 0,75* | 0,999995* |
| protein 4 | 12,27 ± 0,59* | 12,02 ± 0,97* | 0,999202* |
| protein 5 | 4,22 ± 0,66* | 4,35 ± 0,68* | 0,999994* |

[a)] N=5 electrophoreograms from five independent urine aliquots. * Data were analysed by ANOVA test at the $p < 0, 05$ level and compared by Tukey HSD test.

### 8.1.3 Effect of proteases

The quantity of enzymes (including proteases) in an urine in kidney disease increases due to damaged plasma filtration and may unfavourably affect protein analysis results. Figure 4 shows four different urine samples from patients with the nephrotic syndrome. The first three electrophoreograms (A, B, C) are focused on the total urinary protein spectrum comparison and the last one (D) is focused on the area of molecular weight higher than 30 kDa. The study of protein spectra (urine samples from five different patients with glomerular disease) using software Phoretix 2D expression 2005 has confirmed important protein changes observable at two areas of molecular weight, i.e. range 50–100 kDa and 10–20 kDa (Fig. 4 – diagram marked in black).

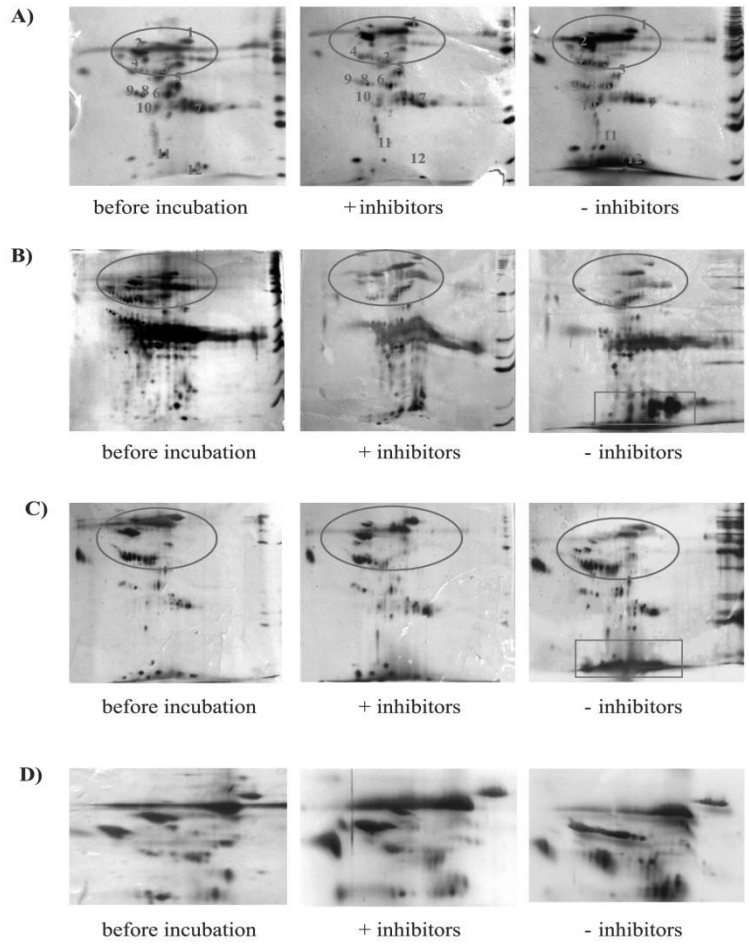


Fig. 4. Changes in the protein map in accordance to the presence of protease inhibitors in the samples of four different patients (A, B, C, D) with nephrotic syndrome. D) detailed comparison of protein map changed in the area of molecular weights higher than 30 kDa. Samples were analysed after incubation using the IEF method on polyacrylamide strips,where 20 µg of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver.

Data of intensity of proteins quantity were evaluated using software 2D expression 2005. (Table III.). We could detect proteolysis in samples without adding any protease inhibitors, as manifested by the increased amounts of small proteins (about 10 kDa). On the other hand, a decrease of proteins was observable in the area with molecular weights about 50 kDa.

Table III. Intensity of quantity of selected proteins before experiment, with addition of proteases inhibitors (SIN) and without addition of proteases inhibitors (BIN)

| **Analyzed data (intensity of quantity of proteins) using Phoretix 2D expression v2005** | | | | | |
|---|---|---|---|---|---|
| Protein | before experiment (mean ± SD)[a] | SIN (mean ± SD)[a] | BIN (mean ± SD)[a] | p-level before experiment vs. SIN | p-level before experiment vs. BIN |
| **1** | 6,69 ± 0,18* | 6,29 ± 0,22* | 5,44 ± 0,19* | 0,080900 | **0,000160** |
| **2** | 5,66 ± 0,29* | 6,82 ± 0,04* | 3,71 ± 0,19* | **0,000160** | **0,000160** |
| **3** | 1,27 ± 0,05* | 1,60 ± 0,37* | 2,64 ± 0,31* | 0,376714 | **0,000160** |
| **4** | 0,62 ± 0,02* | 0,66 ± 0,06* | 0,71 ± 0,06* | 1,000000 | 1,000000 |
| **5** | 2,26 ± 0,06* | 2,45 ± 0,07* | 2,95 ± 0,28* | 0,998383 | **0,000161** |
| **6** | 2,26 ± 0,05* | 2,01 ± 0,14* | 1,25 ± 0,04* | 0,913737 | **0,000160** |
| **7** | 4,46 ± 0,20* | 4,76 ± 0,08* | 5,00 ± 0,16* | 0,648513 | **0,001094** |
| **8** | 0,97 ± 0,01* | 0,94 ± 0,21* | 0,90 ± 0,18* | 1,000000 | 1,000000 |
| **9** | 0,60 ± 0,01* | 0,65 ± 0,07* | 0,81 ± 0,08* | 1,000000 | 0,987023 |
| **10** | 1,38 ± 0,03* | 1,22 ± 0,23* | 0,90 ± 0,18* | 0,999732 | **0,005742** |
| **11** | 1,46 ± 0,04* | 1,26 ± 0,21* | 1,95 ± 0,04* | 0,994046 | **0,005242** |
| **12** | 1,18 ± 0,01* | 1,87 ± 0,15* | 3,88 ± 0,31* | **0,000161** | **0,000160** |

[a)] N=5 electrophoreograms from five independent urine aliquots. * Data were analysed by ANOVA test at the $p < 0, 05$ level and compared by Tukey HSD test. P- levels of proteins, where the changes of intensity quantity were statistically significant are marked in bold.

Therefore, we focused on quantitative changes and found strong elevation and drop of the amount of the proteins in these molecular weight groups. The amount of proteins with the lowest molecular weight about 10 kDa in samples without addition of the inhibitors increased more than twice. On the other hand, the proteins in the area of about 30–50 kDa increased and decreased with lesser contrast in both experiments (with or without inhibitors). Repeat Measures ANOVA test at the $p < 0, 05$ level followed by the Tukey test analysis was not statistically significant for differences between quantities of proteins (1-12) analyse immediately after collection and proteins incubated with proteases inhibitor except protein 2 and 12. (Table III)

We found similar spectra of proteins in samples analysed immediately after the collection and analysed after incubation with protease inhibitors. This indicates that the effect

of proteases can be bypassed either by immediate analysis or by proteolysis inhibition. On the other hand, proteases in the urine may induce marked changes in its protein spectra, namely increase of small molecules (proteins of about 10 kDa) and decrease of molecules with 50–100 kDa (Table III, Fig. 4).

### 8.2 Study of urine proteins in patients with nephrotic proteinuria

Electrophoreograms of urine samples from ten patients from each specific disease (IgA nephropathy, Lupus nephritis, Wegener´s granulomatosis, Membranous nephropathy) with proteinuria 5g / 24h were analysed using software 2D expression 2005. Abundant proteins such as albumin, transferrin, alpha-1-antitrypsin precursor, and alpha-1-microglobulin and transthyretin precursor were identified by MALDI-TOF MS. (Fig. 5)

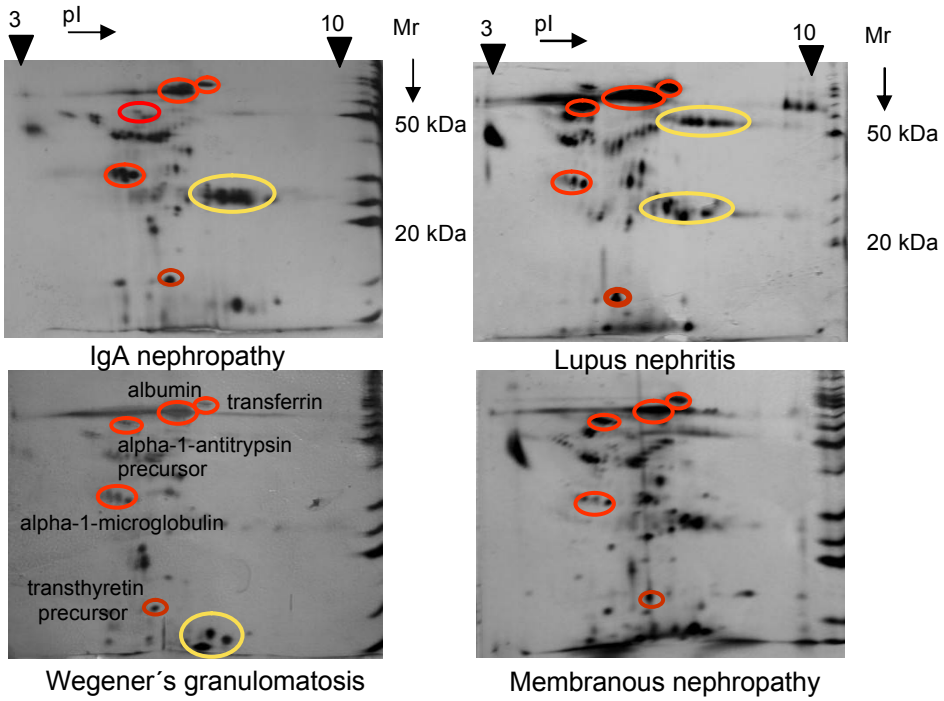


Figure 5 Comparison of protein maps. Ten urine samples from patient with specific disease (IgA nephropathy, Lupus nephritis, Wegener´s granulomatosis, Membranous nephropathy) were analysed immediately after collection using 7 cm polyacrylamide strips pH 3-10L by the IEF method, where 20 µg of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver. The proteins identified by MALDI-TOF MS are marked in red and the groups of proteins, which are characteristic for specific disease, are marked in yellow.

The urinary maps comparison brought out that there are significant changes in proteins´group, which are typical for specific nephropathy. (Fig. 5) Nevertheless, we still have not found out a specific protein, which could be used as a biomarker.

### 8.3 Study of urine protomes in patients with Anderson-Fabry disease

Twenty urine samples collected from AFD patients were analyzed in this study. Two examples of electrophoreograms are shown in Figure 6. Urinary spectra of all the AFD samples included identical proteins, most of them with molecular weight around 20–40 kDa. There was no significant difference in the distribution of proteins on electrophoreograms in AFD patients undergoing ERT, compared to those without therapy or healthy controls (Fig.6). No *a*-gal A activity was detected in the urine samples examined. *a*-gal A activity was measured as published.(Dean et al., 1977) Quantitative analysis revealed significantly increased amount of some proteins with molecular weights about 30 kDa in AFD patients. This finding was then confirmed by software Phoretix 2D expression 2005.

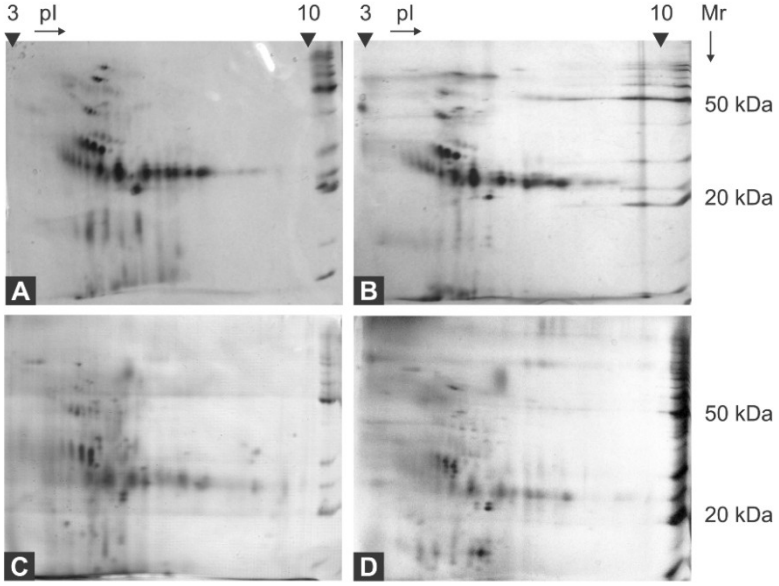


Figure 6. Comparison of 2D electrophoreograms of an urine samples collected from patients with Fabry disease (A, B) and healthy controls (C, D). The urine samples were analyzed immediately after their collection and short-time centrifugation, using 7 cm polyacrylamide strips, pH 3–10 L, and SDS-PAGE. BenchMark™ Protein Ladder (Invitrogen, Glasgow, UK) was used as a molecular mass marker.

We picked out three proteins, the quantities of which were significantly higher in the AFD subjects, compared to the healthy controls; in the AFD samples, the levels of prostaglandin H2 D-isomerase and complement-c1q tumor necrosis factor-related protein were three times higher and the Ig kappa chain V–III levels were more than five times higher, compared to the controls (Table IV.).

Table IV. Intensity of proteins´ quantity in patients with AFD and healthy subjects

| Analyzed data (intensity of quantity) using Phoretix 2D expression v2005 | | | | |
|---|---|---|---|---|
| protein | healthy (mean ± SD)[a] | AFD- after therapy ERT (mean ± SD)[b] | AFD-no therapy ERT (mean ± SD)[c] | p-level* |
| Prostaglandin H2 D-isomerase | 3,11 ± 1,12* | 12,41 ± 1,45* | 12, 45 ± 1,44* | 0,000133 [1)] |
| | | | | 1,000000 [2)] |
| Complement-c1q tumor necrosis factor-related protein | 3,93 ± 1,31* | 12,56 ± 1,35* | 11, 98 ± 1,61* | 0,000133 [1)] |
| | | | | 0,999794 [2)] |
| Ig kappa chain V–III | 4,22 ± 0,89* | 23,04 ± 1,96* | 22, 88 ± 1,99* | 0,000133 [1)] |
| | | | | 1,000000 [2)] |

[a)] N=10 [b)] N=13 [c)] N=7 electrophoreograms obtain from ten and twenty (patients AFD without and after therapy ERT) independent urine aliquots. * Data were analysed by ANOVA test at the $p < 0, 05$ level and compared by Tukey HSD test [1)] healthy vs. Diseased AFD after therapy and [2)] diseased after ERT vs. Diseased without therapy. Change of protein quantity in AFD patients was significantly higher than in healthy subjects.

Repeat Measures ANOVA test at the $p < 0, 05$ level followed by the Tukey test analysis was statistically significant for differences between quantities of proteins in AFD patients and healthy subjects. A detailed Tukey test analysis was also statistically significant for differences between quantities of proteins in AFD patients after therapy and healthy subjects. On the other hand, comparison of p-levels was not statistically significant for differences between quantities of proteins in AFD patients after therapy and AFD patients with no therapy. (Table IV.)

### 8.3.1 Characterization of proteins

Some AFD proteins also included differentially expressed spots. These were then excised and subjected to in-gel tryptic digestion and identification by MALDI-TOF MS. In cases when the sequence coverage was too low, identity of the protein candidate was confirmed by MS/MS peptide sequencing. The abundant proteins in gels were characterized as alpha-1-antitrypsin, alpha-1-microglobulin, prostaglandin H2 D-isomerase, complement-c1q tumor necrosis factor-related protein, Ig kappa chain V–III (Figure 7). Positions of all proteins identified on 2D gels were compared with their theoretical isoelectric points and molecular sizes.

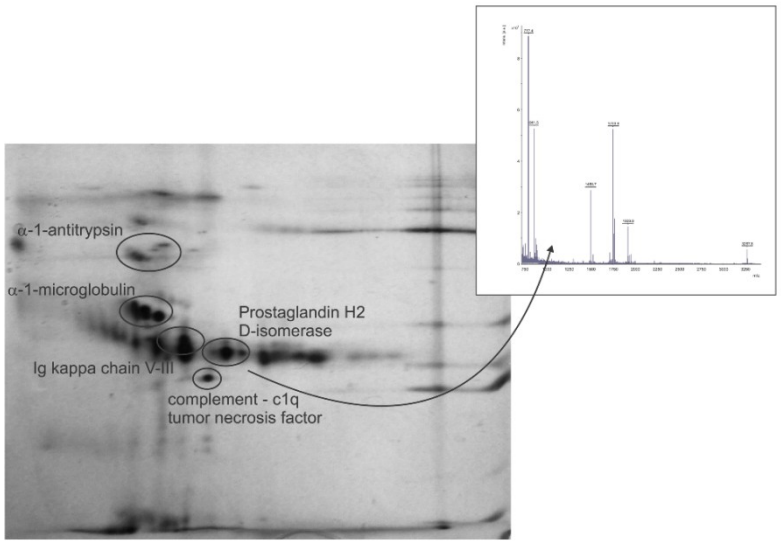


Figure 7. A representative 2D gel image of the Fabry disease proteome, depicting all identified proteins. All urine samples were analyzed immediately after their collection using 7 cm polyacrylamide strips, pH 3–10 L, and SDS-PAGE.

Most of proteins were in the expected range, except prostaglandin H2 D-isomerase. This exception may be caused by glycosylation at Asn51 and Asn78 sites.

The urinary protein in healthy people does not exceed 150 mg/day and its increase above 250 mg/day indicates kidney damage. Proteinuria higher than 3,5 g/ 24h is typical for the nephrotic syndrome, usually indicating severe primary or secondary glomerular damage and also plays roles in progression of renal diseases. The findings of specific biomarker in the urine in nephrotic patients, which would be characteristic for the specific renal disease, would be a big contribution especially for patients, who would not have to undergo unpleasant and invasive treatment. Protein analysis using 2D electrophoresis method, especially when it is followed by MALDI-TOF MS, brings a clear picture of proteins in the sample. Urinary samples from 60 patients with nephrotic proteinuria and other diagnoses were analysed. Their proteinuria varied between 0.23 and 19 g per day. Urinary samples from 20 patients with AFD with proteinuria 0,1 – 1 g za 24h were collected. We optimized 2D method providing a lucid spectrum of urine proteins in nephrotic patients with detectable proteins´changes. The study of protease effect on protein´s spectra has confirmed important protein´s changes observable at two areas of molecular weight, i. e. range 50–100 kDa and 10–20 kDa. Abundant proteins such as albumin, transferrin, alpha-1-antitrypsin precursor, alpha-1-microglobulin and transthyretin precursor were identified in the urine of all groups of patients with nephrotic proteinuria by MALDI-TOF MS.

Quantity of several proteins was substantially different in the AFD group and the control group: Ig kappa chain V–III, complement-c1q tumor necrosis, factor-related protein, and prostaglandin H2 D-isomerase. Also glycosylation at Asn51 and Asn78 was detected in prostaglandin H2 D-isomerase.

## 9. Discussion

Two-dimensional electrophoresis followed by MALDI-TOF MS is a powerful tool for studying protein changes and for their identification. The main aim of most clinical studies using this technique is to identify possible biomarkers typical for specific diseases. In our study,(Vojtova et al., 2007) lucid spectra of proteins were investigated in patients with nephrotic proteinuria and other diagnoses, such as systemic lupus erythematosus with renal involvement (lupus nephritis) and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. The comparison of protein spectra among certain renal diseases, but also patients' protein maps before and after treatment, can lead to detection of biomarkers typical for a specific disease. As mentioned in the Introduction, insufficient kidney function may lead to a higher amount of proteases in an urine. These proteases may affect the true content and composition of proteins in the urine during analysis.

### 9.1 Optimization of 2D electrophoresis technique

The main aim of our study was the urine sample preparation and 2D technique optimization in order to reduce protein loss or change before analysis and to verify the effect of the proteases on patients' protein maps. In most cases, the samples were treated immediately after collection to reduce protein loss due to other factors. Other samples were frozen at –80˚C for later analysis. The sample stability during storage was confirmed by urine sample reanalysis 30 days after the first analysis. Protein spectrum analysis confirmed that freezing and subsequent storage did not affect the presence of proteins in urine samples. Earlier study of protein detection in urine using 2D electrophoresis called attention to the presence of interfering substances, which can negatively affect analysis using isoelectric focusing (Oh et al., 2004).We applied limited amounts of protein to the polyacrylamide strip and analysis was performed by silver detection to avoid these problems without using sample dialysis and subsequent lyophilisation (Oh et al., 2004). Albumin is the main protein in the serum, and therefore also in the urine. Its molecular weight is about 67 kDa. Besides this form, the patient's urine also contains its various fragments. As shown in Fig. 1, separation of albumin caused higher lucidity of the urinary proteomes and higher protein resolution in the area with molecular weight > 30 kDa. This fact was confirmed by 1D SDS-PAGE electrophoresis, where only albumin was contained in the supernatant fraction after centrifugation (Fig. 2). The ammonium sulphate protein precipitation is suitable for analysis

of samples with lower protein content. In this case, the optimal concentration of this salt is 75% (Thonboonkerd et al., 2006). It is possible to separate the albumin from a sample using Affi-Gel Blue agarose kit from the BioRad Company (Oh et al., 2004), where the lucidity of the protein spectra was observed to be similar with our results. Another study (Ahmed et al., 2003) documented similar changes when albumin was isolated from serum samples. These methods do not eliminate those albumin fragments that might have a toxic effect on tubular cells. It was established (Jain et al., 2005) that albumin is not a specific indicator for diagnosis of renal damage. Albumin is common in urine in both healthy and diseased individuals (its amount in healthy subjects is very low). The removal of albumin from the samples does not hinder detection of potential markers for a given disease. The occurrence of proteases and their protein degradation effects were confirmed by a study at physiological temperature 37˚C for 48 h. When a cocktail of protease inhibitors was added to the samples, there were only slight changes of protein spectra compared with samples analysed immediately after collection. On the other hand, a decrease in the quantity of a protein with molecular weight about 55 kDa (protein 2) was observed in all samples studied without addition of the inhibitors, which might be explained by the effect of proteases. On the contrary, the quantity of the same protein in samples with added inhibitors was increased. This could be explained by the thermal effect on proteins with higher molecular weights (e.g. albumin) due to the incubation at 37˚C for 48 h. The temperature effect is not as noticeable as protein cleavage by the enzyme. Therefore, there is no such strong decomposition of proteins into smaller fragments. Their increased quantity is detectable only in the protein marked 2 compared to samples without inhibitors. This fact is especially seen in Fig. 4D. The incubation of samples without addition of the inhibitors caused more extensive protein changes in the area with molecular weight about 50 kDa. Increased proteolysis was visible in proteins with molecular weight lower than 20 kDa, especially in patient B, whose sample contained an unusually large quantity of proteins in the area with molecular weight about 30 kDa. Consequently, we can assume that degradation products occurred mainly in the area of about 10 kDa. This study implies that proteases have probably some effect on protein degradation in tubular processes and may be necessary for protein reabsorption by tubular cells. It is assumed that proteolytic activity may also occur at room temperature; however, it probably proceeds more slowly. It is therefore advisable to analyse the samples immediately or freeze them at –80˚C, because the sample stability established in this way is at least one month. Finally, we should also mention the possibility of increased protease concentration in urine in patients with acute pancreatitis or cancer diseases, as confirmed by the studies of (Sáez et al., 2005)

or (Botchkina et al., 2005), where concentrations of these enzymes were identified as diagnostic markers of the disease.

### 9.2 Composition of proteomes in AFD patients and healthy subjects

The next step of our study was to analyse the composition or structure of proteins in urine samples in AFD patients, compared to that in normal subjects. No significant differences in the number of spots were detected between the healthy controls and the AFD patients; therefore, we may conclude that the disease does not lead to any formation of new proteins or degradation products. This conclusion can also be confirmed by comparison of the spectra obtained from AFD patients with the protein spectra obtained from nephrotic patients, in whom the protein quantity on the gel is substantially higher than in healthy volunteers. Although qualitative changes were not detected, quantitative differences in protein contents were noted. We hypothesize that the increased quantity of proteins in urine is caused by the disease, because AFD is usually associated with impairment of proximal tubular function. This proximal tubular function impairment results in a failure to reabsorb a variety of filtered substances, particularly low-molecular-weight proteins. Most of the identified proteins were of plasma origin and, under normal conditions, would be reabsorbed in the proximal tubule via receptor mediated endocytosis. On the other hand, analysis of quantitative changes revealed substantially increased Ig kappa chain V–III, complement-c1q tumor necrosis factor-related protein, and prostaglandin H2 D-isomerase. The first two protein levels were more than three times higher and the prostaglandin H2 D-isomerase level was five times higher than the respective values in the control subjects. The latter protein plays an important role in the regulation of phospholipid metabolism (Tesar et al., 2003) and its excretion is significantly increased in patients with chronic renal failure. (Ogawa et al, 2005; Oda et al., 2002, Tsuchida et al., 2004) The higher quantity of these three proteins could result from their increased synthesis or from increased glomerular basement membrane permeability. (Tesar et al., 2008; Haraldsson et al., 2008) Impairment of secretion–reabsorption tubular processes because of lysosomal vacuoles affecting the renal tubular system appears to be the most likely cause, as the composition of the proteins, identified in the AFD subjects, appears to be very similar to a proteome found in patients with tubular proteinuria.(Tesar et al., 2003; Vilasi et al., 2007) Other changes in protein excretion were reported in a histochemical study (Utsumi et al., 1999) showing that urinary excretion of integrin *b*3 originating from the vitronectin receptor was significantly increased in the AFD group, compared to a healthy control group.

Finally, the abundant proteins identified on 2D gels were subjected to MALDI-TOF MS analysis. All seven studied proteins (alpha-1-antitrypsin, alpha-1-microglobulin, prostaglandin H2 D-isomerase, complementc1q tumor necrosis factor-related protein, Ig kappa chain V–III) were also found to be present in healthy individuals. Except for H2 D-isomerase, positions of all the other proteins on 2D gels corresponded to their theoretical isoelectric points and molecular sizes. We assumed that modifications such as glycosylation of H2 D-isomerase at Asn51 and Asn78 sites might have occurred, which could result in a change in molecular size of the enzyme and its isoelectric point. The glycosylation changes at the protein sites are of potential relevance and might serve as potential biomarkers. However, this presumption should be confirmed by future studies analyzing the relationship between the above findings and the disease severity and progression. Most of the articles published last year were focused on clinical studies of various types of therapy of the Anderson - Fabry disease patients, (Moran et al., 2003) studies in patients prior to their ERT initiation, (Ortiz et al., 2008) or in patients treated with *a*-gal A (a glycoprotein of molecular size 101 kDa with a homodimeric structure) infusions, aimed at reducing the Gb3 storage in tissues. (Schiffmann et al., 2001) addition, the molecular size of H2 D-isomerase was shown to be modified. These findings should be confirmed by future studies analyzing the relevance of the observed changes for prediction of the disease severity and outcomes.

## 10. Conclusion

1. We optimized the 2D electrophoresis technique for samples of patients with the nephrotic syndrome and other primary glomerulopathies. We found out, that samples are stable at least 6 months, when the sample is stored at -80˚C.

   We recommend applying the method of albumin separation from sample by ammonium sulphate, when albumin concentration in a sample is higher than 3g/ 24h. The separation of albumin from sample resulted in higher lucidity of the urinary proteomes and higher protein resolution in an area with molecular weight > 30 kDa.

   The study of protease effect on protein spectra (urine samples from five different patients with primary glomerular disease) using software Phoretix 2D expression 2005 has confirmed important protein changes observable at two areas of molecular weight, i. e. range 50–100 kDa and 10–20 kDa. We assume that proteases probably play an important role in protein degradation in tubular processes.

2. We focused on the groups of patients with renal disease with different types of heavy proteinuria.We obtained electrophoreograms with proteins´spectra from 60 patients with nephrotic symdrome or other nephropaties. Abundant proteins such as albumin, transferrin, alpha-1-antitrypsin precursor, alpha-1-microglobulin and transthyretin precursor were identified by MALDI-TOF MS. The urinary maps comparison brought out that there are significant changes in proteins´group, which are typical for specific nephropathy.

   Nevertheless, we still have not found out a specific protein, which could be used as a biomarker. The study of urinary protein composition might contribute to the search of relatively specific markers of individual renal diseases and to better understanding of their activity and severity than the conventional determination of proteinuria provides.

3. Our proteomic analysis revealed no significant qualitative differences between the urine samples collected from the treated and untreated AFD subjects and the samples collected from the control subjects. However, quantity of several proteins was substantially different in the AFD group and the control group: Ig kappa chain V–III, complement-c1q tumor necrosis factor-related protein, and prostaglandin H2 D-isomerase. In addition, the molecular size of H2 D-isomerase was shown to be modified. These findings should be confirmed by our future studies analyzing the relevance of observed changes for prediction of the disease severity and outcomes.

At present we are going to continue with our research of the study of proteins using other techniques such as liquid chromatography coupled with MALDI-TOF MS analysis. Especially we would like to focus on glycosylation of prostaglandin H2 D-isomerase in AFD patients and verify this enzyme modification as a potential biomarker of the disease.

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## 12. Publication of the author

### 12.1 Publications with IF

a) publication

**Vojtová L**., Zima T., Tesař V., Kazderová M.: Study of Urinary Proteomes in Patients with Nephrotic Syndrome. Folia Biologica, 2007, 53: 58-65

IF 0,92

**Vojtová L.**, Zima T., Tesař V., Michalová J., Přikryl P., Dostálová G. and Linhart A.: Study of urinary proteomes in Anderson-Fabry disease, Renal Failure, 2010, 32, (10): 1202–1209

IF 0,84

b) oral presentation

**Vojtová L**., Zima T., Tesař V., Michalová J., Přikryl P., Dostalová G., Linhart A.: Study of urinary proteomes in anderson-fabry disease. 34th FEBS Congress, Praha, 4. - 9. 7. 2009. FEBS Journal, 2009, 276, Suppl. 1, OP 8,5-1, s. 84

IF 3,129

c) poster

**Vojtová L.,** Zima T., Tesař V., Kazderová M.: Study of urinary proteomes in patients with nephrotic syndrome by 2D technique, Euromedlab Congress 2007, Amsterdam, 3. - 7. 6. 2007. Clin. Chem. Lab. Med., 2007, 45, Special suppl., s S444

IF 1,89

### 12.2 Publications without IF

a) oral presentation

**Vojtová L**., Zima T., Tesař V., Kazderová M.: Studium změn proteinů u onemocnění ledvin s nefrotickým syndromem, 31. Kongres České nefrologické společnosti, Hradec Králové, 22. - 24. června 2006: Aktuality v nefrologii, 2006, Suppl. 1, s. 23

**Vojtová L.**, T. Zima, V. Tesař, M. Kazderová: Changes in the urinary proteomes in patients with nephrotic syndrome. Sborník abstrakt XX. Biochemický sjezd, Piešťany, 2006, s. 149

**Vojtová L**.: Studium změn proteinů v moči 2D elektroforézou. Sborník abstrakt 6. SVK 1. LF UK, Praha, 2005, s. 27

**Vojtová L:** Studium změn proteinů u nefrotického syndromu, Sborník abstrakt 7. SVK 1. LF UK, Praha, 2006, s. 38

**Vojtová L**.: Study of urinary proteomes in patients with nephrotic syndrome by 2D technique. Sborník abstrakt 8. SVK 1. LF UK, Praha, 2007, s. 31