Protein analyses in the laboratory diagnostics of Crohn's disease

Doctoral (PhD) thesis

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I. Introduction

The prevalence of inflammatory bowel diseases (IBD) shows an increasing tendency all over the world. More than a quarter of freshly diagnosed patients are younger than 18 years old therefore, the management of IBD is challenging in children too.

Crohn's disease (CD), as an inflammatory bowel disease is characterized by a chronic course with relapse and remission periods. Due to the risk of periodical relapses, patients need a life-long gastroenterological care which includes the regular evaluation of inflammatory activity and complications as well as the revision of treatment. Following the principles of personalized therapy for mucosal remission, it is essential to have a more accurate picture of the patients' current condition. Clinical indices enable a rapid and simple assessment however, may not reflect the real extent of intestinal inflammation. Laboratory tests play a key role in giving objective information on CD activity, but the currently used markers do not meet all requirements. At present, the gold standard examination method is endoscopy with histological sampling however, these procedures are invasive and rather stressful especially for children. These factors create a need to strengthen the non-invasive laboratory approaches resulting in an active research for novel, more reliable biomarkers. Besides the extensively investigated blood and fecal markers the diagnostic benefit of urinary proteins has not been well revealed yet however, these may give valuable information on disorders with acute or chronic inflammation.

Orosomucoid (ORM, also called as α-1-acid glycoprotein) is an approximately 43 kDa acute-phase protein. Thanks to its extended carbohydrate component and negative charge, ORM is well soluble in acids and has an exceptional low isoelectric point (pI: 2.8-3.8). Besides hepatocytes, ORM also can be produced by white blood cells, epithelial and endothelial cells. As an immunocalin-type protein, ORM has a double biological function: it binds and transports lipophilic molecules (including drugs) furthermore, its anti-inflammatory and immunoregulatory role are also known. Elevation of serum ORM (se-ORM) has been noted in inflammatory conditions and tumors too however, its clinical utility is ambiguous due to its relatively long half-life (5-6 days). Resulting from its molecular weight ORM is probably filtrated by the glomeruli therefore it can be detected in urine in healthy state as well. However, the exact mechanism of ORM excretion is unclear. Elevated urinary ORM (u-ORM) levels were found in inflammatory disorders (eg. sepsis, diabetes mellitus, SLE, RA) suggesting that u-ORM can be a promising marker for clinical utility however, its possible role regarding CD has not yet been explored.

Cystatin-C (CYSC) found in plasma is a non-glycosylated inhibitor of cysteine proteases with a molecular mass of 13 kDa. It is produced by most nucleated cells at a constant rate and is secreted into the blood then eliminated by the kidneys at a half-life of approximately 2 hours. Serum or plasma CYSC is considered to be a reliable and more accurate indicator of GFR than serum creatinine. CYSC is freely filtrated by the glomeruli then it is reabsorbed and almost completely catabolized by the proximal tubular cells. Therefore, CYSC is a normal component of urinary proteins at very low concentrations. In case of tubular injury the reabsorption and degradation of CYSC might be reduced, leading to increased urinary CYSC (u-CYSC) excretion. Thus, u-CYSC may contribute to the early recognition of either acute renal failure (AKI) or diabetic nephropathy (DN). In addition to the direct clinical significance, the measurement of u-CYSC may also be useful in the study of certain urinary markers, as possible tubular dysfunction may have an effect on their values. Despite the promising potential applicability at present no commercial automated u-CYSC kit is available in the clinical laboratory.

For a long time, perchloric acid (PCA) precipitation has been a well-known method for the separation of heavily glycosylated proteins and other acid-soluble small molecules from serum. This method enables to study acid-soluble molecules as potential biomarkers by further sensitive methods (eg. electrophoretic techniques, mass spectrometry), excluding the interfering effect of major serum proteins. So far, acid-soluble serum proteins have been investigated mainly in malignant diseases where increased concentrations of mucoproteins and changes in their glycosylation structure have been observed. As several acute phase proteins can occur in this group, the analysis of the acid-soluble fraction may provide a number of new diagnostic possibilities in inflammatory diseases (including CD), as well as a starting point for the identification of new biomarkers.

II. Aims

Our central aim was the investigation of novel protein markers which may reflect the activity of CD. On the one hand, we focused on determining the diagnostic value of u-ORM. The examination of specific urinary proteins raised the need for the reliable monitoring of renal tubular function because this factor may have an impact on the urinary concentration of reabsorbed proteins. We planned to check renal tubular function of CD patients by measuring u-CYSC with the objective of setting up a new automated method. Our additional goal was to investigate further clinical applications of the new u-CYSC test. Finally, we analyzed acid-soluble serum proteins by searching for further activity markers of CD.

In details, our aims were the followings:

II.1.

- Development and analytical validation of an automated test for u-CYSC measurements
- Determination of a preliminary reference range for our u-CYSC assay
- Investigation of u-CYSC levels in patient groups with the possibility of acute or chronic renal tubular damage:
    - sepsis related AKI
    - chronic hypertension
    - type 2 diabetes mellitus (DM)

II.2.

- To check glomerular and tubular renal function of CD patients by serum and u-CYSC measurements
- To explore whether u-ORM levels indicate the activity of CD in adult and pediatric patients
- To determine the diagnostic performance of u-ORM in discriminating between active and inactive state
- To search for correlations between u-ORM and conventional inflammatory parameters and clinical indices

II.3.

- To standardize the separation of acid-soluble protein fraction from serum samples of healthy individuals and CD patients by PCA precipitation
    - measurement of the acid-soluble protein concentration
    - optimization of the PCA extraction method for electrophoretic analyses
- To analyse the acid-soluble serum proteins by one-dimensional sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE); to compare and assess patterns of healthy individuals and CD patients with active or inactive phase
- To examine the profiles of acid-soluble serum proteins obtained by microchip gel electrophoresis (MGE)
    - to determine the relative proportion of the separated fractions
    - to explore if a characteristic pattern indicating the active phase of CD can be identified

III. Materials and methods

III.1. Sample collection

Our study protocol was approved by the Regional Research Ethical Committee of the University of Pécs (4327/KK15/2011 ill. 5133/KK15/2013) and was performed according to the ethical guidelines of the 2008 Helsinki Declaration. Every enrolled individual was fully informed and written consent was obtained from all of them. Single blood and spot urine samples were simultaneously obtained from each patient during the morning hours. After centrifugation of urine and clotted blood samples (10 min, 1500 g), serum specimens and supernatants of urine were stored in aliquots at −70 °C until analyses.

III.2. Validation and clinical utility of a novel automated u-CYSC assay

III.2.1. Assay settings and validation process

For u-CYSC measurements, a polystyrene particle-enhanced turbidimetric assay (Cystatin C FS kit, Cat. No. 171589910930, DiaSys Diagnostic Systems GmbH, Holzheim, Germany) was adapted to an open developmental channel of Cobas 8000/c502 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The determinations of u-CYSC were

performed by using polyclonal anti-human goat CYSC immunoparticles and TRIS buffer (100 mmol/L; pH=7.5) included in the kit. Dilution series of TruCal Cystatin C calibrator set (Cat. No. 171509910059, DiaSys) were used for calibrations and proper dilutions of TruLab Cystatin C Level 1 and Level 2 (Cat. No. 598709910046, 598809910046, DiaSys) were applied as controls. For the dilution of the calibrators, controls, patient samples and for blank samples sterile 154 mmol/L NaCl solution was used.

In order to obtain appropriate analytical sensitivity, conditions of the original test were completely changed. The calibrator/control/sample volume was increased to 12 µL, the reagent volume was decreased to 50 µL and the buffer volume was changed to 170 µL. For calibration six standards and the linear regression fitting were applied. The turbidimetric reaction was detected at the wavelength of 505 nm and CYSC concentration was calculated based on the change in absorbance between 42-70 measuring points. The assay was performed at 37°C with 10 minutes of reaction time.

Intra- and inter-assay imprecisions, parameters of analytical sensitivity and linearity were assessed based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. 3 different dilutions of control solution were analyzed 10 times in parallel to determine intra-assay imprecision and 2 times per day for 20 days for inter-assay imprecision. Linearity was checked by measuring a dilution series of a urine sample in duplicates. To determine limits of analytical sensitivity 5 blank samples, 5 urine samples with low CYSC concentration and a dilution series of a urine sample were analyzed.

High dose hook effect was examined in the concentration range of CYSC between 0.0-8.0 mg/L. Recovery was examined based on Westgard guidelines by measuring aliquots of 5 different urine samples spiked with TruLab Cystatin C Level 1 control material or saline. Potential interfering effect of calcium, urea, glucose, albumin and hemoglobin was tested by analyzing aliquots of different urine samples spiked with the interferer solution or saline.

To examine analyte stability 10 aliquoted spot urine samples were studied without any additives. U-CYSC was determined from freshly obtained urine samples and after 2, 4, 6, 24 hours of storage at room temperature and at 4°C. Additionally, the effects of 3 months' storage at -20°C and freezing - thawing cycles on analyte stability were also assessed.

III.2.2. Examined groups

To determine a preliminary reference range of our u-CYSC method, 117 healthy individuals between the age of 10 and 60 years were enrolled. The healthy state was

established based on physical examination and on medical records. Exclusion criteria were any kind of acute or chronic diseases, a serum hsCRP level >5 mg/L and proteinuria. In order to investigate the potential clinical utility of u-CYSC measurements, a group of patients with sepsis-related acute kidney injury (n=33), patients with chronic hypertension (n=43) and type 2 diabetic patients (n=41) were also included. AKI in septic patients was established using the Kidney Disease Improving Global Outcomes (KDIGO) guideline supported by serum CYSC concentrations. Inclusion criteria for the chronic hypertension and diabetic patient groups were: established clinical diagnosis of their treated disease, the lack of acute inflammatory conditions and other chronic disorders such as autoimmune diseases or tumors.

Urinary total protein (u-TP) and creatinine (u-CREAT) were also measured by standard routine laboratory procedures. Besides absolute u-CYSC concentrations, we also reported u-CYSC data referred to u-CREAT (u-CYSC/u-CREAT ratio).

III.3. Investigation of u-ORM levels in Crohn's disease

III.3.1. Examined groups

Previously diagnosed adult (n=55) and pediatric (n=31) CD patients were involved and compared with healthy individuals as controls (adults, n=38; children, n=30). Participants below 18 years of age formed the pediatric groups. Exclusion criteria at CD patients were any kind of underlying kidney disease, malignancy; any identified gastrointestinal infection or the lack of consent. Exclusion criteria at controls were any kind of acute or chronic diseases, a serum hsCRP level higher than 5 mg/L and proteinuria. Patients were classified by their corresponding activity indices (Pediatric Crohn's Disease Activity Index, PCDAI or Harvey-Bradshaw Index, HBI).

III.3.2. Laboratory analyses

U-ORM levels were measured by our previously developed and validated latex particle-enhanced turbidimetric assay using anti-human ORM immunoparticles (ref. no. OA504, Dako Denmark A/S, Glostrup, Denmark) and reaction buffer (ref. no. PO1812, Dako). Detection limit of the test was 0.02 mg/L and the measuring range was 0.08–5.25 mg/L.

HsCRP, ESR, WBC, platelet count (PLT), se-ORM, u-TP, urinary albumin (u-ALB) and u-CREAT were measured by routine procedures. To check renal glomerular and tubular

function, CYSC was measured from serum and urine samples too. U-CYSC levels were determined by our method described in paragraph III.2.

As we analyzed spot urine samples, u-ORM and u-ALB levels were expressed as u-ORM/u-CREAT and u-ALB/u-CREAT ratios to reduce the effect of different hydration states. Additionally, u-ORM/u-TP and u-ALB/u-TP ratios were also determined to examine the proportional change of ORM and albumin among urinary proteins.

III.4. Examination of acid-soluble serum proteins in Crohn's disease

III.4.1. Study groups

Previously diagnosed adult (n=32) and pediatric (n=30) CD patients were enrolled and healthy participants (adults, n=14; children, n=11) as controls were also involved. Classification of CD patients according to disease activity and exclusion criteria were the same as described in paragraph III.3.1.

Serum total protein (se-TP), hsCRP, se-ORM, WBC were measured by routine procedures.

III.4.2. Extraction of PCA-soluble serum proteins

In order to separate acid-soluble proteins, serum samples were mixed with an equal volume of 1.0 M PCA for 10 min at room temperature. After centrifugation (3800g, 6 min), the supernatant was neutralized by adding 1.42 M NaOH in a ratio of 3:2. After 10 min incubation at room temperature and repeated centrifugation (3800g, 6 min), 2 M Tris-HCl buffer was added to the supernatant in a ratio of 10:3 to set the final pH between 8.5 and 8.8. Total protein concentration of the obtained specimens was determined by spectrophotometry at 220 nm (Hitachi U-2910 UV/VIS) then the original serum concentration of acid-soluble proteins was calculated by taking the dilution factor and using a calibration curve.

III.4.3. Analysis of the PCA extracts

Electrophoretic separation of PCA-soluble proteins was carried out by one-dimensional SDS-PAGE according to the method of Laemmli. The used resolving gels contained 7.5% acrylamide polymer crosslinked with methylene bisacrylamide. PCA extracts were mixed with 5x concentrated Laemmli sample buffer (containing SDS and β-mercaptoethanol) in a ratio of 4:1 then the mixture was boiled for 3 min in a 100°C water bath. For the

electrophoresis, 10-10 μL sample volumes were applied. The protein patterns were detected on the gels by Coomassie Brillant Blue (CBB) and silver staining using the method of Willoughby and Lambert. Finally, digital image documentation of the gels was done.

Further analysis of PCA-soluble proteins was performed by electrophoresis in microchips using the High Sensitivity Protein 250 LabChip kit on the Agilent 2100 Bioanalyzer system. This method involved a gel matrix consisting of 4.5% polydimethyl-acrylamide based polymer with linear structure. Samples prior to electrophoresis were mixed with a reactive fluorescent dye containing DMSO and were incubated for 10 min at room temperature. The excess dye was quenched by adding ethanolamine. The fluorescently labeled samples were diluted five times with distilled water then were mixed with denaturing solution in a ratio of 2:1. Afterwards, specimens were boiled for 5 min at 100°C. 6 μL of each sample or molecular mass marker were loaded on the microchip channels. The analysis was performed at 1000 V for 60 s at 30°C. Protein fractions were detected based on the emission of the bound fluorescent dye at 680 nm. Since dye binding is proportional to the amount of protein, the relative proportion of each separated fractions was calculated based on the area under the curve (AUC).

III.5. Statistical analyses

For statistical analysis IBM SPSS Statistics for Windows, Version 22 (IBM Corporation, NY, USA) was used. The distribution of our variables was determined by Shapiro-Wilk method. To assess the differences between groups non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were performed. For analysis of relationships between continuous variables, Spearman's rank correlation test was applied. Receiver operating characteristic (ROC) curves were plotted for analyzing the diagnostic performance and AUC ROC were determined. Data of continuous variables were expressed as medians and as interquartile ranges (IQR, 25-75 percentiles). A p value of $p<0.05$ was considered to be statistically significant.

IV. Results

IV.1. Validation and clinical utility of a novel automated u-CYSC assay

IV.1.1. Method validation, interference and stability studies

The calibrated range for u-CYSC measurements was linear between 0.0 – 1.0 mg/L, as shown on Figure 1A. Samples at above 1.0 mg/L u-CYSC concentration had to be remeasured using an appropriate dilution. Both intra- and inter- assay imprecision at different concentration levels did not exceed 5% of CV. Parameters of analytical sensitivity were found to be satisfactory (limit of detection, LOD=0.017 mg/L; limit of quantification, LOQ=0.037 mg/L). The quantification limit allowed the accurate determination of samples at a concentration down to 0.037 mg/L. The assay showed good linearity (R^2=0.991) in the range of 0.04 – 0.86 mg/L.

Examining the high dose hook effect decrease of delta absorbance was observed above 2.0 mg/L u-CYSC concentration however the security range expanded to 8 mg/L (Figure 1B). The recovery study showed the proportional error to be 2.1% which was within the acceptable error limit.

We found no interference with u-CYSC measurements in spiked urine samples up to 15 mmol/L calcium, 1350 mmol/L urea, 280 mmol/L glucose, 12 g/L albumin and 5 g/L hemoglobin, respectively. We observed that u-CYSC was stable for 24h stored at 4°C, for 6h stored at room temperature, after a 3-month storage at -20°C, even after the 3rd freezing - thawing cycle. However, significant u-CYSC loss was detected in case of 1 or 2 urine samples under each storage conditions.

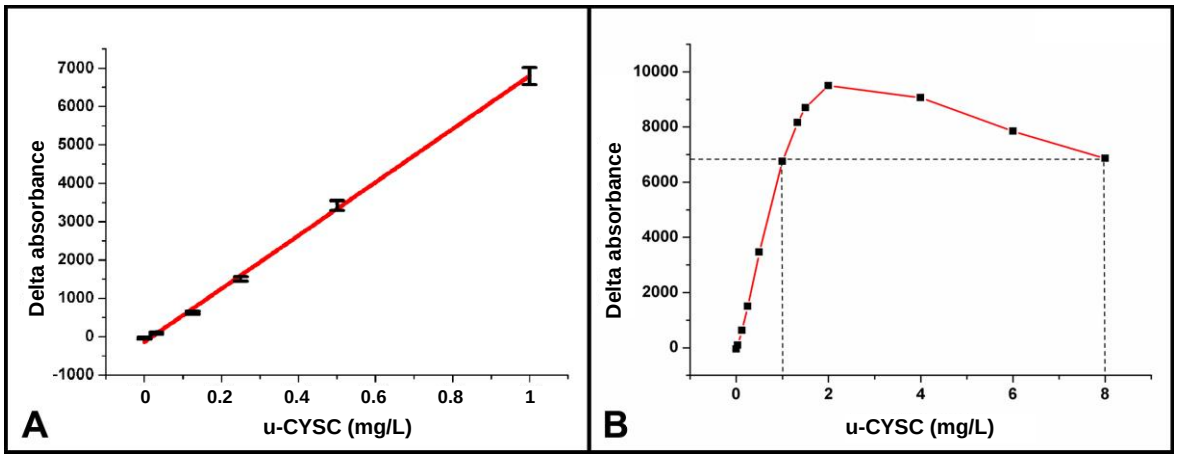

Figure 1. A: Cumulative graph of 30 separate calibrations of the assay in the range of 0.0-1.0 mg/L with 6 points of calibration (linear graph fitting). **B:** Dose response curve in the range of 0.0-8.0 mg/L and determination of high dose hook effect and security range.

IV.1.2. Determination of preliminary reference range

In the group of healthy individuals, we observed no significant differences in u-CYSC excretion and u-CYSC/u-CREAT values between genders. Healthy adolescents (age group 10-18 years) showed higher u-CYSC/u-CREAT values (8.9 mg/mol) compared to adults (6.5 mg/mol, $p<0.001$) but their u-CYSC concentrations appeared to be similar to adults (0.060 mg/L vs. 0,061 mg/L, $p>0.05$). U-CYSC concentrations of a few healthy controls were found to be below the LOQ thus only upper reference limits (<95 percentiles) were defined: <0.14 mg/L for u-CYSC independently of age, <19.7 mg/mol for u-CYSC/u-CREAT for the adolescents and <12.4 mg/mol for adults.

IV.1.3. Application of the u-CYSC test to examine different groups of patients

We found significantly elevated u-CYSC concentrations (0.91 (0.28-3.25) vs. 0.06 (0.04-0.09) mg/L, $p<0.001$) and u-CYSC/u-CREAT ratios (309.6 (123.1-1010.8) vs. 6.5 (5.3-7.8) mg/mol, $p<0.001$) in patients with sepsis-related AKI compared to adult controls. In contrast to these data, values of patients with chronic hypertension (0.05 (0.03-0.09) mg/L; 6.9 (5.4-9.1) mg/mol), and type 2 diabetes (0.06 (0.04-0.08) mg/L; 6.2 (5.0-8.2) mg/mol) did not differ from those obtained for the healthy group.

IV.2. Investigation of u-ORM in relation to the inflammatory activity of CD

From the total of 86 CD patients 38 were defined with active disease and 48 with inactive disease based on the HBI or the PCDAI. 47% of adults and 39% of children were in active phase. We found no differences between females and males in u-ORM/u-CREAT values. Renal function was normal in each group based on serum and urinary CYSC measurements.

Significant elevation of the conventional inflammatory parameters (hsCRP, ESR, se-ORM) indicated the active phase of CD. Additionally, WBC and PLT values of adult patients with active CD were also higher compared with the inactive group ($p<0.01$). Besides traditional markers, u-ORM reflected well the inflammatory activity in patient groups too. In the active CD group proportional increase of u-ORM/u-CREAT ratio was more prominent than that of se-ORM, particularly in children.

U-ORM/u-CREAT values were found 7-times higher in pediatric CD patients with active disease (0.50 (0.33-1.21) mg/mmol) compared with inactive ones (0.07 (0.05-0.17) mg/mmol, $p<0.001$). On the other hand, in adult CD patients with active disease, only a 2-fold elevation was observed (0.32 (0.16-1.54) mg/mmol) when compared with inactive ones (0.14 (0.05-0.33) mg/mmol, $p=0.01$) (Figure 2). No differences were found between children and adults regarding u-ORM/u-CREAT.

In contrast to u-ORM/u-CREAT, u-ALB/u-CREAT values did not differ significantly between active and inactive patient groups (children: 0.52 (0.41-1.09) vs. 0.70 (0.42-1.79), adults: 0.79 (0.31-2.53) vs. 0.61 (0.40-1.16) mg/mmol).

Additionally, taken all patient groups the u-ORM/u-TP ratio was also higher in CD patients with active disease (3.2 (2.0-6.4) %) compared with inactive ones (1.0 (0.6-2.8) %, $p<0.001$) or controls (1.0 (0.6-1.7) %, $p<0.001$). On the other hand, u-ALB/u-TP did not show any difference among the three groups (active CD patients, 6.7 (3.4-14.8) % vs. inactive ones, 8.3 (6.3-10.5) % vs. controls, 6.7 (4.9-12.6) %).

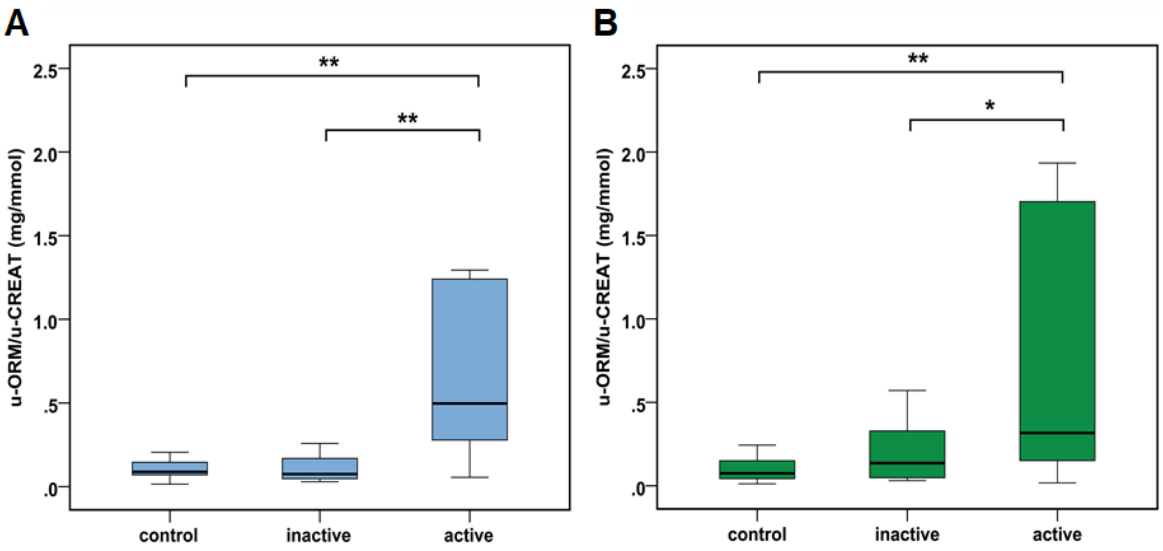

Figure 2. Separately presented u-ORM/u-CREAT data of pediatric CD patients and controls (A), and adult CD patients and controls (B). *p=0.01; **p<0.001

IV.2.3. Spearman's correlation results

U-ORM/u-CREAT showed significant correlations with inflammatory markers and activity indices. The correlation coefficients for hsCRP, se-ORM and ESR were slightly lower in adults than in children (hsCRP: 0.44 vs. 0.55; se-ORM: 0.49 vs. 0.59; ESR: 0.37 vs. 0.47). Moreover, the association with HBI (0.33; p=0.018) was found to be considerably weaker than with PCDAI (0.59; p<0.001).

IV.2.4. ROC analyses

ROC analysis showed that besides hsCRP (AUC: 0.86, p<0.001) and se-ORM (AUC: 0.88, p<0.001), u-ORM/u-CREAT also had similar significant (AUC: 0.88, p<0.001) discriminative power in children to differentiate between active and inactive CD patients. However, among adults the AUC ROC for u-ORM/u-CREAT (0.70, p=0.01) was mildly lower than that of hsCRP (0.84, p<0.001) and se-ORM (0.80, p<0.001) (Figure 3).

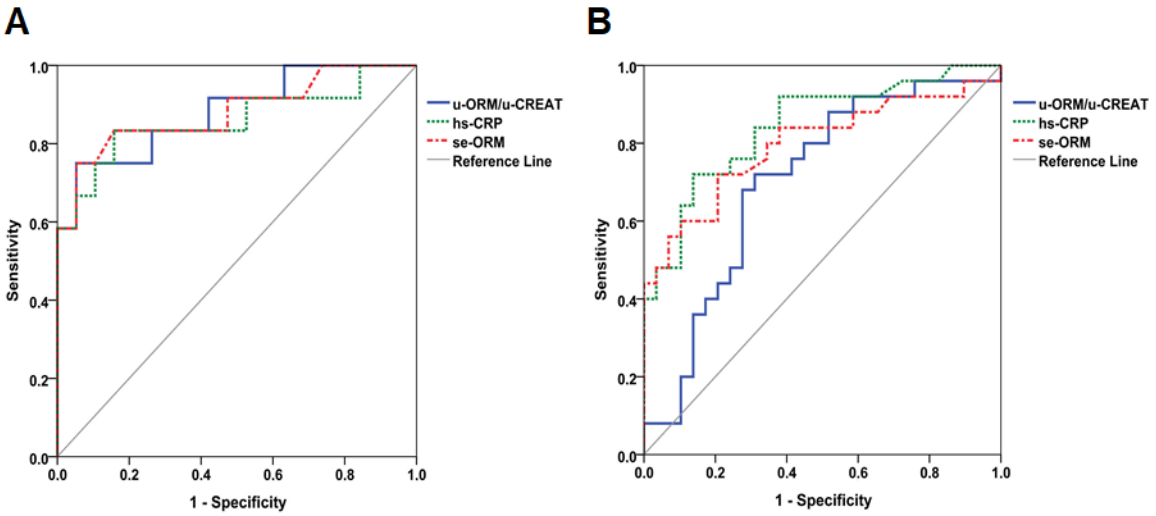

Figure 3. ROC curves for discriminating active from inactive phase in children (A), and in adults with Crohn's disease (B).

IV.3. Examination of acid-soluble serum proteins in Crohn's disease

IV.3.1. Laboratory data of study groups

The conventional inflammatory parameters (WBC, hsCRP, se-ORM) were significantly elevated in the group of 28 patients with active CD compared with the inactive group included 34 patients and healthy controls ($p<0.01$). Se-TP concentration showed a mild decrease in patients with active disease compared with inactive ones ($p<0.05$).

IV.3.2. Analysis of acid-soluble serum proteins

Significantly increased ($p<0.001$) acid-soluble protein levels were observed in patients with active CD (2.9 (2.2-3.7) g/L) compared with the inactive ones (1.7 (1.4-2.1) g/L) and the control group (1.2 (1.0-1.5) g/L).

During the qualitative evaluation of silver stained SDS-PAGE patterns of PCA-soluble serum proteins marked differences were found among the studied groups. In comparison with the pattern of healthy individuals, more fractions could be detected in the cases of active CD furthermore, fractions of the 30-67 kDa region appeared more prominently. Gel images of nonactive CD patients seemed to be very similar to the controls.

By MGE analysis of PCA-soluble serum proteins four characteristic bands were identified for the samples of all three groups at the ~11, ~65, ~85 and ~120 kDa regions. The pattern of patients with inactive CD was similar to that of healthy individuals, while the pattern of

patients in the active period was markedly different (Figure 4). The relative amount of the ~11 kDa band was about three times higher in control (12.2 (7.2-23.8) %) and inactive groups (12.1 (9.5-17.2) %) compared with active CD patients (4.4 (2.9-9.0) %; p<0.001). On the other hand, the relative abundance of fractions at ~65 and ~85 kDa were significantly higher in the active phase of CD (10.8 (6.5-18.5) %; p<0.05 and 11.7 (8.2-17.5) %; p<0.001) compared with the inactive (7.2 (5.3-11.1) % and 3.1 (2.5-5.8) %) and healthy state (5.5 (3.3-10.3) % and 1.7 (1.2-2.9) %). The proportion of the component at ~120 kDa was lower in active disease than in the other groups however this difference was not found to be significant.

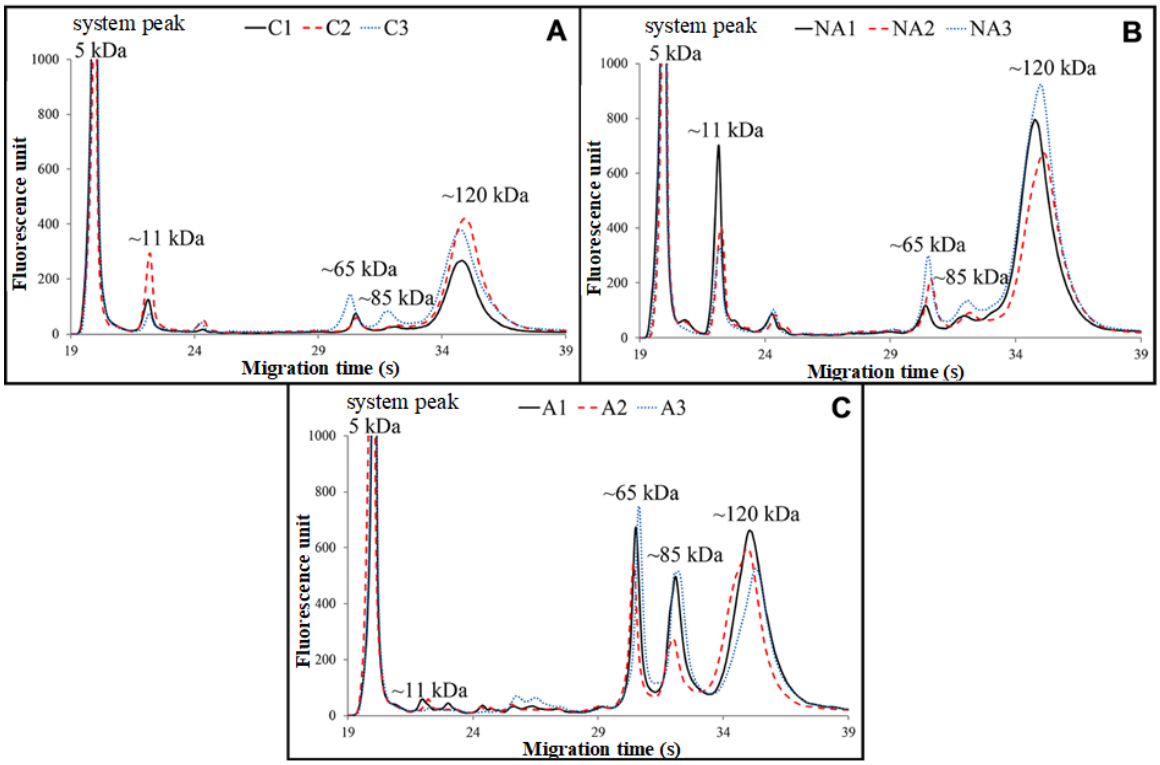

Figure 4. MGE profiles of PCA-soluble proteins (3-3 samples are presented per group). A: Control. B: Inactive CD. C: Active CD.

V. Discussion

V.1. Validation and clinical utility of a novel automated u-CYSC assay

We set up and validated a fast, precise and sensitive, fully automated turbidimetric assay for u-CYSC measurements on Roche cobas 8000/c502 analyzer which is ideal for routine work. Due to the outstanding analytical sensitivity, CYSC can be quantified not only in

urine samples of patients with renal dysfunction but also in a healthy population. In case of an u-CYSC value above the calibrated range (>1 mg/L) repeated measurement is needed after proper dilution. Urine samples of patients with severe AKI should be prediluted because u-CYSC levels exceeding the security zone (>8 mg/L) may occur in association with this state.

Our assay properties seem to be similar or even more advantageous than those of others' turbidimetric tests.

In accordance with former studies, we did not observe considerable interference between u-CYSC and endogenous substances that are present in the urine (albumin, hemoglobin, calcium, urea and glucose).

Our stability studies revealed CYSC being unstable in some urine samples regardless of storage temperature as it was previously suggested by others too. Our data support the recommendation that proteinase inhibitor preservatives should be added to freshly voided urine samples.

U-CYSC does not show circadian rhythm, therefore urine collection is not necessary. Analyzing spot urine samples, it would be reasonable to express results also as u-CYSC/u-CREAT ratio in order to exclude the effect of different hydration states. However, the validity and reliability of absolute concentrations of u-CYSC and u-CYSC/u-CREAT values are subjects of debate therefore both type of data were given simultaneously in our study.

We determined the upper reference limit only as 95^{th} percentiles because in some cases, urine samples of healthy individuals can show lower CYSC concentrations than the LOQ and the very low values have no clinical relevance. The observed u-CYSC and u-CYSC/u-CREAT values among healthy people and our reference range are in agreement with previously reported data. The difference found between u-CYSC/u-CREAT values of adolescents and adults might be due to the age dependency of urinary creatinine excretion associated with the growth of muscle mass during adolescence.

We detected significant elevation of u-CYSC and u-CYSC/u-CREAT ratio in AKI patients compared to the control group, indicating the renal tubular damage in sepsis-related AKI.

Although chronic hypertension is a risk factor for nephropathy, our results suggest that u-CYSC seems not to be affected by chronic hypertension. In the diabetic patient group u-CYSC data suggest that tubular damage has not been developed yet. According to former studies, tubular damage might precede the appearance of microalbuminuria in some cases of diabetes where increased u-CYSC may be useful for the recognition of these patients.

Our results support that u-CYSC is capable – especially in case of AKI – for timely diagnosis of renal tubular damage, creating a need for daily clinical utilization. Our method can easily be adapted to other commercial fully automated analyzers, supporting u-CYSC to be added to the diagnostic arsenal.

V.2. U-ORM, as a possible activity marker of Crohn's disease

Monitoring disease activity is substantial to specify the most appropriate treatment for patients with CD. Due to the partial subjectivity of clinical indices and the invasivity of endoscopic examination, the role of laboratory diagnostics is getting to be more appreciated resulting in a vigorous research for novel, non-invasive biomarkers. The benefit of u-ORM as an inflammatory marker has not been investigated yet in association with CD.

We found that u-ORM/u-CREAT had an ability to discriminate between active and inactive CD and its performance is similar to hs-CRP and se-ORM in children. However, its power seems to be mildly less than that of the serum markers in adult CD patients. Although the groups of patients were rather small, probably this difference may also be related to the score-based categorizing since PCDAI includes more objective factors than HBI. The change of u-ORM/u-TP suggests a mild alteration in the pattern of urinary proteins in relation to CD activity. We previously observed this phenomenon in sepsis related severe acute inflammatory conditions, where a more pronounced change was detected than in the active phase of CD.

Elevated u-ORM excretion is supposed to be in association with the activation of the immune system. The several-fold elevation found in active CD might be in accordance with the former observations related to other acute (sepsis, cardiac surgery) and chronic (SLE, type 2 DM, RA) inflammatory disorders which reported similar or higher u-ORM values. This assumption is confirmed by the significant correlations of u-ORM with conventional inflammatory parameters.

The exact mechanism and factors influencing u-ORM excretion have not been revealed completely. The moderate correlation with se-ORM might mean that besides se-ORM concentration, other factors may have an impact on u-ORM levels too. Previous results of our research group suggested that kidney function may have a major influence on u-ORM excretion. However, based on se-CYSC and u-CYSC analyses, decreased renal function generally does not occur in patients with CD. Renal secretion of ORM was previously

hypothesized which might also contribute to its elevated levels in urine. This assumption is supported by a study in which expression of the ORM gene was induced in the kidney of mice by intraperitoneal LPS injection.

We found that the proportional increase of u-ORM – especially in children – was higher than that of se-ORM, therefore u-ORM seems to be more sensitive for assessment of inflammatory activity in CD than se-ORM. In contrast to former observations, in our study u-ALB/u-CREAT did not reflect the activity of CD suggesting that u-ORM and u-ALB are excreted into the urine by different mechanisms.

Our findings support that urinary quantification of ORM can provide valuable, objective information contributing to a more precise CD management. Thanks to the non-invasive property of sampling and the rapid, automated measurement, u-ORM is ideal for routine diagnostic use.

V.3. Analysis of acid-soluble serum proteins in Crohn's disease

To our best knowledge, our study is the first which investigates the diagnostic value of PCA-soluble serum proteins in relation to the activity of CD.

In patients with CD – especially in case of active phase – the elevated PCA-soluble protein concentration might be related to systemic inflammation, since several heavily glycosylated acute phase proteins (ORM, haptoglobin, alpha-1-antitrypsin) can remain soluble in PCA.

Besides the elevation of positive acute phase proteins, the change in quality may also play a role in the increasing serum concentration of the acid-soluble fraction. It is known that the glycosylation of acute phase proteins can change in various diseases making them more resistant to PCA. Thereby, a higher proportion of these proteins can be included in the acidic extract. Moreover, specific changes in glycosylation may be used as novel potential markers of inflammation and cancer.

Based on former observations of our research group, under catabolic conditions (extended surgery, chemotherapy) elevated acid-soluble protein levels can occur in the blood which may result from the entering of molecules into the circulation originated from cellular metabolic processes and tissue necrosis. Consequently, in CD, a catabolic condition associated with chronic inflammation may contribute to higher levels of acid-soluble proteins.

In septic patients, we found several times higher levels of acid-soluble proteins however, se-TP was markedly decreased compared with healthy individuals. This suggests that the change in acid-soluble protein concentration is not influenced by the change in se-TP.

The differences seen in the SDS-PAGE patterns of our study groups indicate different protein composition of the PCA-soluble fractions. Although, silver accumulation is not proportional to the protein amount, stronger staining of 30-67 kDa molecular weight fractions of patients with active CD reflects higher protein content of acidic extracts. Among others, ORM is also involved in this range (42 kDa), which is well known as a major member of acid-soluble proteins.

By MGE analysis we detected marked alterations in the PCA-soluble protein profiles along with the inflammatory activity of CD. Although, the exact protein composition of the dominant fractions is not known, probably highly glycosylated acute phase proteins (e.g. ORM, α-1 antitrypsin, complement factors) can be responsible for the altered relative proportions. In addition to quantitative variability, MGE profiles may also be affected by changes in the carbohydrate structures of glycoproteins due to inflammation.

In contrast to the time-consuming and qualitative features of SDS-PAGE, MGE is a miniaturized, rapid method applying linear polymer which allows a quantitative evaluation based on relative proportions and a high degree of reproducibility. Identification of PCA-soluble proteins responsible for changes in MGE profile using proteomic methods may reveal new potential biomarkers which can be further investigated for clinical applicability.

VI. Summary, novel findings

We adapted and validated an automated turbidimetric assay to the Cobas 8000/c502 analyzer for u-CYSC measurements which is appropriate for routine clinical use due to its analytical properties (sensitivity, imprecision, short assay time).

We determined a preliminary reference range of our u-CYSC method for adults and adolescents by investigating healthy individuals.

In patients with sepsis related AKI, we found markedly increased u-CYSC excretion which may be an indicator of the tubular component of AKI.

We measured elevated u-ORM/u-CREAT values both in adult and pediatric patients with active CD compared with the inactive state. U-ORM proved to be – particularly in children – a more sensitive inflammatory marker than se-ORM.

Our important finding is that u-ORM/u-CREAT ratio can discriminate between active and inactive phase of pediatric CD with similar performance as conventional inflammatory parameters (hsCRP, se-ORM).

Correlations of u-ORM/u-CREAT with traditional inflammatory parameters and clinical indices of CD suggest an association between u-ORM elevation and inflammatory activity.

Measuring u-ORM may contribute to a more accurate and objective assessment of inflammatory activity in patients with CD, especially in children.

We observed increased acid-soluble protein concentration in sera of patients with active CD.

Analyzing the PCA-soluble serum fraction by SDS-PAGE, we explored a qualitative difference between patterns of patients in the active or inactive phase of CD.

We were the first to analyze acid-soluble serum proteins in CD by MGE. We identified a characteristic profile in patients with active CD, in which the relative proportion of the main fractions markedly differed from that of inactive patients and healthy individuals.

The amount and composition of acid-soluble serum proteins also reflect the inflammatory activity of CD, which may serve as a starting point for further biomarker research.

VIII. List of publications

VIII.1. Articles related to this thesis

Szirmay B, Kustán P, Horváth-Szalai Z, Ludány A, Lakatos Á, Mühl D, Wittmann I, Miseta A, Kovács GL, Kőszegi T. Novel automated immune turbidimetric assay for routine urinary cystatin-C determinations. Bioanalysis. 2018 Mar 1;10(6):377-384. doi: 10.4155/bio-2017-0228. **IF: 2.321**

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Cumulative impact factor of publications related to this thesis: 8.883

Cumulative impact factor of publications not related to this thesis: 14.44

Cumulative impact factor of all publications: 23.323

VIII.3. Book chapters

Kustán P, **Szirmay B**, Mühl D, Ludány A. Human orosomucoid in the clinical laboratory. In: Laboratory techniques with applicability in medical practice. Editors: Tamás Kőszegi, Antonella Chesca. Lambert Academic Publishing, 2015. ISBN-13: 978-3-659-31724-8, ISBN-10: 3659317241.

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