Urinary orosomucoid as inflammatory biomarker in sepsis

PhD theses

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CONTENTS

I. INTRODUCTION

The leading causes of global morbidity and mortality (cardiovascular diseases, malignancies and infections) are all associated with activation of the inflammatory system. Systemic inflammation often results in severe complications. From these disorders, sepsis has to be highlighted, which has been known since the ancient times, however it still remains a challenging healthcare problem with relevant social and economic burden. Sepsis with an approximately 30% mortality rate and high costs of care is one of the most serious conditions of intensive care unit (ICU) admissions.

Sepsis is a heterogeneous, complex clinical syndrome with various etiology, severity and prognosis. Early recognition of sepsis and distinguishing it from systemic inflammatory response syndrome (SIRS) is essential to improve disease outcome. Microbiological and laboratory tests provide a considerable help in the diagnosis. Certain biomarkers might be capable of prognostication, recognition of organ dysfunction and guiding antibiotic therapy as well. In the past few decades approximately 200 sepsis markers have been studied. Most of the biomarkers belong to the mediators of inflammatory response. The latest diagnostic guidelines regarding sepsis dedicated serum procalcitonin (PCT) and high-sensitivity C-reactive protein (hsCRP) measurements if sepsis is suspected, even if these markers also possess some limitations. It is particularly advantageous, if a marker can be measured from a non-invasively obtained sample and if it is suitable for real-time monitoring of the inflammatory activation. Recently urinary proteomics also revealed the importance of several urinary proteins in systemic and in local processes as well.

Orosomucoid (ORM) or α-1-acid glycoprotein is a major positive acute phase protein. Although ORM has been described in 1950, and numerous function of ORM has already been explored, its exact biologic role is not well clarified. ORM is an extensively glycosylated 41–43 kDa glycoprotein with extraordinary structure yielding unique features and functions. As a member of the immunocalin protein family, ORM plays a role in transporting biomolecules and drugs influencing their pharmacokinetics and takes part in the regulation of inflammatory processes with anti-inflammatory and immunomodulating activities.

In vitro studies showed that ORM has several effects on all major leukocyte types: it inhibits lymphocyte proliferation, neutrophil chemotaxis, superoxide generation and platelet aggregation, as well. Animal experiments confirmed that ORM improves the outcome from different types of shock, sepsis, and it is important in maintaining perfusion of vital organs, and regulating capillary permeability.

As an acute phase protein, serum ORM (se-ORM) concentrations can increase up to two-three fold in acute and chronic inflammatory diseases, malignancies and severe infection, thus it serves as a general, non-specific inflammatory marker.

Due to its molecular weight, most probably it is filtrated through the glomeruli, and ORM can also be found in urine of healthy individuals, however the mechanism of urinary excretion of ORM is not well clarified. Only scarce data are available on urinary ORM (u-ORM).

Elevated u-ORM excretion has been described in type II diabetes, in chronic heart failure and in rheumatoid arthritis and is considered to be associated with the ongoing chronic low grade inflammation and endothelial dysfunction. Based on previous studies, monitoring of u-ORM seems to be promising in several systemic inflammatory disorders and it might serve valuable information for daily clinical practice. In spite of favorable literature data on the potential utility of u-ORM, a sensitive, reliable automated test for u-ORM measurement is unavailable yet.

II. AIMS

Goals of our investigations were to explore urinary orosomucoid levels in acute inflammatory conditions, whether u-ORM might be capable for assessing the severity of systemic inflammation.

Our aims were the followings:

- Development and validation of an automated method for u-ORM measurements
- Determination of a reference range for u-ORM by investigating healthy individuals
- To explore effects of chronic disorders on u-ORM excretion by investigating patients with several comorbidities
- Investigation of u-ORM levels among septic patients, hypothesizing that u-ORM is an early inflammatory marker. The following features of u-ORM were studied:
  - Diagnostic value
  - Time course
  - Predictive value for mortality
  - Relation to organ dysfunctions and disease severity
  - Correlation with classic laboratory parameters
- Monitoring of the kinetics of u-ORM in surgery induced systemic inflammation, supposing that u-ORM levels might indicate the magnitude of inflammatory activation as a non-invasive marker. U-ORM excretion was studied in patients undergoing cardiac surgery as follows:
  - Baseline, preoperative u-ORM levels were compared to those of healthy subjects whether ongoing disorders could affect the u-ORM excretion
  - Time course of u-ORM levels after cardiac surgery were monitored
  - Association with the magnitude of inflammatory response after surgery and with conventional inflammatory markers was studied

III. MATERIALS AND METHODS

III.1. Method validation for automated urinary orosomucoid measurements

For u-ORM measurements, a latex particle-enhanced turbidimetric assay was developed and adapted to an open developmental channel of cobas 8000/c502 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

The determination of u-ORM was performed by using anti human rabbit Orosomucoid Immunoparticles (ref. no. OA504, Dako Denmark A/S, Glostrup, Denmark) and Reaction Buffer (ref. no. PO1812, Dako).

Dilution series of N Protein Standard SL for BN II Systems (ref. no. OQIM13, Siemens Healthcare Diagnostics GmbH, Marburg, Germany) with 788 mg/L orosomucoid concentration were used for calibration and two different dilutions (4.125 and 0.825 mg/L, respectively) of N/T Protein Control SL/L (ref. no. OQIN13, Siemens) were applied as controls. All the dilutions of calibrators, controls and samples were made by sterile 154 mmol/L NaCl solution which was also used as blank sample.

The wavelength for the turbidimetric reaction was 546 nm. Two-point end assay type was set and delta absorbance was calculated between 38–70 measuring points. The calibrator/control/sample volume was 7 μL, the latex-antibody volume was 40 μL and buffer volume was 130 μL. Full calibration was performed by applying a six-point standard curve between 0.0-5.25 mg/L applying the spline graph evaluation method. The assay was performed at 37 °C using 10-minute reaction time.

During the validation, analytical limits were determined from data of 30 independent measurements of blank samples. Mean, standard deviation (SD) and coefficients of variation (CV) were calculated. Limit of detection (LOD) was calculated as the blank mean + 3 SD, while limit of quantification (LOQ) as the blank mean value + 10 SD. Functional sensitivity was evaluated from dilution series of a urine sample (0.025-0.390 mg/L) at the lowest u-ORM concentration where the CV reached 20%. Linearity was determined by two parallel measurements of ten different dilutions of a urine sample (0.10–4.68 mg/L).

For imprecision and inaccuracy measurements we used four different dilutions of PreciControl ClinChem Multi 2 (PC2, ref. no. 05117216 190, Roche) with 845 mg/L orosomucoid concentration. For the assessment of intra-assay imprecision ten parallel measurements were performed on the same day and for that of inter-assay imprecision we executed duplicate measurements on ten consecutive days. Accuracy was also calculated from the measured and theoretical value at four levels of PC2 and was expressed as %.

For stability studies, five different urine samples (0.52-4.11 mg/L) without additives were tested. Specimens were stored at 2–8 °C. U-ORM was determined right after sample collection and on the 3rd, 5th, 8th and 10th day. Analyte stability after 5 freezing - thawing cycles was also assessed.

III.2. Patients and sampling

The study was approved by the Regional Ethics Committee of the University of Pécs, Medical School in accordance with the Helsinki declaration (no. 4327.316-2900/KK15/2011). Every patient and control individual was fully informed and written consent was obtained from all of them.

III.2.1. Healthy reference group

In order to determine the reference range for u-ORM, healthy volunteers were recruited between the age of 10 and 60 years (n=72, mean age: 30±16 years, 51% females). Healthy state was assessed if the person had no chronic illness, no complaints, and no symptoms on medical examination and did not take prescribed medicines. Accordingly, exclusion criteria were inflammation (based on hsCRP <5 mg/L, white blood cell count (WBC) <10 G/L and clinical signs), any kind of chronic diseases (based on medical records) or the lack of consent. Spontaneous random urine samples and venous blood were simultaneously obtained from the participants.

III.2.2. Matched control group with comorbidities

Patients with comorbidities (n=30, mean age: 60±12 years, 47% females) were enrolled to investigate the effect of chronic disorders on u-ORM excretion and for age-, gender-, comorbidity-matched control group for critically ill patients. Patients with acute inflammation (WBC <10 G/L, hsCRP <5 mg/L and clinical signs), with chronic kidney failure (medical records and GFR <60 mL/min/1.73m^2) were excluded. Spontaneous random urine samples and venous blood were simultaneously obtained from the participants.

III.2.3. Critically ill patients from ICU (SIRS, sepsis)

Patients with SIRS (n=13, mean age: 63±9 years, 31% females) and severe septic patients (n=43, mean age: 65±14 years, 40% females) from ICU were enrolled. Only patients with negative blood culture were enrolled into the “SIRS group”. Diagnostic criteria for severe sepsis included SIRS plus microbiologically proven or presumed infection, elevated serum

PCT levels (> 2 ng/mL) and at least one organ dysfunction induced by sepsis. We applied the current definitions and guidelines of sepsis care and severity and prognostic scores (SAPS II, APACHE II, SOFA) were assessed, too.

Exclusion criteria were chronic kidney disease, urosepsis and withdrawal of consent.

We performed a follow-up study, the first sample was obtained within 24 h after clinical diagnosis then on the 2nd, 3rd and 5th consecutive days in sepsis, whereas at patients with SIRS the sampling was carried out only on the 1st and 2nd days because of the shorter ICU treatment.

Data of patients with SIRS and sepsis were compared to data of matched controls, because of the similar demographic characteristics.

III.2.4. Patients underwent cardiac surgery

The effect of surgery induced systemic inflammation was investigated by monitoring of patients undergoing elective on-pump cardiac surgery (n=38, mean age: 66±8 years, 40% females). Patients suffering from acute or chronic inflammatory disorders, autoimmune diseases, tumors, infection, kidney disease, acute coronary syndrome or recent surgical interventions were excluded to avoid their potential influence on u-ORM levels. We performed a follow-up study, samples were obtained on the day before surgery and on the 1st, 3rd and 5th postoperative days.

Data of patients underwent cardiac surgery were compared to data of healthy adults from Reference group (n=42, mean age: 43±11 years, 57% females)

III.2.5. Laboratory analyses

Simultaneously obtained blood and urine samples were analyzed. After centrifugation (1500 x g, 10 minutes) serum and urine aliquots were stored at -70 °C until use. From serum samples inflammatory parameters (hsCRP, PCT, se-ORM) and kidney function markers (creatinine, cystatin-C) were measured and from urine total protein (u-TP), albumin (u-ALB) and creatinine (u-CREAT) were determined by routine procedures. U-ORM was measured by the novel turbidimetric assay. Since urine was obtained by spontaneous micturition, u-ORM levels were referred to u-CREAT (u-ORM/u-CREAT, mg/mmol) to reduce the influence of urine volume on their concentrations. To reveal the relative changes of ORM among urinary proteins, we expressed the proportional data as u-ORM/u-TP (%), too.

III.3. Statistical analyses

Statistical analyses were performed by IBM SPSS, Version 22 (IBM Corporation, NY, USA). After normality testing (Shapiro-Wilk test) our variables were found to be non-normally distributed, therefore non-parametric tests were used. For comparison of groups Mann-Whitney U test (2 groups) or Kruskal-Wallis test (more groups) was carried out. Differences during the follow-up period were investigated by Wilcoxon sum rank test (2 days) and by Friedman's test (more days). Receiver operating characteristic (ROC) curves with area under the curve (AUC ROC) were used for analyzing the diagnostic and predictive values. Logistic regression analyses were applied to evaluate the clinical impact of variables, odds ratios and corresponding 95% confidence intervals (95% CI) were calculated. Spearman's tests were performed for correlation analyses. Statistical significance was considered at $p < 0.05$.

Continuous variables were expressed as medians (25-75 percentiles), and reference interval for u-ORM, u-ORM/u-CREAT and u-ORM/u-TP were determined as 2.5-97.5 percentiles.

IV. RESULTS

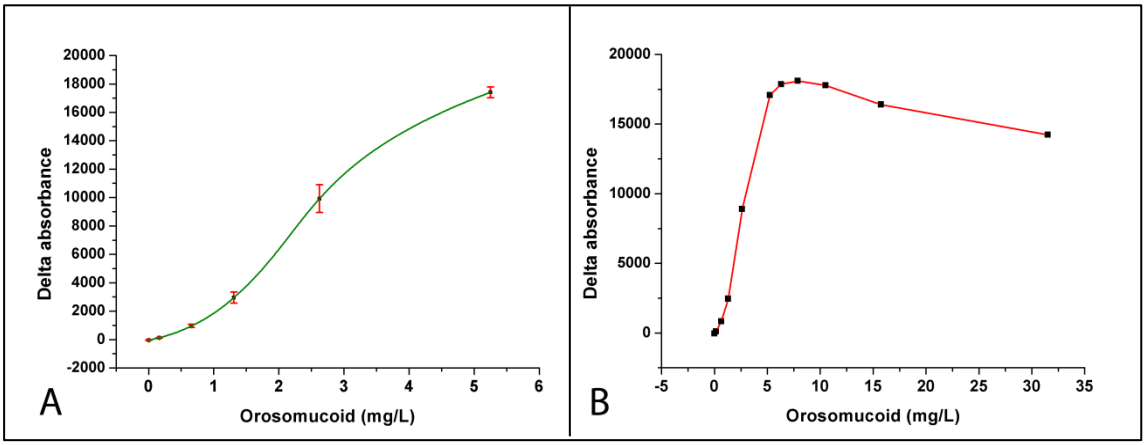
IV.1. Method validation for urinary orosomucoid measurements

The calibrated working range for u-ORM measurements was between 0.16–5.25 mg/L (Figure 1/A). Investigating a wide concentration range (0.1–31.5 mg/L) we observed a narrow security zone (Figure 1/B), therefore samples at above 5.25 mg/L u-ORM concentrations had to be diluted due to the hook effect experienced. Samples under the lowest calibration point (0.16 mg/L) of the assay could be determined down to the LOQ.

The analytical limits of the assay were satisfactorily low (LOD=0.02 mg/L, LOQ=0.08 mg/L). The functional sensitivity of our assay was assessed to be 0.03 mg/L. The assay showed good linearity (R^2=0.999) in the range of 0.1–4.68 mg/L. Both intra- and inter- assay imprecision was determined to be less than 5% of CV and even accuracy of our u-ORM assay was found to vary between 95.69 - 102.51%.

During 10 days of storage at 2–8 °C the u-ORM concentrations of the urine samples remained stable (97.2–109.2%). Furthermore, we found no significant decrease in u-ORM concentrations after repeated freeze-thaw cycles.

Figure 1. Calibration (**A**) and dose response (**B**) curve of the u-ORM assay



A: Cumulative graph of a 6-point calibration curve of the assay in the range of 0.16–5.25 mg/L with spline graph fitting. Mean±SD from 22 separate calibrations are represented

B: Dose response curve in the range of 0.1-31.5 mg/L.

IV.2. Reference range

From the enrolled 72 healthy individuals 3 age groups were created (10-20, 21-40, 41-60 years). We found no significant differences in u-ORM excretion between the three age-groups, therefore we determined a common reference interval for all cases between 10–60 years: u-ORM: 0.13-2.96 mg/l; u-ORM/u-CREAT: 0.01-0.24 mg/mmol; u-ORM/u-TP: 0.21-3.58 %. There was no significant difference in u-ORM/u-CREAT values between females and males in either of three groups.

IV.3. Matched control group

Members of matched control group suffered from several chronic diseases (hypertension, diabetes, COPD, etc.) treated with medication, and were older than individuals from reference group. Compared to healthy individuals we observed significantly higher ($p<0.001$) u-ORM excretion in this control group (0.08 (0.05-0.15) vs 0.2 (0.1-0.3) mg/mmol, respectively). Likewise hsCRP levels were also elevated ($p=0.03$), however se-ORM concentrations did not differ between these groups.

U-ORM/u-CREAT values did not show any differences with respect to gender and various comorbidities.

IV.4. Critically ill patients from ICU (SIRS, sepsis)

In our study more patients were admitted to ICU after surgical interventions than due to non-surgical causes. SIRS patients required an average of only 2 days of ICU treatment while septic patients were treated on average of 6 days. During the 5-day follow-up, 28% of the severe septic patients died and the total 28-day mortality was 47%. As complication, acute kidney injury (AKI) was developed in 22 cases, 9 patients required acute dialyses.

All the enrolled SIRS patients had negative blood cultures and in 9 cases of sepsis the microbes remained unidentified. Most frequently mixed microbial infections (53%) caused sepsis.

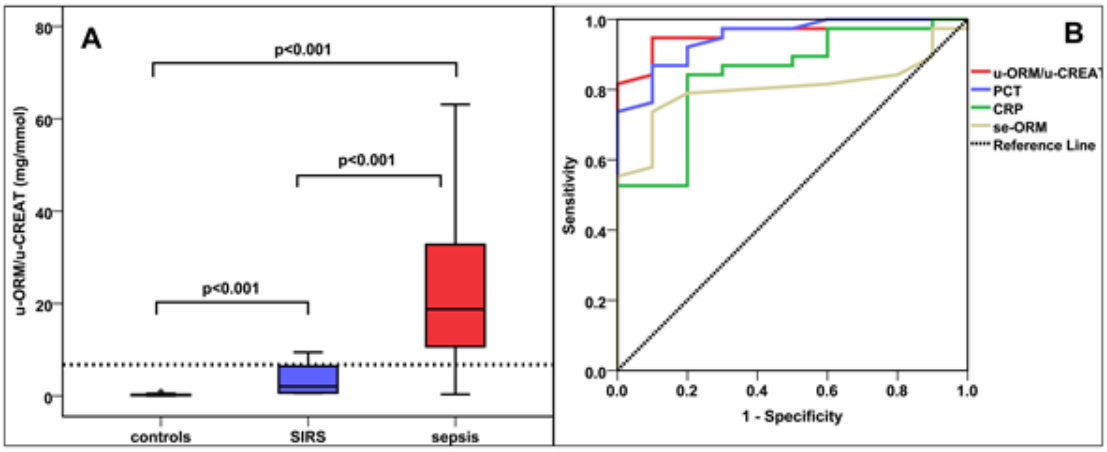
Admission laboratory data to distinguish SIRS from sepsis

Besides the conventional inflammatory markers (WBC, hsCRP, PCT), serum and urinary ORM levels were also significantly higher ($p<0.001$) in SIRS and in sepsis than in controls. About 10-fold higher u-ORM/u-CREAT levels were found in sepsis than in SIRS (19.20 (11.43-32.78) vs 2.06 (0.72-6.36), $p<0.001$).

Based on ROC analyses, a cut-off value for u-ORM/u-CREAT with 94.7% sensitivity and 90% specificity could be set at 6.75 mg/mmol to distinguish SIRS from sepsis (Figure 2/A). The AUC ROC was found to be 0.954 for u-ORM/u-CREAT, similarly to PCT (0.949) and it was higher than that for hsCRP (0.845) and for se-ORM (0.804) (Figure 2/B).

Logistic regression analysis showed that increased u-ORM/u-CREAT, PCT, and hsCRP levels were significant ($p<0.05$) indicators of sepsis: u-ORM/u-CREAT: 1.56 (95% CI: 1.14–2.14), PCT: 2.37 (95% CI: 1.23–4.57) and hsCRP: 1.02 (95% CI: 1.01–1.02).

Figure 2. Diagnostic value of admission levels of u-ORM/u-CREAT in sepsis



A: Comparison of u-ORM/u-CREAT values between controls, SIRS and sepsis. Dotted line shows the cut off value at 6.75 mg/mmol.

B: ROC analyses to distinguish SIRS from sepsis.

Monitoring of urinary orosomucoid in sepsis

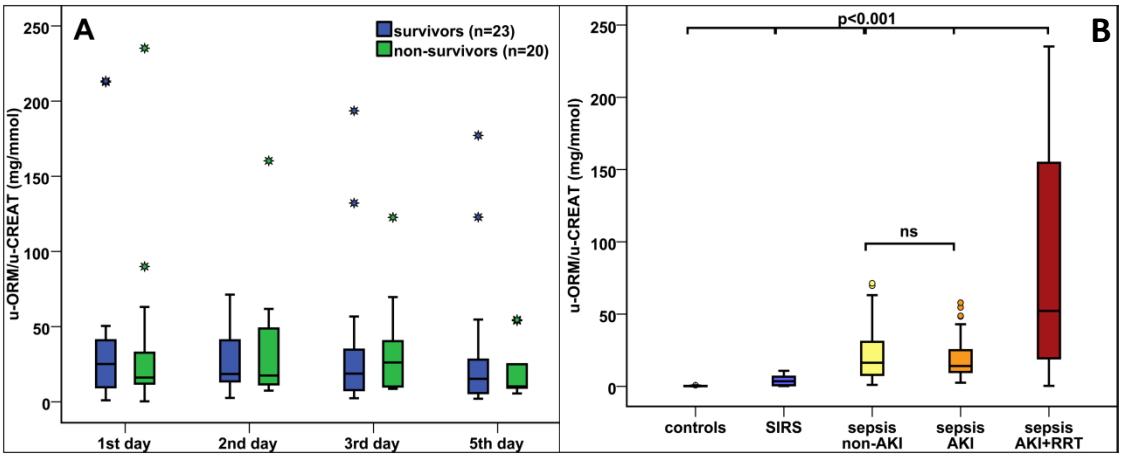
We did not observe any significant changes in u-ORM/u-CREAT values during the 2-day monitoring of SIRS and the 5-day follow-up of sepsis. Moreover, in our study period the u-ORM/u-CREAT levels of survivor septic patients (n=23) did not differ significantly from those of non-survivors (n=20) (Figure 3/A).

The prognostic performance for 28-day mortality prediction of u-ORM/u-CREAT levels in sepsis (AUC ROC: 0.414) was lower than that of the used prognostic scores (SAPS II: 0.731, APACHE II: 0.676, SOFA: 0.698).

U-ORM/u-CREAT values were similarly high in septic patients independently from the pathogen and from source/origin of infection. Also the u-ORM/u-CREAT levels of patients with septic shock or with more than three organ dysfunction did not differ significantly from patients without these conditions, and also no relation with the use of vasopressors or diuretics

could be found. However, extremely elevated (p<0.001) u-ORM/u-CREAT levels were found in the urine samples obtained from patients with dialysis requirement (52.2 (19.4-154.7) mg/mmol) compared to non-dialyzed patients (14.1 (9.9-25.0) mg/mmol). There were no differences in u-ORM/u-CREAT values between non-dialyzed AKI and non-AKI patients (Figure 3/B).

Figure 3. Monitoring of u-ORM/u-CREAT in sepsis



A: 5-day monitoring of u-ORM/u-CREAT in sepsis. No significant differences were found during the follow-up period. Stars represent the extreme values.

B: Differences of u-ORM/u-CREAT levels between acute kidney injury (AKI) and non-AKI groups. RRT, renal replacement therapy (dialysis); ns, non-significant.

Albuminuria and orosomucoiduria during inflammation

Changes in urinary protein composition during inflammation were also analyzed. The u-ORM proportion (u-ORM/u-TP) also showed a significant elevation both in SIRS and in septic patients compared to controls (p<0.001). In controls the u-ORM accounted for only 1.6%–4.0% of urinary proteins, in SIRS it reached 3.6%–11.9%, while in sepsis u-ORM/u-TP ranged from 10.2-34.0%. In contrast, the u-ALB/u-TP proportion did not change significantly during systemic inflammation.

Correlation analyses

We found significant (p<0.001) correlation between u-ORM/u-CREAT and conventional inflammatory parameters: se-ORM (0.693), hsCRP (0.600), WBC (0.407) and PCT (0.348). Additionally u-ORM/u-CREAT directly (p<0.001) correlated with kidney function markers: cystatin-C (0.488), creatinine (0.318), but no associations were observed with other organ dysfunction parameters.

IV.5. Patients underwent cardiac surgery

From the total 38 patients 20 underwent coronary artery bypass grafting (CABG) and 18 required atrial valve replacement (AVR) surgery. The basic demographic and operative data were comparable between CABG and AVR patients. Most patients required only 1 day intensive care after surgery. During the 5-day follow-up none of the patients developed AKI or sepsis. Within a 60-day period after surgery no death was observed.

Baseline preoperative data

On the day before cardiac surgery, the patients baseline u-ORM/u-CREAT and hsCRP levels were moderately elevated compared to healthy individuals ($p<0.001$). Oppositely, se-ORM concentrations did not differ from healthy reference persons.

The baseline u-ORM/u-CREAT values were similarly elevated in patients independently from ongoing chronic disorders. There were no differences in u-ORM/u-CREAT values between CABG (0.27 (0.17-0.36) mg/mmol) and AVR (0.33 (0.13-0.49) mg/mmol) patients and we found no effect of age or gender.

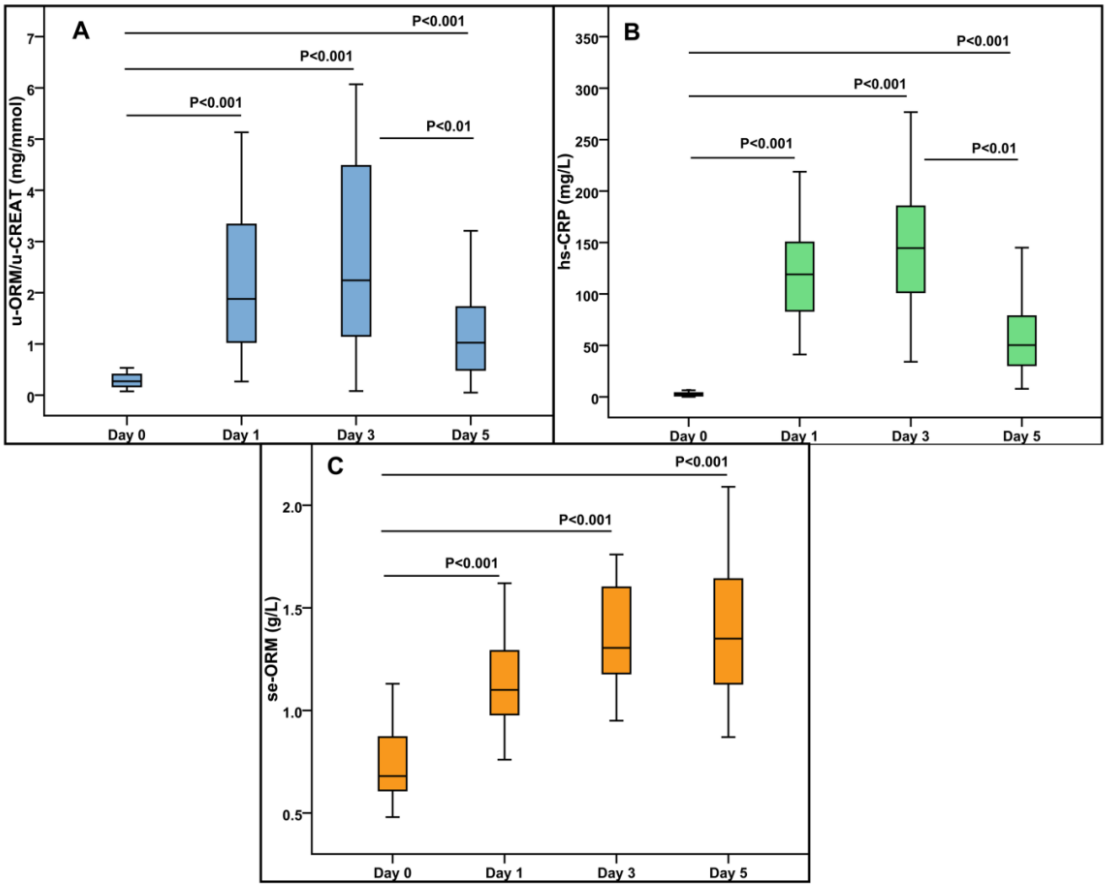
Monitoring of inflammation after cardiac surgery

The perioperative changes of u-ORM/u-CREAT showed similar pattern to that of hsCRP levels. Approximately 10-fold increased u-ORM/u-CREAT values were found after cardiac surgery which remained also high on the 3rd postoperative day then on the 5th day they significantly decreased, however remained higher than the baseline values (Figure 4/A,B). The u-ORM concentrations (mg/L) and the proportional changes, as u-ORM/u-TP (%) ratios also showed similar trends. Nevertheless, se-ORM concentration showed an increasing tendency up to the 5th postoperative day with a 2-fold elevation, and no decrease was observed in its values (Figure 4/C).

Correlation analyses

After cardiac surgery u-ORM/u-CREAT levels showed significant ($p<0.001$) positive correlations with inflammatory parameters: hsCRP (0.724), se-ORM (0.633) and WBC (0.461). However, no association was discovered with kidney function markers, serum cystatin-C or creatinine concentrations.

Figure 4. Monitoring of inflammatory parameters after cardiac surgery



A: u-ORM/u-CREAT levels, **B**: hsCRP values, **C**: se-ORM concentrations. Day 0 indicates the day before surgery.

V. DISCUSSION

We studied urinary orosomucoid levels in systemic inflammatory conditions. Our results might help to reveal the potential role of u-ORM in acute inflammation.

We elaborated a fast, sensitive and precise turbidimetric approach for u-ORM measurements on a cobas 8000/c502 analyzer which is ideal for routine work. Due to the extraordinarily low quantification limit and the good functional sensitivity this assay enables the quantitative u-ORM determination in all healthy individuals and after proper dilution in patients suffering from systemic inflammatory diseases. However, due to the narrow security zone, a 20-fold predilution of all urine samples is suggested in order to automatically extend the measurable concentration limit up to 105 mg/L.

Our assay settings allow more sensitive and precise u-ORM determination compared to previously reported methods.

In agreement with other studies we also proved that ORM in urine is stable *in vitro*, thus urine samples can be stored for later u-ORM analyses.

We found a low urinary orosomucoid excretion rate in healthy persons. The reference interval for u-ORM/u-CREAT determined by investigation of 72 healthy persons is in accordance with former studies. In case of spot urine samples, u-ORM is suggested to be expressed as urinary orosomucoid/creatinine ratio in order to reduce the influence of urine volume, moreover u-ORM/u-CREAT levels seem to be independent from age and gender.

Compared to healthy persons, we observed 2-3 fold increased u-ORM/u-CREAT values in individuals with comorbidities, which revealed the impact of chronic disorders on u-ORM excretion. This moderate elevation was most likely caused by low-grade inflammation related to the comorbidities, which is also supported by mildly elevated hsCRP levels. The association between chronic inflammation and elevated u-ORM values has been supposed previously in chronic disorders, cardiovascular diseases, diabetes, atherosclerosis, heart failure. The observed elevation of u-ORM/u-CREAT among matched controls is in accordance with the preoperative findings of cardiac surgical patients who also suffered from chronic diseases and showed moderately increased hsCRP. On the other hand, se-ORM concentrations did not differ between healthy individuals and patients with chronic conditions, therefore u-ORM/u-CREAT might be a more sensitive marker of low grade inflammatory activation than se-ORM.

Significantly increased u-ORM excretion was found in severe systemic inflammation as well. Compared to controls we described 100-fold elevated u-ORM/u-CREAT levels in sepsis and

it was 10 times higher than in SIRS, too. The cut-off value at 6.75 mg/mmol for u-ORM/u-CREAT might be able to distinguish sepsis from SIRS, which was also confirmed by logistic regression analyses. Based on our results, u-ORM/u-CREAT as early inflammatory marker seems to be promising for the detection of sepsis, and prompt diagnosis is of utmost importance for improving the outcome. Analyzing se-ORM too, we also found elevated admission levels but the diagnostic as well as the prognostic capacity of it was much lower than those of u-ORM.

So far only Magid et al. published similarly increased u-ORM excretion in sepsis, although they monitored altogether 7 septic patients.

However u-ORM/u-CREAT values showed an early rise in sepsis, they did not change significantly during 5-day follow-up period. This phenomenon can be explained by the ongoing severe inflammatory condition and heterogeneity of the patients, but the relatively long, 5 days half-life of ORM presumably may not account for it.

We observed increased u-ORM excretion in sepsis independently from the source of infection or from the cause of admission, which might be a good advantage in sepsis diagnostics contrast to PCT.

In spite of the fact that u-ORM excretion seems not to be influenced by the severity of sepsis or organ dysfunctions, kidney function may have an important impact on the urinary concentration of u-ORM as shown by the extremely increased u-ORM/u-CREAT levels in dialyzed patients and is confirmed by the correlation between kidney function parameters and u-ORM. The observed extreme values in dialyzed patients suggest that u-ORM/u-CREAT might indicate renal insufficiency and the necessity of dialysis, however further studies should conduct to investigate this issue. In accordance to our results, previously Devarajan et al. published extremely increased u-ORM excretion after cardiac surgery in children as the early marker of postoperative AKI.

In healthy individuals u-ORM accounts for only 1-5% of urinary proteins, normally it appears 5-10 fold lower than albumin, while in sepsis u-ORM exceeds the u-ALB levels and it becomes a considerable protein fraction. Based on our results, the relative increase of u-ORM is much higher than that of u-ALB during the inflammatory process. It can suggest that different mechanisms are responsible for their excretion and u-ORM seems to be a better indicator of inflammation, which was also suggested by Magid et al.

Major surgical procedures such as on-pump cardiac surgery trigger systemic inflammatory response through activation of immune system similarly to sepsis.

Our results demonstrated that u-ORM/u-CREAT might indicate the magnitude of systemic inflammatory activation likewise to hsCRP. Interestingly, the time course of u-ORM/u-CREAT levels after cardiac surgery followed the alterations of hsCRP and not the kinetics of se-ORM. The relatively long, about 5-day half-life of se-ORM can explain its time course, however other factors should influence the u-ORM levels. The kinetics of u-ORM after cardiac surgery might explain the non-decreasing tendency of u-ORM/u-CREAT levels observed in SIRS patients during the first 2 days of intensive care, because they declined 3 days after stimuli. The currently available data regarding the kinetics of u-ORM after surgery is limited. In accordance to our results, previously Magid et al. described that the changes of u-ORM mirrored those of CRP by monitoring u-ORM excretion in 6 patients after abdominal surgery. Devarajan et al. reported that u-ORM levels could elevate within 2 hours after cardiac surgery in children.

The pathmechanism of u-ORM excretion is not well clarified. Correlation between serum and urine levels of ORM suggests that circulating ORM appears in urine, however the increased se-ORM level itself may not explain that alone, probably it is not a result of a simply overflow mechanism.

Alteration in kidney function, presumably both glomerular and tubular dysfunctions may be involved. Furthermore, a possible local renal production due to systemic manifestation of inflammation is also suspected. Extrahepatic, local ORM synthesis in kidneys might contribute to maintain organ function and reduce tissue damage caused by inflammation. Based on correlation analyses u-ORM/u-CREAT levels are associated with the magnitude of inflammatory response, the strong correlation between inflammatory parameters and u-ORM/u-CREAT values suggested that systemic inflammation seems to be mainly responsible for increased u-ORM excretion, highlighting its immunomodulatory activity.

To conclude, urinary orosomucoid is a more sensitive inflammatory marker than serum orosomucoid and u-ORM might be ideal for real-time monitoring of the inflammatory response.

Consequently, non-invasively obtained urine samples could be a possible alternative to blood sampling and u-ORM may be capable of routine clinical usage for monitoring systemic inflammatory conditions, especially in the form of a rapid point of care test.

VI. NOVEL FINDINGS-THESES

- We developed a fast, sensitive, precise, and fully automated particle enhanced turbidimetric assay for u-ORM measurements, which is ideal for routine clinical use.
- By investigating healthy individuals, we determined a reference interval, which is applicable among adolescents and adults as well.
- Healthy persons showed low u-ORM/u-CREAT values, while we found moderately increased u-ORM excretion in individuals suffering from chronic disorders, which can be an indicator of chronic low-grade inflammation related to comorbidities.
- The u-ORM values showed early and considerable elevation in sepsis, and are able to distinguish SIRS from sepsis. U-ORM/u-CREAT levels above 6.75 mg/mmol could distinguish SIRS from sepsis with 94.7% sensitivity and 90% specificity.
- However u-ORM/u-CREAT values seem to be independent from sepsis severity and outcome, extreme values might be potential indicators of kidney failure and requirement of dialysis.
- The kinetics of u-ORM/u-CREAT after cardiac surgery demonstrated that u-ORM is a rapid, sensitive marker of systemic inflammatory activity.
- U-ORM/u-CREAT levels show early and significant increase after cardiac surgery and follow a similar time course to hsCRP, and changes of se-ORM seem not to influence u-ORM/u-CREAT values.
- The strong correlation between conventional inflammatory parameters and u-ORM/u-CREAT suggest that systemic inflammation plays an important role in the elevation of u-ORM excretion.
- When compared to serum orosomucoid, urinary orosomucoid is a more sensitive, early inflammatory marker and is capable for non-invasive monitoring of systemic inflammatory activation.

VII. PUBLICATIONS

Publications related to the Theses

Kustán P, Szirmay B, Kőszegi T, Ludány A, Kovács GL, Miseta A, Mühl D, Németh B, Kiss I, Németh Á, Szabados S, Ajtay Z: Monitoring of urinary orosomucoid in patients undergoing cardiac surgery: A promising novel inflammatory marker. *Clin Biochem.* 2017 Jul 21. pii: S0009-9120(17)30445-9. [Epub ahead of print] doi: 10.1016/j.clinbiochem.2017.07.010. **IF: 2.434**

Kustán P, Szirmay B, Horváth-Szalai Z, Ludány A, Kovács GL, Miseta A, Kőszegi T, Mühl D: Urinary orosomucoid: a novel, early biomarker of sepsis with promising diagnostic performance. *Clin Chem Lab Med.* 2017;55(2):299-307. **IF: 3.432**

Kustán P, Szirmay B, Horváth-Szalai Z, Ludány A, Lakatos Á, Mühl D, Christensen PH, Miseta A, Kovács GL, Kőszegi T: Urinary orosomucoid: validation of an automated immune turbidimetric test and its possible clinical use. *Biochem Med*. 2016;26(3):421–30. **IF: 2.934**

Publications not related to the Theses

Horváth-Szalai Z, **Kustán P**, Szirmay B, Lakatos Á, Christensen PH, Huber T, Bugyi B, Mühl D, Ludány A, Miseta A, Kovács GL, Kőszegi T: Validation of an automated immune turbidimetric assay for serum gelsolin and its possible clinical utility in sepsis. *J Clin Lab Anal.* 2017 Sep 5. [Epub ahead of print] doi: 10.1002/jcla.22321. **IF: 1.521**

Kustán P, Horváth-Szalai Z, Mühl D: Nonconventional markers of sepsis. *E-JIFCC*. 2017;28(2):122-33. **IF: -------**

Horváth-Szalai Z, **Kustán P**, Mühl D, Ludány A, Bugyi B, Kőszegi T: Antagonistic sepsis markers: Serum gelsolin and actin/gelsolin ratio. *Clin Biochem*. 2017;50(3):127-33. **IF: 2.434**

Nemeth B, Kiss I, Jencsik T, Peter I, Kreska Z, Koszegi T, Miseta A, **Kustan P**, Boncz I, Laczo A, Ajtay Z. Angiotensin-converting Enzyme Inhibition Improves the Effectiveness of Transcutaneous Carbon Dioxide Treatment. *In Vivo*. 2017;31(3):425-8. **IF: 0.953**

Németh B, Kiss I, Péter I, Ajtay Z, Németh Á, Márk L, Csorba A, Kőszegi T, Mühl D, **Kustán P**: Monitoring of L-arginine and endogenous dimethylarginines in survivor septic patients – a pilot study. *In Vivo.* 2016 09-10;30(5):663-9. **IF: 0.953**

Németh B, **Kustán P**, Németh Á, Lenkey Z, Cziráki A, Kiss I, Sulyok E, Ajtay Z: Aszimmetrikus dimetilarginin: a cardiovascularis betegségek prediktora? *Orvosi hetilap*. 2016;157(13):483-7. **IF: 0.349**

Kustán P, Horváth-Szalai Z, Németh B, Török C, Ragán D, Kőszegi T, Mühl D: A szepszis diagnózisa napjainkban. *Magyar Epidemiológia*. 2015;12(1-2):59-66 **IF: -------**

Cumulative impact factor of publications related to the Theses: 8.80

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Book chapters

Péter Kustán, Balázs Szirmay, Diána Mühl, Andrea Ludány: Human orosomucoid in the clinical laboratory. In: T. Kőszegi (Ed.) *Laboratory Techniques with Applicability in Medical practice.* LAP Lambert Academic Publishing, Saarbrücken, Germany, 2015. pp:101-120 (ISBN: 978-3-659-31724-8)

Zoltán Horváth-Szalai, **Péter Kustán**, Tamás Kőszegi: New laboratory findings in sepsis. In: T. Kőszegi (Ed.) *Laboratory Techniques with Applicability in Medical practice.* LAP Lambert Academic Publishing, Saarbrücken, Germany, 2015 pp:57-76 (ISBN: 978-3-659-31724-8)

Zoltán Horváth-Szalai, **Péter Kustán**, Tamás Kőszegi: Laboratory diagnostics of sepsis. In: A. Chesca (Ed.) *Methods for Diseases Diagnostic with Applicability in Practice*. LAP Lambert Academic Publishing, Saarbrücken, Germany, 2014. pp. 27-52. (ISBN: 978-3-8473-4502-2)

Péter Kustán, Zoltán Horváth-Szalai, Tamás Kőszegi: Biochemical markers of systemic diseases. In: A. Chesca (Ed.) *Methods for Diseases Diagnostic with Applicability in Practice*. LAP Lambert Academic Publishing, Saarbrücken, Germany, 2014. pp. 69-104. (ISBN: 978-3-8473-4502-2)

Kustán Péter: Vizelet orosomucoid, mint lehetséges szepszis biomarker. In: Szamonek Vera (szerk.) *XI. Grastyán konferencia kötet*. Pécs, 2013. pp. 223-229. (ISBN: 978 963 642 547 0)

Oral presentations

Kustán Péter, Horváth-Szalai Zoltán, Szirmay Balázs: Urinary Orosomucoid as a Potential Diagnostic Marker of Sepsis. 13th International Medical Postgraduate Conference, Hradec Kralove, 2016.11.24-25.

B. Szirmay, **P. Kustán**, P.H. Christensen, A. Ludány, T. Kőszegi: An automated immune turbidimetric test for urinary orosomucoid: validation and clinical usage. *Magyar Laboratóriumi Diagnosztikai Társaság 58. Nagygyűlése*, Szeged, 2016.08.25-27. IN: *Clin Chem Lab Med* 2016;54(10):eA186.

Péter Kustán, Balázs Szirmay, Zoltán Horváth-Szalai, Andrea Ludány, Attila Miseta, Diána Mühl, Tamás Kőszegi: Urinary orosomucoid- automated immunoturbidimetric test and its clinical relevance. *The 8th Conference of PhD Students*, Marosvásárhely, 2015.12.09-10. IN: A*cta medica marisiensis* 2016;61(7):8.

Horváth-Szalai Z, **Kustán P**, Mühl D, Kőszegi T: New protein biomarkers in sepsis. *International CEEPUS Summer School on Complex Diseases*, Portoroz, 2015. 07.23-29.

Szirmay B MD, Kövér A MD, **Kustán P** MD: Informational value of orosomucoid in acute and chronic inflammatory diseases. *International CEEPUS Summer School on Complex Diseases*, Portoroz, 2015. 07.23-29.

Kustán P, Horváth-Szalai Z, Németh B, Ludány A, Mühl D, Kőszegi T: Sepsis and oxidative stress. *International CEEPUS Summer School on Complex Diseases*, Portoroz, 2015.07.23-29.

Balázs Szirmay, **Péter Kustán**: Human orosomucoid in the clinical laboratory. *7th International Student Medical Congress,* Kassa, 2015. 24-26.

Kustán P: Urinary proteins in sepsis. *14th International Congress of Medical Sciences*, Szófia, 2015. 05.07-10.

Horváth-Szalai Z, **Kustán P**: Promising markers of sepsis: serum actin and gelsolin. *14th International Congress of Medical Sciences*, Szófia, 2015.05.07-10.

Dr. Szirmay Balázs, **Dr. Kustán Péter**, Dr. Ludány Andrea: Humán orosomucoid a klinikai laboratóriumi diagnosztikában. *Tavaszi Szél Konferencia,* Eger, 2015.04.10-12.

Dr. Kustán Péter: Vizelet fehérjék szepszisben. *Tavaszi Szél Konferencia,* Eger, 2015.04.10-12.

Horváth-Szalai Z, **Kustán P,** Kőszegi T: Unusual protein markers of sepsis: serum actin and gelsolin. *XX. Korányi Frigyes Tudományos Fórum,* Budapest, 2015.03.12-13.

Kustán P, Horváth-Szalai Z, Ludány A, Mühl D, Kőszegi T: Orosomucoid in urine. A useful biomarker? *International CEEPUS Summer School*, Portoroz, 2014.08.23-29.

Horváth-Szalai Z, **Kustán P**, Ludány A, Mühl D, Bugyi B, Kőszegi T: Serum actin and gelsolin: new potential biomarkers in sepsis? *International CEEPUS Summer School*, Portoroz, 2014.08.23-29.

P. Kustán, Z. Horváth-Szalai, A. Ludány, T. Kőszegi, D. Mühl: Urinary orosomucoid and sepsis. *10th János Szentágothai Transdisciplinary Conference and Student Competition*, Pécs, 2013.11.04-05.

Z. Horváth-Szalai, **P. Kustán**, T. Kőszegi, A. Ludány, D. Mühl: New potential sepsis biomarker. *10th János Szentágothai Transdisciplinary Conference and Student Competition*, Pécs, 2013.11.04-05.

Balazs Szirmay, Anna Kover, **Peter Kustan**: Method for the examination of urinary and tear orosomucoid. *HMAA Balatonfüredi nyári konferencia,* Balatonfüred, 2013.08.16-17. IN: Archives of the Hungarian Medical Association of America (ISSN: 1070-0773).

Kustán Péter: Vizelet orosomucoid, mint lehetséges szepszis biomarker. *V. Nemzetközi és XI. Országos Interdiszciplináris Grastyán Konferencia*, Pécs, 2013.04.17-19.

Szirmay Balázs, Kövér Anna, **Kustán Péter**: Módszer a vizelet és könny orosomucoid vizsgálatára. *XXXI. OTDK Orvos- és egészségtudományi szekció*, Szeged, 2013.04.02-05. IN: *Előadáskivonatok.* Szeged, 2013. pp:294 (ISBN: 978-963-306-203-6)

Szirmay Balázs Gábor, Kövér Anna, **Kustán Péter**: Módszer a vizelet és könny orosomucoid vizsgálatára. *PTE ÁOK Tudományos Diákköri Konferencia*, Pécs, 2013.02.07-08.

Kustán Péter: Vizelet orosomucoid mérések szeptikus betegek mintáiban. *PTE ÁOK Tudományos Diákköri Konferencia*, Pécs, 2012.04.17-18.

Poster presentations

Kustán Péter, Szirmay Balázs, Horváth-Szalai Zoltán, Ludány Andrea, Mühl Diána, Kőszegi Tamás: Monitoring of novel urinary protein markers in sepsis. *EuroMedLab Athens 2017*. Athén, 2017.06.11-15. IN: *Clin Chem Lab Med.* 2017;55(Spec.Suppl):s588.

Horváth-Szalai Zoltán, **Kustán Péter**, Szirmay Balázs, Mühl Diána, Ludány Andrea, Kőszegi Tamás: Serum Gc globulin and gelsolin as potential early predictors of sepsis. *EuroMedLab Athens 2017*. Athén, 2017.06.11-15. IN: *Clin Chem Lab Med.* 2017;55(Spec.Suppl):s585.

Szirmay Balázs, **Kustán Péter**, Horváth-Szalai Zoltán, Ludány Andrea, Kőszegi Tamás: Urinary cystatin-c: a new automated particle-enhanced immune turbidimetric test for the routine evaluation of kidney tubular function. *EuroMedLab Athens 2017.* Athén, 2017.06.11-15. IN: *Clin Chem Lab Med.* 2017;55(Spec.Suppl):s803.

Németh Ádám, **Kustán Péter**, Kőszegi Tamás, Kovács L Gábor, Miseta Attila, Mühl Diána, Németh Balázs, Kiss István, Cziráki Attila, Szabados Sándor, Ajtay Zénó: Vizelet orosomucoid monitorozás szívműtéten átesett betegeknél. *Magyar Kardiológusok Társasága 2017. évi Tudományos Kongresszusa,* Balatonfüred, 2017.05.11-13.

Németh Balázs, Kiss István , Péter Iván, Kreska Zita, Kőszegi Tamás, **Kustán Péter**, Ajtay Zénó: Az ACE gátló adása javítja a szén-dioxid kezelés hatékonyságát. *Magyar Kardiológusok Társasága 2017. évi Tudományos Kongresszusa,* Balatonfüred, 2017.05.11-13.

Peter Kustan, Balazs Szirmay, Zoltan Horvath-Szalai, Daniel Ragan, Andrea Ludany, Diana Mühl, Tamas Koszegi: Monitoring of novel urinary protein markers in sepsis. *4th Joint EFLM-UEMS Congress*, Varsó, 2016.09.21-24. IN: *Clin Chem Lab Med.* 2016;54(10):eA324.

Balazs Szirmay, **Peter Kustan**, Per Hjort Christensen, Andrea Ludany, Tamas Koszegi: An automated particle-enhanced immune turbidimetric test for urinary orosomucoid: validation and clinical usage. *4th Joint EFLM-UEMS Congress*, Varsó, 2016.09.21-24. IN: *Clin Chem Lab Med*. 2016;54(10):eA365.

Zoltán Horváth-Szalai, **Péter Kustán**, Balázs Szirmay, Beáta Bugyi, Diána Mühl, Andrea Ludány, Tamás Kőszegi: Synergistic, predictive protein markers in sepsis: serum Gc globulin and gelsolin. *4th Joint EFLM-UEMS Congress*, Varsó, 2016.09.21-24. IN: *Clin Chem Lab Med.* 2016;54(10):eA365.

P. Kustán, B. Szirmay, Z. Horváth-Szalai, D. Ragán, A. Ludány, D. Mühl, T. Kőszegi: Novel urinary protein markers in sepsis. *Magyar Laboratóriumi Diagnosztikai Társaság 58. Nagygyűlése*, Szeged, 2016.08.25-27. IN: *Clin Chem Lab Med.* 2016;54(10):eA199.

Z. Horváth-Szalai, **P. Kustán**, B. Szirmay, B. Bugyi, D. Mühl, A. Ludány, T. Kőszegi: Serum Gc globulin and gelsolin as sepsis markers. *Magyar Laboratóriumi Diagnosztikai Társaság 58. Nagygyűlése*, Szeged, 2016.08.25-27. IN: *Clin Chem Lab Med.* 2016;54(10):eA200.

A. Szijjártó, **P. Kustán**, B. Szirmay, E. Györgyi, F. Kilár, T. Kőszegi, A. Ludány, L. Makszin: Microchip electrophoretic analysis of acid soluble serum proteins of patients. *Magyar Laboratóriumi Diagnosztikai Társaság 58. Nagygyűlése*, Szeged, 2016.08.25-27. IN: *Clin Chem Lab Med*. 2016;54(10):eA207.

Z. Horváth-Szalai, **P. Kustán**, D. Mühl, A. Ludány, T. Kőszegi: Unusual biomarkers in serum and urine of septic patients. *21st IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine*, Párizs, 2015.06.21-25. IN: *Clin Chem Lab Med.* 2015;53(Spec.Suppl):s547.

Kustán P, Kőszegi T., Ludány A., Mühl. D: Vizeletfehérjék szepszisben. *Magyar Aneszteziológiai és Intenzív Terápiás Társaság 43. Kongresszusa*, Siófok, 2015.05.28-30. IN: *Aneszteziológia és Intenzív Terápia* 2015;45(suppl.1.):33.

Horváth-Szalai Z, **Kustán P**, Mühl D, Kőszegi T: Nem szokványos szepszis markerek: szérum aktin és gelszolin. *Magyar Aneszteziológiai és Intenzív Terápiás Társaság 43. Kongresszusa*, Siófok, 2015.05.28-30. IN: *Aneszteziológia és Intenzív Terápia* 2015;45(suppl.1.):41.

P. Kustan, A. Ludany, D. Muhl, Z. Horvath-Szalai, T. Koszegi: Urinary orosomucoid in sepsis: Laboratory approaches. *22nd International Congress of Clinical Chemistry and Laboratory Medicine IFCC-WordLab*, Isztambul, 2014.06.22-26. IN: *Clin Chem Lab Med.* 2014;52(Spec.Suppl):s1368.

Z. Horvath-Szalai, A. Ludany, D. Muhl, **P. Kustan**, B. Bugyi, T. Koszegi: Serum actin and gelsolin: new biomarkers in sepsis? *22nd International Congress of Clinical Chemistry and Laboratory Medicine IFCC-WordLab,* Isztambul, 2014.06.22-26. IN: *Clin Chem Lab Med.* 2014;52(Spec.Suppl):s1365.

Kustán P, Horváth-Szalai Z, Ludány A, Kőszegi T, Mühl D: Vizelet orosomucoid szepszisben. *Magyar Aneszteziológiai és Intenzív Terápiás Társaság 42. Kongresszusa*, Siófok, 2014.05.22-24. IN: *Aneszteziológia és Intenzív Terápia* 2014;44(suppl.1.):28.

A. Ludany, **P. Kustan**, T. Koszegi, G. Woth, D. Muhl, G.L. Kovacs: Urinary orosomucoid in sepsis. *20th IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine*, Milánó, 2013.05.19-23. IN: *Biochimica Clinica* 2013;37(3):S299.

Péter Kustán: Urinary orosomuucoid as a marker of sepsis. *9th International Biomedical Croatian Student Summit*, Zágráb, 2013.03.20-23.

T. Kőszegi, **P. Kustán**, A. Ludány, E Györgyi, G Woth, D. Mühl, G.L. Kovács: Urinary orosomucoid in sepsis. *Magyar Laboratóriumi Diagnosztikai Társaság 56. Nagygyűlése*, Budapest, 2012.08.30-09.01. IN: *Clin Chem Lab Med.* 2012;50(8):eA33.

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