SERUM AND URINARY BIOMARKERS DETERMINATION AND THEIR SIGNIFICANCE IN DIAGNOSIS OF KIDNEY DISEASES
ODREĐIVANJE BIOMARKERA U SERUMU I MOKRAČI I NJIHOV ZNAČAJ U DIJAGNOSTICI BOLESTI BUBREGA

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Summary: Chronic kidney disease (CKD) is becoming a major public health problem worldwide due to the epidemic increase of patients on renal replacement therapy and their high cardiovascular morbidity and mortality. The only effective approach to this problem is prevention and early detection of CKD. In addition, despite significant improvements in therapeutics, the mortality and morbidity associated with acute kidney injury (AKI) remain high. A major reason for this is the lack of early markers for AKI, and hence an unacceptable delay in initiating therapy. Therefore, there is a pressing need to develop biomarkers (proteins and other molecules in the blood or urine) for renal disease, which might assist in diagnosis and prognosis and might provide endpoints for clinical trials of drugs designed to slow the progression of renal insufficiency. Besides serum creatinine, promising novel biomarkers for AKI include a plasma panel (neutrophil gelatinase-associated lipocalin-NGAL and cystatin C) and a urine panel (NGAL, kidney injury molecule-1, interleukin-18, cystatin C, alpha1-microglobulin, Fetuin-A, Gro-alpha, and meprin). For CKD, these include a similar plasma panel and a urine panel (NGAL, asymmetric dimethylarginine, and liver-type fatty acid-binding protein). Increased plasma and urinary TGF-β1 levels might contribute to the development of chronic tubulointerstitial disease, indicating the possible therapeutic implications. Furthermore, to differentiate lower urinary tract infection and pyelonephritis interleukin-6 and serum procalcitonin levels were introduced. It will be important in future studies to validate the sensitivity and specificity of these biomarker panels in clinical samples from large cohorts and in multiple clinical situations.

Keywords: chronic kidney disease, acute kidney injury, biomarkers in the blood or urine

Introduction

Chronic kidney disease (CKD) is a worldwide public health problem with an increasing incidence and prevalence, poor outcome and high treatment costs. Due to the asymptomatic nature of the disease,
CKD is frequently detected in its advanced stage, resulting in lost opportunities for influencing on the course and outcome of the disease. On the other hand, as acute kidney injury (AKI) is characterized by high mortality despite the permanent improvement of AKI treatment, the investigations of methods that could have beneficial effects on AKI outcome continue. Progression of AKI to CKD and CKD itself to renal failure or other adverse outcomes could be prevented or delayed through its early detection and treatment (1, 2).

Wide range of insults including infections, toxins, ischaemia, hypertension, genetic or metabolic disorders, autoimmune diseases or allograft rejection can cause kidney damage. The effects of these insults may induce AKI, which is clinically defined as a sudden reduction in renal function or urine output (3), or they may induce CKD, in which kidney structural or functional alterations persist for at least 3 months (4). Biological markers (biomarkers) which identify normal or pathogenic processes are a valuable tool for determining a patient’s condition.

Biomarkers can be used to assess a predisposition towards an illness or detect biological abnormalities, but are more often used to diagnose and measure a pathological condition, to evaluate reaction to particular therapy or to make a prognosis about the disease. Recent studies indicated that particular biomarkers could be used as predictors of AKI, progression of CKD and cardiovascular events. Therefore, the investigation of diagnostic and prognostic value of well-known and new biomarkers for kidney disease is the focus of interest.

The characteristics of a biomarker need to be carefully considered before its potential usefulness can be determined. Some important criteria for selecting renal biomarkers are listed in Table I. Ideally, these biomarkers should be obtainable by procedures which are either non-invasive or have minimal effects on patients (routine blood and urine collections). Consequently, large efforts have been made to identify reliable biomarkers of renal injury in serum, plasma and urine.

Growing number of potential renal biomarkers in the serum and urine of patients and animal models of kidney disease have been identified. Many of these are still awaiting further testing and clinical validation. It is becoming clear, however that these renal biomarkers can be grouped into different categories (Table II), which represent different types of renal injury. These categories are discussed individually below.

### Biomarkers of renal function

Blood urea nitrogen (BUN) and creatinine clearance are routinely established biomarkers of renal function. Both urea and creatinine are products of protein metabolism which are cleared almost entirely by the kidneys. BUN is routinely measured in serum,
however, its levels are affected by non-renal influences such as protein intake, dehydration, liver function, gastrointestinal bleeding and steroid use (5). In addition, BUN assays often underestimate renal function due to interfering chromogens. Creatinine levels in serum and urine can be measured by a variety of assays (Jaffe rate reaction, creatinonase method, HPLC method), but are most commonly assessed by the Jaffe rate reaction, which is cheap and easy to perform. However, HPLC is the most sensitive method for assessing creatinine levels and is not affected by chromogen interference (6). Creatinine levels are also affected by non-renal influences such as muscle mass, age, gender and liver function (5). Creatinine clearance is one of the most common assessments of renal function, but it lacks sensitivity when renal impairment is mild and can be affected by tubular secretion of creatinine when the glomerular filtration rate is declining.

Cystatin-C has recently emerged as a reliable alternative biomarker of renal function. It is a cysteine protease inhibitor which is constantly produced by nucleated cells and released into the blood, where it is normally reabsorbed and catabolised by kidney tubules without re-entering the blood stream (8). Serum levels of cystatin-C can currently be measured by immunonephelometry or ELISA and are affected by steroid use or thyroid dysfunction (8). Cystatin-C is particularly sensitive at detecting changes in kidney function when renal impairment is mild (9), and is better than creatinine for assessment of acute kidney injury due to its shorter half-life (10). In 85 patients at high risk to develop AKI in the ICU, a 50% increase in serum cystatin C predicted AKI one to two days before serum creatinine with AUC of 0.97 and 0.82 respectively (10), and was moderately good predicting the need for renal replacement therapy one day later with an AUC of 0.75 (11). However, like other functional markers, cystatin C cannot differentiate among different causes of AKI or CKD.

Some other potential biomarkers of renal function are also worthy of note. *Uric acid* is normally excreted through the kidney but circulating levels increase during renal impairment in CKD. Animal model studies have shown that hyperuricaemia activates the renin angiotensin system (RAS), induces oxidative stress and reduces renal function (12). Increased levels of serum uric acid have been detected in patients with CKD by colorimetric assay and predict a greater risk of end stage renal disease (ESRD) (12). Urinary levels of angiotensinogen detected by ELISA have been reported to be a specific index of the intrarenal RAS and correlate with blood pressure and glomerular filtration rate in CKD (13). Therefore, urine angiotensinogen appears to be a potential biomarker of renal function in kidney diseases which are dependent on hypertension.

The *fractional excretion of magnesium* (FE Mg) is considered to be a measure of tubular function because tubules normally reabsorb magnesium filtered by glomeruli (9). Levels of magnesium can be measured in serum and urine by atomic absorption spectroscopy. Elevations in the FE Mg are thought to indicate the loss of peritubular capillary flow resulting from tubulointerstitial damage (9).

**Biomarkers of renal oxidative stress**

Oxidative stress is known to play a pathological role in animal models of CKD (15). Increased oxidative stress is also present in patients with moderate to severe CKD (16). Some serum and urine biomarkers have been shown to reliably measure the level of renal oxidative stress in patients and animal models. During oxidative stress, oxidized guanine in cellular DNA is spliced out by DNA repair enzymes, releasing a metabolically stable product 8-hydroxy-2-deoxyguanosine (8-OH-dG) into the urine. Increased levels of 8-OH-dG can be detected in urine by ELISA during CKD (17). Peroxidation of lipids also occurs during oxidative stress, resulting in the formation of 8(F2a)-isoprostane and 4-hydroxy-2-nonenal. Levels of 8-isoprostane and 4-hydroxy-2-nonenal can be measured in serum or urine by ELISA or HPLC and are elevated in CKD (18–20). In addition, renal oxidative stress produces peroxynitrite which nitrates protein tyrosine residues to form stable 3-nitrotyrosine (3-NT) peptides. A recent study has indicated that levels of 3-NT peptides can now be accurately measured in serum or urine using liquid chromatography and mass spectroscopy, which may prove to be useful for assessing both oxidative and nitrosative stress in kidney disease (21).

**Advanced glycation end products (AGEs)** are proteins modified by oxidative stress which accumulate in the blood of diabetic and uremic patients. These circulating AGEs can deposit in the kidney and cause cellular dysfunction and renal damage. Elevated serum and urine levels of the AGE pentosidine can be detected by HPLC or ELISA and help to predict the development of diabetic nephropathy (20). In addition, plasma levels of pentosidine have been shown to increase with loss of residual renal function in patients on peritoneal dialysis and to decrease with patients recovering renal function after transplantation (22, 23).

**Biomarkers of kidney structural and cellular injury**

The excretion rate of albumin is the most commonly used biomarker of renal injury. Albumin is the most abundant protein in the circulation and during normal kidney function very little intact albumin is excreted by the kidney (<30 mg/day in humans). However, following renal injury, glomerular filtration of albumin is increased and the reabsorption and degradation of albumin by tubules is decreased,
resulting in increased levels of intact albumin in the urine. Patient albuminuria is usually classified by ranges of severity, which include: microalbuminuria (30–300 mg/day), macroalbuminuria (300 mg – 3 g/day) and nephritic range proteinuria (>3.5 g/day).

Albuminuria is commonly used as an early marker of renal injury because it often precedes a decline in renal function. However, it cannot distinguish different types of proteinuric kidney disease and has a limited ability to predict disease progression and determine therapeutic efficacy. Albuminuria is commonly measured by immunological techniques, which include: immunonephelometry, immunoturbidimetry, radioimmunoassay and ELISA (24). These techniques are good for assessing albumin excretion which is distinctly higher than normal. However, newer HPLC based methods (Accumin Test) can identify both immunoreactive and nonimmunoreactive albumin providing greater sensitivity than conventional immunological methods for distinguishing microalbuminuria from normal albumin excretion (25, 26).

Podocyte injury is a feature of many kidney diseases which is postulated to increase glomerular filtration of albumin. Severely damaged podocytes can detach from the glomerular basement membrane and be collected in the urine sediment. Analysis of the urine sediment by quantitative PCR or ELISA can determine mRNA or protein levels of podocyte-specific molecules (eg. nephrin, podocin, podocalyxin) as markers of podocyte injury. Increased urine sediment levels of nephrin and podocin have been detected in patients with diabetic nephropathy and active lupus nephritis (27, 28). Similarly, increased levels of podocalyxin have been found in the urine sediment of patients with IgA nephropathy, lupus nephritis and post-streptococcal glomerulonephritis (29).

Sensitive markers of tubular injury have been identified in acute and chronic kidney diseases. N-acetyl-beta-D-glucosaminidase (NAG) is a proximal tubular lysosomal enzyme which is released during damage to proximal tubules. Increased urine levels of NAG can be detected by enzymatic assay in kidney diseases which involve tubulo-interstitial damage (30, 31).

Kidney injury molecule-1 (KIM-1) is a transmembrane protein which is expressed on the luminal surface of proximal tubules during injury after ischemic and nephrotoxic injury. Increased urine levels of KIM-1 can be detected by ELISA, microbead-assay or immunochromatographic dipstick in patients with tubulo-interstitial damage and correlate with renal expression (31–33). In a preliminary study of 40 patients, only 9 of which had AKI, urinary KIM-1 could distinguish ischemic ATN from pre-renal azotemia and CKD. It was not detected in contrast nephropathy (34). In a modestly larger unpublished study of 67 subjects presented at ASN in 2005, KIM-1 could distinguish AKI from CKD and normal with an AUC of 0.94 (35). In 47 patients undergoing abdominal aortic aneurysm repair, KIM-1 increased 12 hrs after surgery but well before serum creatinine, and also modestly predicted the need for dialysis even after adjustment for disease severity and gender (36, 37).

Liver type-fatty acid binding protein (L-FABP) is a marker which is shed by proximal tubular cells in response to hypoxia from decreased peritubular capillary flow. Urine levels of L-FABP are a sensitive indicator of acute and chronic tubulointerstitial injury (38, 39). In chronic kidney diseases, increasing urine levels of L-FABP correlate with declining renal function (39). In an animal model of cisplatin-induced AKI, there was increased shedding of urinary L-FABP within the first 24 hours, whereas a rise in serum creatinine was not detectable until after 72 hours of cisplatin treatment (40). In a study involving liver-related kidney transplant patients immediately after reperfusion of their transplanted organs, a significant direct correlation was found between urinary L-FABP level and both peritubular capillary blood flow and the ischemic time of the transplanted kidney (41).

L-FABP expression and urinary excretion are also increased in the setting of CKD. In subjects with nondiabetic CKD, urine L-FABP levels correlated with urine protein and serum creatinine levels. Notably, L-FABP levels were significantly higher in the group of patients who progressed to more severe disease (42). Additional studies are needed to demonstrate LFABP’s ability to predict CKD and its progression in cohorts with CKD of multiple etiologies.

Neutrophil gelatinase associated lipocalin (NGAL), also known as lipocalin-2, is an iron-transporting protein which is almost entirely reabsorbed by tubules in the normal kidney. NGAL levels in the urine increase following acute nephrotoxic and ischemic insults, indicating defects in proximal tubular reabsorption and the distal nephron (43). Urine levels of NGAL can be measured by ELISA and are a very sensitive marker of acute kidney injury which can increase up to 1000-fold in patients (44). Urinary NGAL has also been used as a triaging tool to randomize patients with AKI to treatment (45). In addition, serum and urine NGAL levels have been found to be independent risk markers of CKD (46). Rise in serum NGAL was observed as early as 2 hours after PCI and lasted up to 4 hours. Urine NGAL increased after 4 hours and remained significantly elevated in comparison to baseline even 48 hours after PCI without changes in serum creatinine (47). Additionally, in a prospective study of children receiving cardiopulmonary bypass, urinary NGAL levels greater than 50 μg/L 2 hours after surgery had 100% sensitivity and 98% specificity for the subsequent diagnosis of AKI (48). These findings were confirmed in a prospective study of adults who had cardiac surgery, in whom the urinary NGAL level increased rapidly after surgery (postoperative AUC, 74% at 3 hours and 80% at 18 hours (49)).
Biomarkers of immune responses within the kidney

Molecular components of humoral immunity (eg. immunoglobulin, complement components) and cellular immunity (eg. chemokines, leukocyte adhesion molecules, proinflammatory cytokines and their soluble receptors) are known to play significant roles in the development of renal inflammation. The serum or urine levels of these molecules can be detected by ELISAs and some have been shown to be sensitive markers of the immune response in the injured kidney.

Urine excretion of immunoglobulins can predict the development of immune-mediated kidney diseases. The fractional excretion of IgG has been shown to predict the progression of primary FSGS and the response of this disease to treatment (50). Similarly, urine levels of IgA can be an indicator of the severity of renal damage in IgA nephropathy and are known to correlate with proteinuria, serum creatinine and glomerulosclerosis in this disease (51). In comparison, urine levels of IgM are a strong predictor of disease progression for patients with anti-nuclear cytoplasmic antibody (ANCA)-associated vasculitis (52). Furthermore, because IgM has a high molecular weight (600 kDa) and is usually not filtered by healthy glomeruli, its levels in urine are a stronger predictor of ESRD than the more readily filtered albumin (68 kDa) in a number of glomerular diseases (53). However, these filtration properties of IgM suggest that it is better associated with advanced glomerular injury and is not a specific or sensitive marker of early renal damage.

Levels of complement C3d, C4d and complement factor H have been identified as potential biomarkers of complement-mediated injury in renal diseases. Increased urine levels of C3d are found in tubulointerstitial nephritis, membranous nephropathy and non-membranous glomerular diseases (54). In patients with glomerular diseases, the urine excretion of C3d correlates with the progression or remission of proteinuria and is independent of the underlying glomerular disease (55). A study has also shown that serum C4d and urine C3d correlate with moderate to severe disease activity in lupus nephritis (56). In addition, urine levels of factor H (a regulator of the alternative pathway of complement) are elevated in patients with IgA nephropathy and idiopathic membranous nephropathy and are associated with disease activity (56, 57).

During a renal inflammatory response, leukocytes are recruited into the kidney by chemokines. The urine levels of some chemokines increase with the development of renal inflammation and correlate with kidney leukocyte numbers. Monocyte chemotactic protein-1 (MCP-1), also known as CC-chemokine ligand 2 (CCL2), is considered to be the most potent chemokine for recruiting monocyte/macrophages. It is expressed by many cell types in diseased kidneys, but is produced mostly by glomerular and tubular epithelial cells (58). Urine levels of MCP-1 correlate with kidney MCP-1 expression and interstitial macrophage accumulation in lupus nephritis and diabetic nephropathy (59, 60). Interferon-inducible protein 10 (IP-10), also known as CXC-chemokine ligand 10 (CXCL10), is produced by many renal cell types and is a soluble chemoattractant for activated T-cells. Urine IP-10 levels are increased in patients with diabetic nephropathy and renal allograft rejection (61, 62). In addition, urine levels of IP-10 correlate with the incidence of renal allograft rejection and predict allograft function (63). CXC-chemokine ligand 16 (CXCL16) is another chemoattractant for activated T-cells which correlates with T-cell accumulation in acute and chronic renal diseases. Transmembrane CXCL16 is present on glomerular and tubular cells during injury and is released after proteolytic cleavage by metalloproteinases. Urine levels of soluble CXCL16 are increased in patients with lupus nephritis or renal allograft rejection (64, 65). Macrophage migration inhibitory factor (MIF) is a molecule which is produced at sites of inflammation and inhibits further macrophage migration in response to chemokines, thereby allowing macrophages to accumulate at the inflammatory site. MIF can also enhance the activity of macrophages and T cells at sites of injury. Increasing levels of MIF in urine correlate with kidney leukocyte accumulation and the severity of renal damage in proliferative forms of glomerulonephritis (66). In addition, elevated MIF levels in urine can predict episodes of acute renal allograft rejection and discriminate from cyclosporine nephrotoxicity (67).

There are also other proinflammatory mediators that can identify inflammation in the injured kidney. Vascular cell adhesion molecule-1 (VCAM-1) is expressed by renal vessels and some kidney cells during renal inflammation and facilitates transendothelial leukocyte migration. Some of this VCAM-1 is enzymatically cleaved and excreted into the urine. Urine levels of soluble VCAM-1 are elevated during active periods of ANCA vasculitis and lupus nephritis (64, 68), and are useful for determining the severity and type of renal allograft rejection (69).

Interleukin-18 (IL-18) is a proinflammatory cytokine which is produced by leukocytes, vessels and kidney tubules. During acute renal injury, there is a substantial increase in IL-18 production by tubules. Elevated urine levels of IL-18 are a relatively sensitive and specific marker of ATN and delayed graft function in the post-ischaemic kidney (70). Urine levels also correlate with disease activity in idiopathic nephritic syndrome (71).

Tumour necrosis factor receptor-1 (TNFR1) is one of the major receptors for the proinflammatory cytokine TNF-α, which is expressed on infiltrating leukocytes and some resident kidney cells during renal inflammation. The soluble form of TNFR1 is more stable and easier to detect in serum and urine than TNF-α and it can serve as a surrogate marker of
TNF-α activity in kidney disease. Serum and urine levels of soluble TNFR1 are increased during acute and chronic renal inflammation and correlate with the progression of acute renal failure, lupus nephritis and diabetic nephropathy (61, 64, 72).

Another recent inclusion to this family of biomarkers is soluble human leukocyte antigen (sHLA)-DR. Urine levels of sHLA-DR are a sensitive and highly specific marker of acute renal allograft rejection, which can be detected up to 5 days before the clinical signs of acute cellular or vascular rejection are evident (73).

**Biomarkers of renal fibrosis**

The development of renal fibrosis is dependent on excessive production of profibrotic growth factors and extracellular matrix which can be detected in urine by ELISA. Transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) are two of the major growth factors which promote renal fibrosis. Urine levels of TGF-β1 and CTGF increase with the progression of chronic kidney diseases (74–76). Some profibrotic molecules which are induced by TGF-β1, such as TGF-β inducible gene H3 (βig-H3) and plasminogen activator inhibitor-1 (PAI-1), are also detectable in urine and can act as surrogate markers of renal TGF-β1 activity. Urine levels of βig-H3 are about approximately 1000 times greater than TGF-β1 in diabetic patients and can be detected before the onset of albuminuria (77), indicating that βig-H3 is an early and sensitive marker of renal fibrosis during diabetes. In the small study of Djukanovic and coworkers, plasma and urine TGF-β1 levels were measured in patients in the early stage of Balkan endemic nephropathy (BEN) and in those with manifestened BEN and compared with the levels in patients with primary GN and healthy subjects. Median plasma TGF-β1 concentration was similar in all examined groups but the greatest range of individual plasma TGF-β1 levels was found in BEN patients. Urinary TGF-β1 excretion was significantly higher in both BEN groups and the GN group than in healthy controls, suggesting a role for TGF-β1 in the initial events related to development of interstitial fibrosis (78).

**Identifying kidney diseases with proteomics**

Recent advances in proteomic analysis incorporating mass spectrometry have led to the identification of novel biomarkers of kidney injury in urine, which include: 1) intact or fragmented proteins that are selectively increased or decreased in kidney disease; 2) protein patterns which are indicators of specific types of kidney disease; and 3) protein patterns which can predict the progression of acute or chronic kidney diseases. Proteomic studies of patients with diabetic nephropathy have identified exosomal fetuin-A as an early biomarker of acute kidney injury (81), cleaved forms of β2-microglobulin as markers of acute renal allograft rejection (82), and a ubiquitin fusion protein (UbA52) as a potential specific marker of diabetic nephropathy (83). Other researchers have focused on urine proteomic patterns as a means to predict the progression of kidney diseases with high sensitivity and high specificity. A urinary polypeptide pattern has been shown to distinguish IgA nephropathy from normal controls (90% specificity) and from patients with membranous nephropathy, minimal change disease, focal segmental glomerulosclerosis or diabetic nephropathy (100% specificity) (84). Another urine proteomic study found that two proteins in a mass spectrometer signature can distinguish active and inactive lupus nephritis with 92% specificity (85). In addition, a clinical analysis has identified a 12 peak proteomic mass spectrometer signature that can predict cases of diabetic nephropathy in 74% of type 2 diabetic patients before the onset of albuminuria (86). Similarly, a more complex panel of 65 biomarkers has been shown to predict the development of diabetic nephropathy in patients with albuminuria (97% sensitivity) and differentiate from other chronic renal diseases (91% specificity) (87).

**Conclusion**

Recent advancements in molecular analysis have resulted in the identification of a wide range of potential serum and urine biomarkers for assessing renal function and injury and predicting the development of kidney disease. Many of these biomarkers can be grouped according to their association with a particular type of injury (eg. podocyte or tubular injury) or a mechanism of damage (eg. oxidative stress, inflammation, fibrosis).

There is growing evidence from small clinical studies that the progression of kidney diseases may be predicted by evaluating a combination of serum and urine biomarkers together with other risk factors such as age and hypertension. In the future, this analysis process may also include urine proteomic patterns and genetic biomarkers. However, larger clinical studies will be required to compare panels of biomarkers and achieve agreement on which combination offers the most useful and cost-effective clinical information.

**Conflict of interest statement**

The authors stated that there are no conflicts of interest regarding the publication of this article.
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