**Charles University in Prague**

**1st Faculty of Medicine**

PhD Thesis Summary

# Novel Biomarkers in Patients with Renal Disease

**MUDr. PhDr. Oskar Zakiyanov**

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|---|---|
| Board: | **Biochemistry and pathobiochemistry** |
| Board chairperson: | **Prof. MUDr. Stanislav Štípek, DrSc.** |
| Workplace: | **Institute of Clinical Biochemistry and Laboratory Diagnostics** |
| | **1st Faculty of Medicine, Charles University in Prague** |
| | **and General University Hospital in Prague** |
| | **U nemocnice 2, Praha 2, 128 08** |
| Supervisor: | **Prof. MUDr. Marta Kalousová, Ph.D.** |

The full text of the thesis will be available at least five days before the PhD. defense at the Department of Science and Research and International Relations of the 1st Faculty of Medicine, Charles University in Prague.

# Contents

# Abstrakt

Cílem disertační práce bylo studium nových biomarkerů, jejich vztah k renální funkci, chronickému zánětu, případně zvýšenému kardiovaskulárnímu riziku. Studovány byly: placentární růstový faktor (PlGF), s těhotenstvím asociovaný protein A (PAPP-A), matrixová metalloproteináza 2 (MMP-2), matrixová metalloproteináza 9 (MMP-9), solubilní receptor pro konečné produkty pokročilé glykace (sRAGE), protein vázající vápník S100A12 – nově identifikovaný extracelulární protein vázající se na receptor pro konečné produkty pokročilé glykace (EN-RAGE) a amfoterin (HMGB-1) u pacientů s chronickým renálním onemocněním, u hemodialyzovaných, pacientů s akutním poškozením ledvin a zdravých kontrol pro srovnání.

První studie odhalila, že hladina PlGF je zvýšená u pacientů se sníženou funkcí ledvin. Druhá studie zjistila spojitost hladin MMP-2 a PAPP-A s proteinurií u pacientů s chronickým renálním onemocněním. MMP-2, MMP-9 a PAPP-A se výrazně lišily u pacientů s nefropatiemi. Hladiny EN-RAGE nebyly zvýšeny v souvislosti se sníženou funkcí ledvin, ale byly spojeny se zánětlivými stavy. U pacientů s akutním renálním poškozením byly hladiny PAPP-A, EN-RAGE a HMGB-1 zvýšené, ale PlGF a sRAGE nebyly zvýšené. PAPP-A korelovalo s markery nutrice, PlGF, EN-RAGE a HMGB-1 vykazovaly korelaci se zánětlivými parametry.

V souhrnu tyto studie prokázaly možnost využití nových biomarkerů u pacientů s onemocněním ledvin.

# Abstract

The aim of the thesis was to study novel promising biomarkers, their relationship to kidney function, chronic inflammation and/or cardiovascular risk – placental growth factor (PlGF), pregnancy associated plasma protein A (PAPP-A), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), soluble receptor for advanced glycation end products (sRAGE), calcium binding protein S100A12 or extracellular newly identified RAGE binding protein (EN-RAGE), and high mobility group box protein-1 (HMGB-1) in patients with renal diseases including CKD, hemodialysis (HD), AKI patients, and healthy controls for comparison.

First study revealed that PlGF is elevated in patients with decreased renal function. Second study demonstrated the association of MMP-2 and PAPP-A with proteinuria in patients with CKD. Moreover, serum MMP-2, MMP-9 and PAPP- EN-RAGE levels are not elevated in patients with CKD, but are related to inflammatory status. MMP-2, MMP-9, PAPP-A levels significantly differed in patients with various nephropathies. PAPP-A, EN-RAGE and HMGB-1 levels are significantly elevated, but sRAGE and PlGF levels are not increased in AKI patients. Whereas PAPP-A correlates with markers of nutrition; PlGF, EN-RAGE and HMGB-1 are related to inflammatory parameters in AKI patients.

Taken together, these studies identified the novel biomarkers to be useful in patients with renal disease.

# 1. Introduction

Chronic kidney disease (CKD) may arise due to multitude of different insults to renal function. However, substantial loss of nephrons provokes a common syndrome characterised clinically by proteinuria and progressive decline in glomerular filtration rate [1]. Acute kidney injury (AKI) is now recognized as a disease with long-term sequelae, including increased risk for death and CKD progression [2]. There is unequivocal evidence linking cardiovascular risk (CVR) and endothelial dysfunction in CKD and haemodialysis (HD) patients [3]. Several examples of testing potential markers of inflammation, oxidative stress and endothelial dysfunction have been presented [4]. Validation of candidate biomarkers in a broad range of populations with renal disease prior into implementation into routine management of patients with renal disease is needed.

Placental growth factor (PlGF), which is a member of the vascular endothelial growth factor (VEGF), stimulates angiogenesis and growth of collateral vessels in ischemic tissues via VEGF receptor-1 (Flt1) [5]. PlGF is upregulated in atheromatic lesions, and antiFlt1 suppresses atherosclerotic process and plaque vulnerability [6]. It was reported that elevated levels of circulating PlGF might be associated with worsening atherosclerosis in patients with decreased renal function [7].

Pregnancy associated plasma protein-A (PAPP-A) is a high-molecular-weight zinc-binding metalloproteinase belonging to metzincin superfamily of metalloproteinases and was originally identified in the plasma of pregnant women [8]. PAPP-A was found to be abundantly expressed in eroded and ruptured vascular plaques, but is only minimally expressed in stable plaques [9]. PAPP-A levels are elevated in chronic HD patients and have been identified as an independent mortality predictor in long-term hemodialysis patients [10].

Matrix metalloproteinase-2 (MMP-2) is a metalloproteinase which can remodel ECM proteins, leading to a change in the balance between ECM synthesis and degradation, which may result in an accumulation of ECM molecules [11]. MMP-2 is zinc-dependent and is known as gelatinase A. MMP-2 degrades ECM proteins in the kidney and vessels including fibronectin, laminin and collagens [12]. Recent studies have shown that MMP-2 is involved in the development and progression of CKD and CVD inducing tissue remodeling via structural alterations in the glomerular and tubular areas; MMP-2 also play roles in blood vessel injury [12, 13].

Matrix metalloproteinase-9 (MMP-9) is a zinc-dependent metalloproteinase and is also known as gelatinase B. Gelatinases have three repeats of the fibronectin-binding domain that

allow them to bind to gelatine, collagen, and laminin. MMP-9 has significantly more specificity towards types IV and V collagen [12]. It was suggested that the increased concentrations of MMP-9 were related to inflammation in atherosclerotic plaques, which in aggregate might be related to the extent of coronary artery disease (CAD) [14]. An identification of renal disease progression and its associated CV risk has become increasingly important.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface proteins that interacts with a wide range of ligands, including advanced glycation end products, modified low-density lipoproteins, amyloid fibrils, amphoterin (HMGB-1) and various S100 proteins [15]. C-truncated RAGE (endogenous secretory RAGE; esRAGE) isoforms circulate in the plasma, where they can act as a decoy for RAGE ligands. These secreted variants together represent the total amount of soluble RAGE (sRAGE) that can be detected in the bloodstream [16].

The calcium-binding protein S100A12, also known as EN-RAGE (extracellular newly identified RAGE-binding protein), is a ligand for RAGE that is expressed on macrophages, lymphocytes and the endothelium. Binding of S100A12 to RAGE activates the intracellular signaling cascades leading to proinflammatory responses in target cells including upregulation of adhesion molecules on the endothelium [17]. S100A12 is overexpressed at sites of local inflammation, and serum concentrations of S100A12 correlate with an individual's disease activity [18].

HMGB-1 is a 30-kDa nuclear and cytosolic ubiquitous protein, a DNA – binding protein, known as a transcription and growth factor [19]. It has been implicated as a putative danger signal involved in the pathogenesis of a variety of inflammatory conditions [20]. HMGB-1 has been reported to trigger cellular signaling through toll-like receptor (TLR) 2, TLR4, and TLR9 and receptor for advanced glycation end products, leading to the recruitment of inflammatory cells and the release of proinflammatory cytokines and chemokines that cause organ damage[21].

Measuring promising biomarkers of inflammation, oxidative stress and endothelial dysfunction may define the risk profile of patients with renal disease, including the underlying biochemical and pathophysiological processes, and/or cardiovascular risk.

## 2. Aims of the study

1. To compare serum PlGF levels in patients with CKD, HD patients and healthy controls. To detect the PlGF levels in the urine of CKD patients. To evaluate possible relationships of serum PlGF concentrations and markers of inflammation and atherosclerosis

2. To compare serum EN-RAGE levels in patients with CKD, HD patients and healthy controls. To assess possible relationships of serum EN-RAGE levels to inflammatory markers.
3. To compare serum MMP-2 and PAPP-A at each levels of CKD. To determine biochemical determinants of MMP-2 and PAPP-A in CKD patients stages 1-5
4. To compare circulating serum levels of MMP-2, MMP-9 and PAPP-A in patients with various biopsy proven nephropathies
5. To evaluate PlGF, PAPP-A, s-RAGE, EN-RAGE, and HMGB-1 levels in patients with AKI. To establish possible relationships of studied biomarkers to inflammatory markers and markers of nutrition

# 3. Materials and methods

## 3.1 Parameters of clinical study

45 patients (28 males and 17 females with mean age 61±15 years) with CKD not yet dialyzed (CHRI group), 31 long-term HD patients (15 males and 16 females, mean age 59±15 years), and 38 age matched healthy subjects (16 males and 12 females, mean age 57±8 years) were included in this one centre study. 46 patients (mean age 59±16 years) of CKD patients not yet dialyzed (CHRI group), the same group of HD patients as in the first study was used and 24 healthy subjects were studied. 159 white patients at different stages of CKD were included in a cross-sectional study. Patients were separated into five CKD groups according to their estimated glomerular filtration rate [eGFR; Modification of Diet in Renal Disease (MDRD)], based on Kidney Dialysis Outcomes Quality Initiative (K/DOQI), as follows: stage 5 (n = 15; eGFR < 15 ml/min), stage 4 (n = 31; eGFR 15-30 mL/min), stage 3 (n = 42; 30-60 mL/min). Patients with CKD stage 1 and 2 were analysed together (n = 71; GFR > 60 mL/min). The control group consisted of 44 healthy subjects. 173 subjects were studied, including 128 patients with various types of glomerular disease (GN), defined by kidney biopsy. 33 had IgA glomerulonephritis (IgA), 23 membranous glomerulonephritis (MN), 11 focal segmental glomerulosclerosis (FSGS), 7 minimal change nephrosis disease (MCNS), 22 lupus nephritis (LN), and 32 anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (AAV). The control group embraced 45 healthy subjects randomly selected from local population. 40 patients with AKI determined using the RIFLE (Risk, Injury, Failure, Loss, and End stage kidney) staging criteria for changes in the serum creatinine within one week. Blood tests and physiological parameters were obtained for each patient at the time of enrolment but before initiation of RRT. The

aetiologies of AKI were ischemia (39.8%), nephrotoxicity (22%), and multifaceted factors (38.2%). 42 patients with CKD stage 5 with glomerular filtration rate (eGFR < 15 ml/min/1.73 $m^2$) at the onset of RRT were included. The aetiology of CKD was vasculits (11%), chronic glomerulonephritis (23%) hypertension (19 %) and diabetes (12 %). The same group of HD patients as in the first and second study was used for comparison.

Written informed consent and laboratory samples were obtained from all subjects according to ethical guidelines. The study was performed in adherence to the principles of the Declaration of Helsinki and approved by the Institutional Ethical Committee.

## 3.2 Methods

PlGF MMP-2, MMP-9 and sRAGE were measured by means of sandwich ELISA (enzyme-linked immunosorbent assay) using standard kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Results of PlGF and sRAGE are given in picograms per milliliter (pg/mL). Results of MMP-2 and MMP-9 are given in nanograms per milliliter (ng/mL). EN-RAGE (Circulex$^{TM}$, CycLex Co. Ltd., Nagano, Japan) and HMGB-1 (IBL International GmbH, Hamburg, Germany) were measured by means of a sandwich ELISA using standard kits according to the manufacturer's protocol. Results of EN-RAGE are given in nanograms per milliliter (ng/mL). Results of HMGB-1 are given in picograms per milliliter (pg/mL). PAPP-A was assessed immunochemically with TRACE (Time Resolved Amplified Cryptate Emission) by the KRYPTOR analyzer (Thermo Fischer, Henningsdorf, Germany). The results are expressed in mIU/L. Routine biochemical parameters were assessed by commercially available kits using certified biochemical techniques with automated analyzers Modular (Roche Diagnostics, Germany) and Beckman Coulter LH750 Hematology analyzer (Beckman Coulter, USA). The eGFR was calculated using the MDRD formula [22]. Blood count and serum concentrations of routine biochemical parameters were determined in fresh samples. For special biochemical analysis, blood was collected into tubes without anticoagulant and was centrifuged for 10 minutes at 1.450 g (4 °C). Sera were stored at -80 ºC until analysis.

*Statistical analysis.* Results of biochemical parameters are expressed as mean±standard deviation (SD). Statistical analysis of group differences was performed by unpaired t-test Mann-Whitney test, and ANOVA (one-way analysis of variance) followed by posttest analysis. Variables with non-normal distributions were log-transformed where appropriate. Association between parameters was determined by using Spearman's or Pearson's coefficients. Stepwise multivariate regression analysis was used to assess independent predictors of studied biomarkers For statistical analyses, the InStat software (GraphPad Software) and Statistics Toolbox™

(MATLAB® software) were used. All results were considered statistically significant at p less than 0.05.

# 4. Results

**PlGF levels in patients with decreased renal function:** PlGF levels were significantly elevated in both CHRI and HD groups compared to healthy subjects, without significant differences between CHRI and HD patients (10.5±3.3 pg/mL in CHRI patients and 11.5±3.4 pg/mL in HD patients versus 8.1±1.8 pg/mL in controls; both $p < 0.0001$ versus controls) (Fig1).

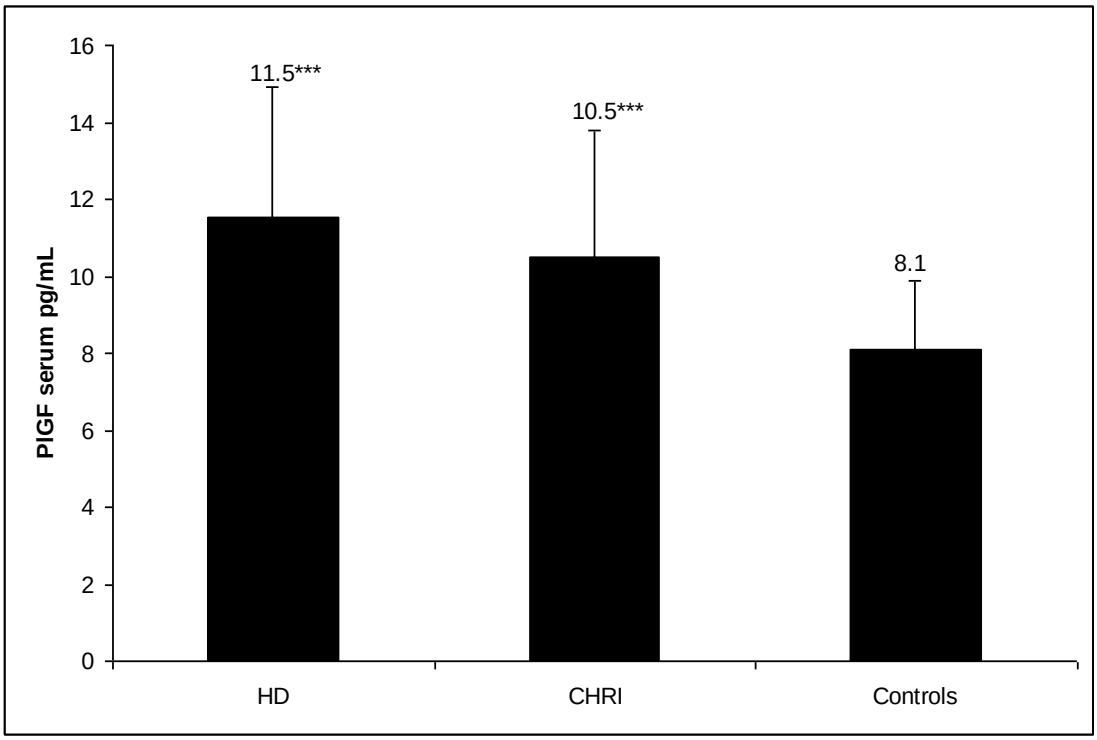


Fig. 1 Serum levels in CHRI, HD patients and healthy controls. Results expressed as mean ± SD. *** $p < 0.0001$, HD and CHRI versus controls

In 15 of 19 patients with nephropathy PlGF was detectable in urine samples (mean 5.8±5.4 pg/mL) and correlated with its serum levels $r=0.45$, $p < 0.05$. Higher levels of PlGF were found in CHRI patients with CVD, compared to those free of such complication (12.1±3 pg/mL versus 10.0±3.2 pg/mL, $p=0.03$). We found that PlGF in CHRI group correlated significantly with triglyceride concentrations ($r=0.32$, $p < 0.05$), and in HD group correlated significantly with low density lipoprotein concentrations ($r=0.36$, $p < 0.05$). PlGF also correlated with age in CHRI group ($r=0.35$, $p=0.02$), correlations in controls ($r=0.30$) was of borderline significance ($p=0.06$). PlGF was not associated with CRP levels in any of the studied groups.

**EN-RAGE levels in patients with decreased renal function:** S100A12 levels were not different in CHRI and HD patients compared to controls (166±140 ng/ml in CHRI patients and

127±101 ng/ml HD patients versus 126±106 ng/ml in controls, p=0.27, non-significant). Comparing patients with higher and lower CRP levels, higher levels of S100A12 were found in patients with higher CRP values in both studied groups. In 17 of 19 patients with nephropathy S100A12 was detectable in urine samples (mean 453.1±445.6 ng/ml) and correlated with serum creatinine levels r=0.53, p = 0.03. We performed a stepwise multivariate regression analysis after adjustment for age of contributing factors predicting S100A12 levels: orosomucoid in CHRI patients; CRP, leukocyte count, fibrinogen and negatively sRAGE in HD patients, and leukocyte count in healthy controls.

**Determinants of circulating MMP-2 and PAPP-A in patients with CKD:** Mean serum MMP-2 concentrations at different stages of CKD were 228 ± 99 ng/mL. Compared with healthy controls, MMP-2 levels (195 ± 76 versus 255 ± 77 ng/mL, $p < 0.0001$) were significantly lower in CKD patients 1-2. Mean serum PAPP-A levels at different stages of CKD were 10.3 ± 7.5 mIU/L. From CKD 4 we noted an increase in PAPP-A levels (12.1 ± 8.5 versus. 9.3 ± 2.2 mIU/L $p = 0.001$). Multivariate analysis revealed that PAPP-A ($p < 0.0001$), proteinuria ($p = 0.001$) (Fig.2), alpha-2-macroglobulin ($p = 0.01$), and negatively albumin ($p = 0.02$), haemoglobin ($p = 0.0002$) were independent correlates of MMP-2 after adjustment for age and eGFR ($R^2 = 0.45$).

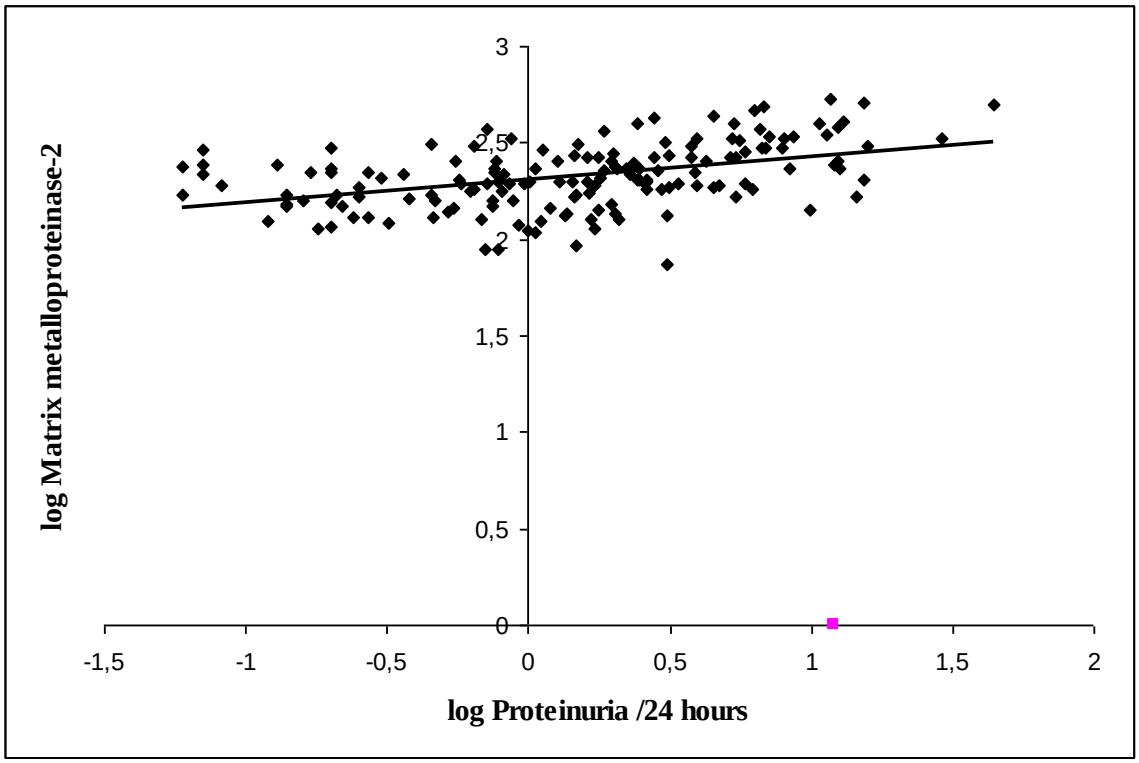


Fig. 2. Correlation of MMP-2 and proteinuria in CKD 5 patients ($r = 0.52$, $p < 0.0001$).

Proteinuria (p = 0.02), creatinine (p < 0.0001), and negatively albumin (p = 0.01) were independent correlates of PAPP-A adjusted for age and eGFR ($R^2$ = 0.25).

**Changes of levels of MMP-2, MMP-9, and PAPP-A in patients with various nephropathies**

Mean serum levels of total MMP-2, total MMP-9, and PAPP-A in the six groups of chronic nephropathies and controls are shown in Table 1.

Table 1

| | Controls | IgAN | MN | MCNS/ FSGS | LN | AAV |
|---|---|---|---|---|---|---|
| MMP-2 ng/ml, (p vs controls) | 255 ± 77 | 183 ± 89 (0.0003) | 237 ± 61 (0.32) | 257 ± 116 (0.95) | 183 ± 64 (0.0003) | 229 ± 64 (0.12) |
| MMP-9 ng/ml, (p vs controls) | 430 ± 280 | 830 ± 550 (< 0.0001) | 800 ± 550 (0.0005) | 600 ± 391 (0.06) | 220 ± 160 (0.002) | 570 ± 400 (0.0.8) |
| PAPP-A mIU/l, (p vs controls) | 9.3 ± 2.2 | 8.2 ± 3.5 (0.08) | 10.2 ± 6.4 (0.41) | 13.1 ± 9.9 (0.65) | 7.1 ± 2.4 (0.0005) | 10.5 ± 3 (0.06) |

Data expressed as mean ± SD.

Abbreviations: AAV – anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, FSGS – focal segmental glomerulosclerosis, IgAN – Immunoglobulin A associated glomerulonephritis, MCNS – minimal change nephrosis disease, MN – membranous glomerulonephritis, MMP-2 – matrix metalloproteinase-2, MMP-9 – matrix metalloproteinase-9, LN – lupus nephritis, PAPP-A – pregnancy-associated plasma protein-A

Compared with controls, IgAN patients exhibited a significant decrease in serum levels of MMP-2 contrasted with increased MMP-9 and unchanged PAPP-A levels. In LN patients exhibited a parallel significant decrease in serum MMP-2, MMP-9 and PAPP-A levels. In the MCNS/FSGS group, unchanged MMP-2, MMP-9 and PAPP-A levels were observed. In MN patients, significantly increased MMP-9 levels contrasted with unchanged MMP-2 and PAPP-A levels. In AAV patients, unchanged serum levels of MMP-2, MMP-9 and PAPP-A were found (all p< 0.05).

In all GN groups, eGFR values inversely correlated with serum MMP-2 (r= - 0.36, p < 0.0001) and PAPP-A (r= -0.32, p < 0.0001) concentrations.

**Evaluation of novel biomarkers PlGF, PAPP-A, sRAGE, EN-RAGE and HMGB-1 in patients with acute kidney injury**

Serum PlGF, PAPP-A, sRAGE, EN-RAGE, and HMGB-1 determined from blood was obtained in AKI, CKD 5, HD and control groups. PlGF was not increased in AKI (11.7 ± 7.4 pg/mL) compared with controls (8.5 ± 2.4 pg/mL, n.s.), but was elevated (p < 0.05) in HD

(11.5 ± 3.8 pg/mL, $p < 0.05$) versus controls. PAPP-A was elevated in AKI (20.0 ± 16.9 mIU/L) CKD 5 (20.2 ± 28.1 mIU/L) and HD (20.8 ± 10.1 mIU/L) compared with controls (9.1 ± 2.3 mIU/L, $p < 0.001$). sRAGE was not elevated in AKI (2400 ± 1400 pg/mL) compared with controls (1760 ± 730 pg/mL, n.s), but was lower compared with CKD 5 (3200 ± 1500 pg/mL, $p < 0.05$). sRAGE was increased in CKD 5 (3200 ± 1500 pg/mL) and HD (2700 ± 1200 pg/mL) versus controls. EN-RAGE was elevated in AKI (480 ± 450 ng/mL) in comparison with controls (60 ± 62 ng/mL), CKD 5 (190 ± 120 ng/mL), and HD (120± 100 ng/mL), all $p < 0.001$. Similarly, HMGB-1 was increased in AKI (5.8 ± 7.5 ng/mL) versus controls (1.7 ± 1.4 ng/mL), CKD 5 (3.2 ± 3.1 ng/mL) and HD (2.5 ±2.1 ng/mL), all $p< 0.001$, as well as HMGB-1 was higher in CKD 5 and HD in comparison with controls.

In AKI group, sRAGE levels were inversely correlated with haemoglobin ($r = - 0.44$, $p = 0.001$). In multivariate regression analysis: PAPP–A levels were associated with transferrin ($p <0.001$), negatively with albumin ($p < 0.01$) and prealbumin ($p < 0.05$); PlGF levels were associated with C - reactive protein ($p < 0.001$). EN-RAGE levels were associated with ferritin ($p < 0.01$) and orosomucoid ($p=0.02$) and HMGB-1 levels with leukocyte count ($p < 0.01$) and negatively with proteinuria ($p = 0.02$) (Fig. 3).

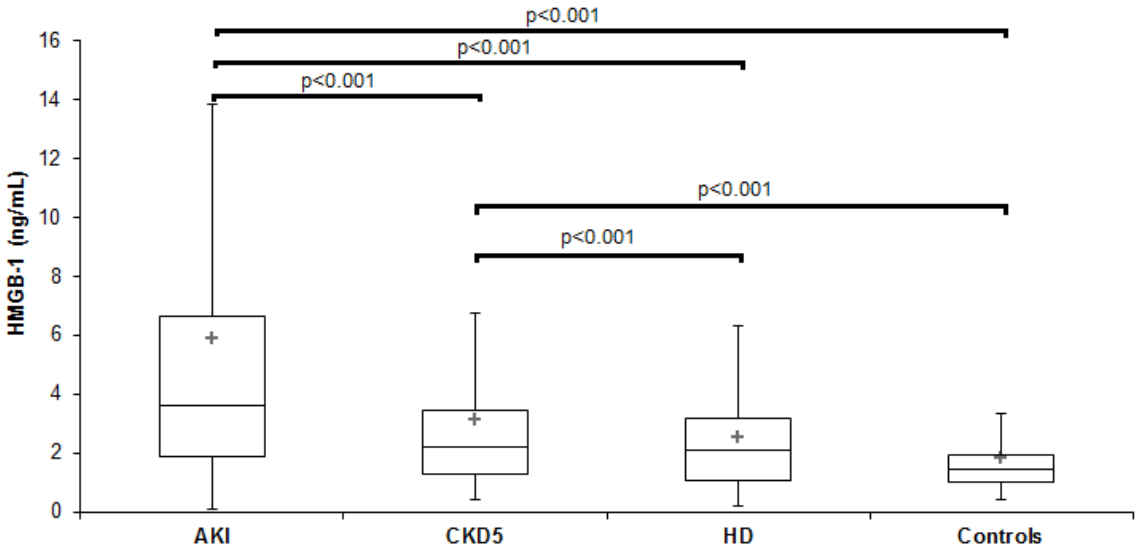


Fig. 3 Serum HMGB-1 levels of AKI, CKD 5 , HD patients and healthy subjects.

# 5. Discussion

### PlGF in patients with decreased renal function

In this study in 45 patients with CKD with different degrees of renal insufficiency not-yet-dialyzed and 31 hemodialysis patients we noted elevated PlGF levels compared to healthy

controls. The serum levels of PlGF in HD patients do not differ from those PlGF concentrations in patients with various degrees of decreased renal function not-yet-dialyzed. Additionally, this is the first study where PlGF levels were measured simultaneously in serum and urine in patients with decreased renal function. PlGF is present in the urine of patients with renal impairment and serum and urine concentrations of PlGF are significantly interrelated in patients with decreased renal function. Given that PlGF is a small dimeric protein with a molecule (≈ 50 kDa) [23], it is readily filtered into urine even in the absence of renal damage [24]. Serum PlGF levels in non-pregnant subjects (both males and females) are much lower in comparison with pregnant women. PlGF-mRNA is present in very small amounts in heart, lung, thyroid, goiter, and skeletal muscle. It is not expressed, however in kidney and pancreas [25].

In addition, our study showed higher levels of PlGF in CKD patients not-yet-dialyzed with CVD compared to those patients without CVD. This finding suggests that PlGF might be an indicator of CVD and atherosclerotic complications in patients with decreased renal function. This finding is in line with finding of Onoue et al. [7], where the PlGF/sFlt-1 ratio was significantly higher in patients with multivessel coronary artery disease than in patients with single-vessel or no coronary disease.

This study suggested that elevated PlGF levels might be associated with subsequent risk of coronary heart disease. In our study we found a modest relation between plasma PlGF levels and low density lipoproteins in hemodialysis patients and triglyceride concentrations in patients with chronic kidney disease. The finding that PlGF is linked to classic risk factors of atherogenesis in our cohort of patients with decreased renal function is a novel finding suggesting that this growth factor might play a role in atherosclerosis in these patients.

**Serum S100A12 (EN-RAGE) levels in patients with decreased renal function and subclinicial chronic inflammatory disease**

This study shows that levels of S100A12 were not different in not-yet-dialyzed CKD patients with different degrees of renal insufficiency and HD patients compared to healthy controls. The patients were all in stable clinical status at the time of the study without signs of acute infection or acute cardiac problems. However, in a subgroup of patients with higher CRP levels in contrast to the whole studied population the S100A12 values were increased. Thus, we suggest that although serum S100A12 levels are not increased at basal conditions, the presence of subclinical inflammation though is likely to result in the higher levels of S100A12 in CKD and HD patients. In addition, we detected S100A12 in the urine of CKD patients which correlated with serum creatinine, but not with serum S100A12 levels.

In line with previous report [26] our study confirms that serum sRAGE levels are elevated in patients with decreased renal function in both patients with CKD and HD patients as compared with those in healthy controls. Also the S100A12/sRAGE ratio appears to be more favorable in CKD and HD patients than in the controls. sRAGE concentrations increase with declining renal function and significantly decrease after renal transplantation [27]. In HD patients, levels of sRAGE are elevated in comparison with healthy subjects, and might modulate vascular and inflammatory reaction during dialysis. Other causes such as stimulation of neutrophils on dialysis membrane, puncture of arteriovenous fistula, and heparin administration might participate in increased serum S100A12 levels in HD patients [28].

In stepwise multivariate regression analysis after adjustment for age, S100A12 remained correlated with inflammatory markers in all studied groups: orosomucoid in CHRI patients; CRP, leukocyte count, fibrinogen and inversely sRAGE in HD patients; and leukocyte count in healthy controls. It is conceivable that S100A12 might contribute to some of the risk for inflammation associated with renal impairment. These correlations of serum S100A12 levels with inflammatory parameters support the possibility that S100A12 might be involved in an inflammatory process of CKD and HD patients. Interaction of EN-RAGE (S100A12) with cellular RAGE on the endothelium, mononuclear phagocytes, and lymphocytes triggers cellular activation with generation of key proinflammatory mediators [17]. Recently, Nakashima et al. [29] showed that S100A12 and sRAGE are elevated and have opposite associations with inflammation in prevalent HD patients.

In accordance with the study of Basta et al. [30], we also found a close association between hsCRP and serum S100A12 levels in CKD patients. Hasegawa et al. [31] have demonstrated that levels of mRNA/protein of S100A12 were enhanced by another pro-inflammatory molecule i.e. IL-6 in cultured THP-1 cells. Atherosclerosis has an inflammatory aetiology and elevated CRP in patients with atherosclerosis not only serves as a biomarker for cardiovascular disease risk but it also functions as an active mediator of atherosclerosis by its direct proatherogenic effects on vasculature [32]. Moreover, CRP and orosomucoid were shown to be the inflammatory markers in different stages of CKD [33]. Mahajan N et al. [34] suggested that CRP is able to augment mRNA expression of both RAGE and S100A12 genes. Augmented expression of S100A12 in the presence of CRP points to close correlation between S100A12 and activation of granulocytes and monocytes under inflammatory conditions. Also orosomucoid displays several activities on one cell type: on neutrophils it influences chemotaxis, superoxide generation and aggregation [35]. Orosomucoid, being an acute-phase reactant, contributes to the

general function of the acute phase response as a coordinated system that modulates host immune response during periods of intense inflammation and tissue destruction.

**Biochemical determinants of PAPP-A and MMP-2 in patients with CKD**

Three important findings emerge from our study. First, there were not marked significant differences in the serum levels of MMP-2 between patients with different levels of kidney function and healthy controls. On the other hand, serum PAPP-A levels are increased from CKD 4, whereas in CKD 1-3 they are not different. Second, and more importantly, serum MMP-2 and PAPP-A levels are the independent correlates of proteinuria in CKD patients. Third, MMP-2 and PAPP-A are interrelated and correlate negatively with albumin, MMP-2 is also related to alpha-2-macroglobulin, a MMP inhibitor. These observations suggest that MMP-2 and PAPP-A are associated with proteinuria, indicating a contribution of these proteases to renal and vascular damage in patients with CKD.

Although, MMP-2 and PAPP-A correlated with estimated GFR, MMP-2 levels were not different between patients with different levels of renal function in this study. PAPP-A values were increased in late stages of CKD only. Indeed, a number of studies evaluated MMPs in patients with CKD [36, 37]. Recently, it was indicated that serum levels of MMP-2 are one of the independent correlates of proteinuria and could be associated with intima media thickness and atherosclerotic plaque in patients with CKD. Increased PAPP-A levels are known to reflect changes in renal function [38] and could be a prognostic marker in dialysis patients [10, 39]. The association of PAPP-A and serum creatinine in our multivariate model supports this notion. Also, it was suggested that PAPP-A may be produced by activated cells in atherosclerotic plaques and released to ECM [9]. The changes in proteases that occur in CKD patients may mediate both degradation of extracellular matrix components and cell proliferation and facilitate leukocyte function [12, 40].

Several factors were associated with MMP-2 and PAPP-A levels in our multivariate model; among them is proteinuria, supporting the concept that MMP-2 and PAPP-A are involved in proteinuria and suggesting that MMP-2 and PAPP-a levels could be markers of renal injury in patients with CKD. The novelty of our observation was the fact that serum levels of PAPP-A were associated with proteinuria in CKD patients. It is well known that proteinuria represents an independent risk factor for poor outcome in most types of glomerular disease and is related to the severity of tubulointerstitial injury [41]. In this study, both serum MMP-2 and PAPP-A were negatively associated with albumin. Indeed, glomerular basal membrane is the site where albumin is lost into urine in CKD; it is also a site for MMP related damage and activation of inflammation and promotion of glomerular sclerosis, fibrosis, and hypertrophy in CKD. Over-

expression of MMP-2 and MMP-9 is related with progressive kidney damage. It was reported that albumin up-regulated the expression of MMP-2 and MMP-9 in podocytes at gene and protein levels in a time and dose dependent manner [42].

Another independent determinant of MMP-2 levels in this cohort of patients is alpha-2-macroglobulin. Alpha-2-macroglobulin is a known MMP inhibitor [13, 43]. MMPs bound to alpha-2-Macroglobulin are unable to act on protein substrates. Alpha-2-Macroglobulin is also an inflammatory, acute-phase protein.

Another interesting finding in this study is the interrelationship between MMP-2 and PAPP-A in CKD patients (1-5) not yet dialyzed. This association might represent a marker of an enhanced inflammatory state in asymptomatic CKD patients.

We observed a close association between declining haemoglobin and MMP-2. To the best of our knowledge, we are the first to describe this association. It is not clear how haemoglobin levels influence either the production or clearance of MMP 2. Haemoglobin is a major component of blood and a potent mediator both of oxidative stress and antioxidant function. Reduced tissue oxygenation associated with anaemia may elicit gelanolytic activity of MMP-2. This association raises a possible role of declining haemoglobin in kidney injury partly through MMP-2 activation.

**Changes in serum levels of MMP-2, MMP-9 and PAPP-A in various nephropathies**

We found that patients with various nephropathies have significant, marked differences in the serum levels of MMP-2, MMP-9 and PAPP-A compared with healthy subjects. Moreover, serum patterns of MMPs and PAPP-A considerably differed between various histopathological types of glomerulonephritis and thus seems to be characteristic of each type of GN. Furthermore, with the exception of MMP-9, serum MMP-2 and PAPP-A concentrations inversely varied with the levels of renal function.

In IgAN patients, we observed decreased levels of MMP-2 contrasting with increased MMP-9 level as compared with healthy controls. PAPP-A levels in this study were comparable with those found in healthy controls. In MN patients, we observed an increase in serum MMP-9, whereas levels of serum MMP-2 and PAPP-A were not different in comparison with healthy control subjects. Though previous study [44] reported increased MMP-2 and decreased MMP-9 level, the finding of increased MMP-9 serum concentrations in human MN nephritis is a novel finding, implicating that MMP-9 may play a role in glomerular disease in membranous nephropathy. In our MCNS/FSGS nephrosis group, the serum MMP-2 and PAPP-A levels were unchanged, and there was a trend of higher serum MMP-9 concentrations as compared with controls. Our data support the notion that also in MCGN/FSGS group the specific changes in

serum levels of MMPs and PAPP-A are distinct from those found in other patients with chronic nephropathy in this study. In LN patients, we observed decreased levels of serum MMP-2, MMP-9 and PAPP-A levels as compared with healthy controls. These findings support the notion that levels of circulating MMPs fluctuate in SLE, and raised levels of MMPs probably reflect an increased inflammatory process, whereas lower concentrations of MMP-2, MMP-9 and PAPP-A can result from the accumulation of MMPs in inflamed blood vessels and tissues. In AAV patients, the levels of serum MMP-2, MMP-9 and PAPP-A were comparable with those found in healthy control subjects. There was a trend of higher serum levels of PAPP-A in AAV patients.

In the present study, an inverse correlation between both serum levels of MMP-2 and serum concentrations of PAPP-A, on one hand, and the degree of renal dysfunction as evaluated by eGFR, on the other, was found in the whole GN cohort. There was no correlation between serum MMP-9 and eGFR. In addition, serum PAPP-A was loosely correlated with eGFR in all GN groups.

**Evaluation of novel biomarkers PlGF, PAPP-A, sRAGE, EN-RAGE and HMGB-1 in patients with acute kidney injury**

This is the first study where we demonstrate the circulating levels of PLGF, PAPP-A, sRAGE, EN-RAGE and HMGB-1 levels in patients with AKI requiring RRT. Significantly higher levels of PAPP-A, EN-RAGE and HMGB-1, but not increased levels of sRAGE and PlGF were observed in the serum of patients with AKI as compared with controls. Further, this study demonstrates significant independent associations of PAPP-A with markers of nutrition, and the associations of PlGF, EN-RAGE, and HMGB-1 with inflammatory parameters in these patients for the first time.

Although PlGF levels in AKI patients were not elevated, PlGF was significantly correlated with inflammatory markers CRP and fibrinogen and inversely with a negative inflammatory marker prealbumin. However, only CRP was positively associated with PlGF levels by multivariate analysis. A recent study has suggested that the level of the ratio of CRP to prealbumin was associated with mortality of AKI patients [45]. Moreover, lower serum prealbumin levels were strongly associated with a higher risk of death independent of AKI severity [46]. On the other hand, serum fibrinogen is independently predictive of cardiovascular and all-cause mortality in end-stage kidney disease [47] and in patients with CKD [48]. In AKI serum fibrinogen levels were comparable with those found in healthy controls [49]. It is thus conceivable that PlGF is released from endothelial cells, among others, in response to inflammation in AKI.

PAPP-A levels were increased in AKI patients in comparison with healthy controls, but were comparable to those found in CKD 5 and HD patients. In line with previous report, PAPP-A is elevated in HD patients [50] and is a prognostic marker in dialysis patients [10]. The PAPP-A levels were also significantly decreased in dialysis patients after successful kidney transplantation, but remained higher than in control group [51]. The mechanisms of PAPP-A increase most probably include the increased synthesis, but also the decreased clearance of PAPP-A in patients with decreased renal function, including the patients with AKI. In this study, PAPP-A levels were independently associated with markers of nutrition: transferin and negatively with albumin and prealbumin. These results permit the conclusion that PAPP-A levels are elevated in patients with AKI and related to markers of nutrition, but are not related to inflammatory markers, as in HD patients in this and previous studies [38].

We provide here evidence that sRAGE levels are increased but not significantly in the setting of AKI. An explanation for the comparable sRAGE levels in AKI might be an enhanced consumption of this molecule. sRAGE acts as an anti-inflammatory "decoy'' by binding and preventing their interaction with cell surface RAGE, suppresses the RAGE mediated inflammatory response [26]. The ligands EN-RAGE and HMGB-1 binding to sRAGE might influence the levels of sRAGE and increase the propensity towards inflammation. RAGE ligands therefore have better binding across to cell membrane receptor, the binding of which activates the inflammatory pathways. Interestingly, in a recent study in septic AKI patients sRAGE levels were elevated [52]. In CKD and HD patients serum sRAGE levels were also increased in this and the previous study and was inversely related to inflammation [53]. The correlation revealed in our AKI patients between serum sRAGE levels and declining haemoglobin suggest that reduced tissue oxygenation associated with anaemia may contribute to the formation of AGEs and activation of RAGE with possible toxic effect of them on haematopoiesis, while sRAGE might inhibit their pathological effect.

In the present study, EN-RAGE levels were significantly increased in AKI patients, but not in CKD5 and HD patients. These results are in line with our previous study where the serum concentrations of CKD patients and HD were not elevated in comparison with healthy controls [54]. Similarly as in CKD, HD and peritoneal dialysis patients [55] also in AKI patients a relation of serum EN-RAGE levels to markers of inflammation was found. Specifically, EN-RAGE concentrations were independently associated with orosomucoid and ferritin.

Plasma EN-RAGE triggers the RAGE pathway as proinflammatory ligand activating key inflammatory signals such as NF-κβ and MAP kinase and stimulates cell adhesion molecules. Circulating EN-RAGE is associated with CVD events and CVD-related mortality in HD patients,

which partly explained by its link to inflammation [56], and is related to mortality of HD patients due to infection [57]. Orosomucoid, being an acute phase protein, contributes to immune response in inflammatory states modulating chemotaxis of neutrophils, superoxide generation and aggregation [35]. On the other hand, a recent study in a murine model of acute renal failure has shown that orosomucoid partially restored activity of clotting and complement systems in acute renal failure [58]. This effect may be due to accumulation of orosomucoid in renal tissue and its protective action in situ.

In the present study all AKI patients in our study had elevated circulating HMGB-1 levels as compared with controls. We could also show that HMGB-1 levels were independently associated with leukocyte count and negatively with proteinuria in AKI setting. Although, we could not exclude patients with high CRP levels in AKI patients, in multivariate analysis no relationship to CRP levels were found. HMGB-1 is one of the high-affinity ligands for RAGE/sRAGE, a potent cytokine playing an important role in the pathogenesis of inflammation. In addition, passive diffusion from necrotic cells might occur [59]. Another interesting finding is the negative association of HMGB-1 and proteinuria in AKI setting, supporting the concept that HMGB-1 could be a marker of renal injury in patients with AKI.

# 6. Conclusions

- We have observed that PlGF, an endogenous proatherogenic cytokine, is elevated in patients with decreased renal function. PlGF is present in the urine, and its serum and urine levels are interrelated. It is higher in CKD patients with cardiovascular disease.
- We have shown that S100A12 (EN-RAGE), a sensitive and specific marker of localized inflammatory process, is not elevated in patients with decreased renal function at stable clinical status without signs of overt inflammation. Even in these basal conditions, it is significantly related to inflammatory markers. In addition, this study also shows that S100A12 is present in the urine, and urine levels are higher in comparison with those in serum and correlate with renal function
- We have demonstrated that increased levels of MMP-2 are associated with PAPP-A, alpha-2-Macroglobulin, proteinuria, lower haemoglobin and lower albumin in CKD (1-5) patients of various nephropathies not yet undergoing dialysis. Similarly, the determinants of PAPP-A in CKD (1-5) patients are proteinuria, serum creatinine, and lower albumin. Both MMP-2 and PAPP-A are associated with proteinuria, a significant marker of renal damage and an independent risk marker for CVD as well
- We have provided the first insight into levels of circulating PlGF, PAPP-A, sRAGE, EN-RAGE and HMGB-1 in patients with AKI. The PAPP-A, EN-RAGE and HMGB-1 levels are significantly elevated, but sRAGE and PlGF levels are not increased in AKI patients. Whereas PlGF, EN-RAGE, and HMGB-1 levels are significantly related to inflammatory markers, PAPP-A levels are associated with markers of nutrition in AKI setting

This thesis demonstrated potential clinical use of several candidate biomarkers for risk stratification in patients presenting with CKD, AKI, ESRD treated by HD and for improved understanding of the biochemistry and pathophysiology of patients with reduced renal function. Concerns that have been addressed for biomarkers reflecting pro-inflammatory, anti-inflammatory, metabolic, nutritional and proatherogenic factors will need to be validated in future studies.

# 7. References


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List of original articles

## 1. Publications *in extenso* related to the thesis

### a) with IF

1. Zakiyanov O**,** Kalousová M, Zima T, Tesař V. Placental growth factor in patients with decreased renal function. Ren Fail. 2011;33(3):291-7. **IF: 0.824**

2. Zakiyanov O**,** Kalousová M, Kříha V, Zima T, Tesař V. Serum A100A12 (EN-RAGE) levels in patients with decreased renal function and subclinical chronic inflammatory disease. Kidney Blood Press Res 2011;34:457–464 **IF: 1.464**

3. Zakiyanov O**,** Kalousová M, Kratochvilová M. Kříha V, Zima T, Tesař V. Determinants of Circulating Matrix Metalloproteinase-2 and Pregnancy-Associated Plasma Protein-A in Patients with Chronic Kidney Disease. Clin Lab 2012; 58(5-6):471-480. **IF: 0.920**

4. Zakiyanov O**,** Kalousová M, Kratochvilová M. Kříha V, Zima T, Tesař V. Changes in levels of matrix metalloproteinase-2 and -9, pregnancy-associated plasma protein–A in patients with various nephropathies. J Nephrol 2013; 26 (3):502-509. **IF: 1.640**

5. Zakiyanov O, Kříha V, Vachek J, Zima T, Tesař V, Kalousová M. Placental growth factor, pregnancy associated plasma protein – A, soluble receptor for advanced glycation end products, extracellular newly identified receptor for receptor for advanced glycation end products binding protein and high mobility group box 1 levels in patients with acute kidney injury: a cross sectional study. BMC Nephrol 2013; 14 (1):245 **IF: 1.640**

## 2. Publications *in extenso* with different objectives

### a) with IF

1. Kratochvilová M, Zakiyanov O, Kalousová M, Kříha V, Zima T, Tesař V. Associations of serum levels of advanced glycation end products with nutrition markers and anemia in patients with chronic kidney disease. Ren Fail. 2011;33(2):131-7. **IF: 0.824**

**b) without IF**


1. Zakiyanov O., Mertová J., Šaková R., Polakovič V. Maligní hypertenze jako závažná komplikace koarktace aorty diagnostikovaná v pokročilém věku. Prakt. Lék. 2005, 85 (9):503-505

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3. Vachek J, Zakiyanov O, Tesař V. Farmakoterapie diabetus mellitus 2. typu u pacientů s chronickým onemocněním ledvin. Kazuistiky v diabetologii 9, č. 5:10-13, 2011

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