

FOLIA MEDICA CRACOVIENSIA

Vol. LV, 4, 2015: 21–33

PL ISSN 0015-5616

Role of new biomarkers for the diagnosis of nephropathy associated with diabetes type 2

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Abstract: In twenty first century, the incidence of type 2 diabetes mellitus (DMt2) dramatically increases, followed by the number of patients suffering from its complications. Currently, diabetic kidney disease (DKD) is the leading cause of renal replacement therapy. Often, DMt2 is diagnosed after several years of duration, and irreversible organ damage can develop during that period. On the other hand, the early diagnosis of DKD in the preclinical phase, when glomerular filtration rate (GFR) is still maintained and there are no evident changes in urinalysis, gives the possibility of implementing the nephroprotective treatment that can significantly delay the progression of the disease. However, the diagnostic tests available in clinical practice, i.e. serum creatinine, estimated glomerular filtration rate (eGFR) and albuminuria have important limitations. There is a need for new, early and non-invasive biomarkers specific for kidney injury, allowing for differentiation between glomerular and tubular injury, and changing dynamically in response to the degree of kidney damage. Hereby, we review the current knowledge about the novel and emerging biomarkers of kidney injury and their used for the diagnosis of DKD.

Key words: diabetic kidney disease, biomarkers, diabetes mellitus type 2.

Introduction

It is estimated that nearly 387 mln people in the world suffer from diabetes. Its prevalence exceeds 8,3% and gradually increases [1]. Diabetic kidney disease (DKD) has been regarded as one of the chronic complications of diabetes resulting from microangiopathy.

DKD is the most common cause of chronic kidney disease (CKD), leading over time to the necessity for renal replacement therapy [2]. Among patients with type 2 diabetes mellitus (DMt2), CKD is one of the most common causes of hospitalisation, associated with cardiovascular events, cardiovascular mortality, and also total mortality [3]. It is also the only complication which incidence has not decreased despite the improvement in the methods of diabetes control during the last 20 years [4]. Thus, the strategy aiming at the decrease of CKD incidence and progression in DMt2 needs to be improved.

Current criteria of DKD diagnosis

In nearly 70% of DMt2 patients, the coexisting insulin resistance and the diabetic environment, exert different effects on kidneys in comparison with the changes observed in type 1 diabetes [5]. The long term hyperglycemia in poorly controlled diabetes, together with the coexisting hypertension and hyperlipidemia are considered the main causes of DKD progression in patients with DMt2 [5]. The recommendations published by the Kidney Disease Outcomes Quality Initiative (KDOQI) working group in 2002 [6] introduced the term DKD as one of the CKD forms, and the albumin concentration measured in 24-hour urine sample has been considered a standard for the assessment of kidney disease in the course of diabetes. However, urine albumin to creatinine ratio (UACR) measured in a spot urine sample may be used in routine care in order to estimate urine albumin excretion. Since 2007, KDOQI has recommended that increased UACR (formerly termed “micro-” or “macroalbuminuria”) should be confirmed by at least 2 further measurements performed in first morning urine samples [7].

Albuminuria is not specific for kidney injury in the course of DMt2. Increased urine albumin excretion is observed in glomerulopathies irrespective of the cause, as well as in medical conditions associated with increased vascular permeability, i.e. severe infections or heart failure [8]. Moreover, increased albuminuria does not have to be caused by kidney injury, but may be associated with hyperfiltration as observed in febrile patients, in those with urinary tract infections, or in healthy people following significant physical activity.

At present, the terms “microalbuminuria” and “macroalbuminuria” are not further recommended. The loss of albumin in urine in quantities of 30–300 mg/day should be considered a moderately increased albuminuria. Higher albumin loss in urine is consequently called severely increased albuminuria. The former term “microalbuminuria” has incorrectly suggested the presence of “microalbumine” in urine, or could have been interpreted as the finding of minimal clinical importance, therefore it has been abandoned. According to the recommendations of Polish Diabetological Association published in 2015 [8], the laboratory tests that should be performed for the diagnosis and assessment of DKD include urine albumin excretion and serum creatinine mea-

surements followed by the estimation of glomerular filtration rate (GFR) according to Modification of Diet in Renal Diseases (MDRD) or Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations. Screening towards increased albuminuria should be done in each patient with DMt2 at least once a year starting from the diagnosis of diabetes [8].

Pathophysiology of kidney damage in DMt2

The morphological changes in the course of DKD involve the structures of glomeruli, including endothelial cells, basement membrane, podocytes and slit membrane. The mesangial expansion, increased numbers of interstitial cells and tubular lesions have also been observed [5]. However, there is only a weak association between the severity of morphological changes in kidneys and the routinely used laboratory and clinical markers of kidney injury (i.e. increased urine albumin and total protein, increased serum creatinine and decreased eGFR, high blood pressure) [5]. Moderately increased albuminuria (30–300 mg/g creatinine) is a first sign of DKD only in a part of patients. In about 30% of DMt2 patients, the decrease in GFR occurs without increased albuminuria [9]. Moreover, it must be remembered that DKD is not the only cause of CKD among patients with diabetes. For a proper diagnosis, imaging tests, and in selected cases, histological examination of kidney biopsy may be indicated apart from albuminuria and serum creatinine. Unfortunately, in routine clinical practice such tests may be difficult to perform. According to the data from the Polish registry of nephropathies diagnosed based on kidney biopsy (2009–2012) concerning DMt2 patients, DKD has been diagnosed in about 2% of biopsies, whereas 60% of biopsies resulted in the diagnosis of other nephropathies [10] (it must be remembered, however, that kidney biopsy as an invasive procedure is not indicated in typical course of DKD). It should also be noted that obesity (often preceding DMt2) causes focal segmental glomerulosclerosis [11]. Thus, it would be interesting to investigate the existing and emerging biomarkers of kidney injury in the association to the specific lesions described following kidney biopsy and histological examination. On account of composite pathomechanism of kidney injury in DMt2 and in order to systematise knowledge about these processes, markers of DKD are divided into bioindicators of injury of kidney tract, injury of renal glomeruli, oxidative stress and inflammation. Figure 1 presents the role of particular biomarkers for the pathogenesis of DKD in DMt2.

The ideal biomarker of kidney injury in DMt2

The diagnostic procedures used in kidney diseases, based on serum creatinine, urinalysis, and imaging tests may sometimes be inaccurate. Kidney biopsy, in turn, is associated with significant invasiveness. Serum creatinine concentrations depend not only on GFR, but

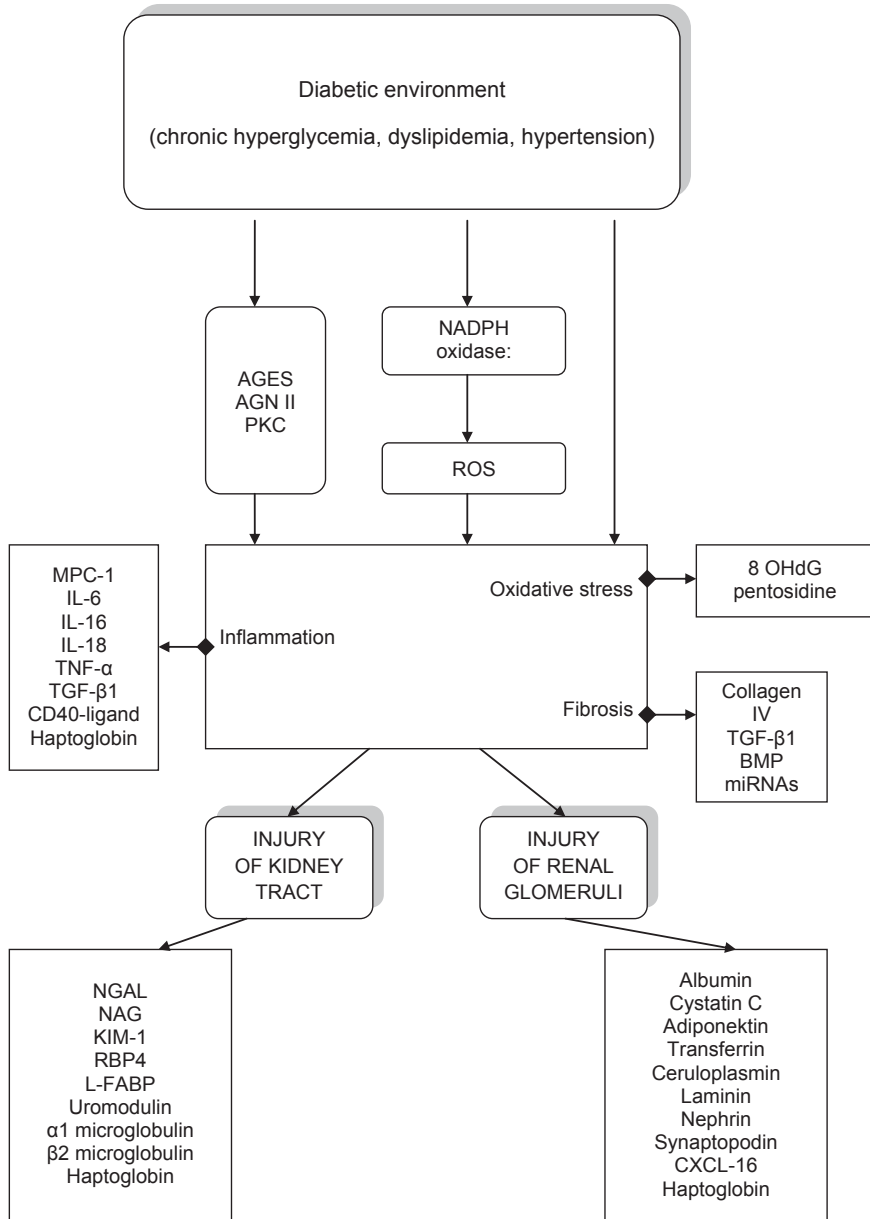


Fig. 1. Role of particular biomarkers for the pathogenesis of DKD in DMt2.

AGES — advanced glycation end-products; AGN II — angiotensin II; BMP — bone morphogenetic protein; CXCL-16 — chemokine (C-X-C motif) ligand 16; DKD — diabetes kidney disease; DMt2 — diabetes mellitus type 2; IL-6 — interleukin 6; IL-16 — interleukin 16; IL-18 — interleukin 18; KIM-1 — kidney injury molecule-1; L-FABP — liver type fatty acid binding protein; MPC-1 — monocyte chemoattractant protein-1; miRNAs — microRNAs; NAG — N-acetylo-b-D-glucosaminidase; NGAL — neutrophil gelatinase-associated lipocalin; NADPH — nicotinamide adenine dinucleotide phosphate (reduced); NOX 1-5 — NADPH oxidase 1-5; 8 OHdG pentosidine — 8-hydroxy-deoxy guanosine pentosidine; PKC — protein kinase C; RBP4 — retinol binding protein 4; ROS — reactive oxygen species; TGF-β1 — transforming growth factor beta 1; TNF-α — tumor necrosis factor α

also on many additional factors, such as muscle mass, sex, age, diet or pharmacological treatment [12]. Also, creatinine in urine comes not only from glomerular filtration, but it is also excreted by the proximal tubule, which leads to falsely increased estimates of GFR based on creatinine clearance [12]. The ideal marker of GFR should be freely filtered in glomerulus, and neither excreted, nor absorbed in kidney tubules. Its concentrations should be easy to determine. It should be specific for kidney damage, and dynamically respond to the changes in GFR, also to the improvement in kidney function following treatment. It is expected that such a marker will be able to predict cardiovascular changes. A good biomarker of decreased GFR, together with a proper marker of tubular injury, would allow for the diagnosis of DKD (or the risk for DKD) in DMt2 patients without increased albuminuria before the kidneys are irreversibly damaged.

Albuminuria in the course of DMt2

In the course of DKD, a damage to the filtration barrier leads to increased leak of plasma proteins with a molecular mass below 40 kDa, including albumin and transferrin. Such proteins are also filtered by the healthy renal glomeruli, although in lower amounts, and are reabsorbed by a proximal tubule [13]. Thus, increased excretion of such proteins in urine may be due to increased filtration in the or due to diminished reabsorption in the initial part of the nephron [13].

Currently, albuminuria is the gold standard in the diagnosis of DKD and the evaluation of its advancement [8]. Albuminuria should be regarded as a marker of an ongoing kidney damage, not as a risk factor for DKD [13]. Albuminuria is the independent predictor of cardiovascular and renal risk in diabetic patients [8]. However, about 30% of DMt2 patients with renal failure do not have albuminuria [9]. Among DMt2 patients without albuminuria or proteinuria, advanced glomerulosclerosis has been observed in kidney biopates [14]. Also, the cut-off value for increased albuminuria is a matter of debate. The cut-off value for UACR of 30 mg/g seems too high, and many researchers consider UACR above 15 mg/g as a sign of kidney pathology or kidney risk [15]. Most recommendations for early nephroprotection in DMt2 are aimed at controlling albuminuria. Facing the increasing numbers of patients with DKD, there is a need for the early biomarkers of kidney injury, that may be used in patients without increased albuminuria, or that would precede the increase in albuminuria.

New biomarkers of DKD

The promising biomarkers in the field include urinary neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), serum cystatin C (CysC), uromodulin (UMOD), urinary N-acetylo-beta-D-glucosaminidase (NAG), liver type fatty acids binding protein (L-FABP) and serum interleukin 18 (IL-18).

Neutrophil gelatinase-associated lipocalin

NGAL, also known as lipocalin 2, is a 25 kDa secretory protein belonging to the lipocalin family, detected in neutrophils' granules [16]. In response to renal ischemia or toxic injury, NGAL expression significantly increases in the ascending limb of the loop of Henle. Urine NGAL concentrations are also correlated with its serum concentrations [17]. NGAL has been studied mainly as a biomarker of acute kidney injury (AKI). In AKI, increased urinary NGAL (uNGAL) concentrations have been observed already after 4–6 hours from injury, thus NGAL has been regarded a “renal troponin” [18–20]. In CKD, including DKD, NGAL is considered as a diagnostics and prognostic marker. Bolignano *et al.* [21] observed that uNGAL increased and correlated with the advancement of kidney disease in patients with DKD both with and without albuminuria. In the Chronic Renal Insufficiency Cohort (CRIC) Study [22], in patients with CKD stage G2-G4, increased uNGAL reflected the risk of cardiovascular complications. However, Helmersson *et al.* [23] observed high biological variation of uNGAL among patients with CKD due to tubular disorders, therefore they recommend both the assessment of uNGAL concentration and the calculation of uNGAL to urine creatinine ratio (uNGAL/uCr). In pathophysiology of DKD, NGAL may play an important role in adaptation of tubular cells to the damaging influence of diabetic environment. Summarizing, uNGAL can become a useful and non-invasive tool for the evaluation of kidney function, overtaking the classic markers, i.e. albuminuria or eGFR [21, 24]. It seems that a useful strategy in an evaluation of kidney disease in DMt2 patients would include the determination of an initial uNGAL concentration and uNGAL/uCr value and then the monitoring of their changes [24].

Kidney injury molecule-1

KIM-1 seems to be another promising biomarker of tubular injury. It is a type 1 transmembrane glycoprotein containing in its extracellular part the immunoglobulin and the mucous domains [25]. It is expressed in proximal renal tubule in response to hypoxia or injury, and is not detected in healthy people [26, 27]. Urinary KIM-1 is increased in AKI from the first day after toxic or ischemic injury [28]. However, KIM-1 expression in renal tubules has been also observed in the course of the focal glomerulosclerosis, IgA nephropathy, or membranoproliferative glomerulonephritis [29, 30]. On the basis of these observations it has been suggested that KIM-1 can serve as a biomarker of chronic tubular injury [29, 30]. Currently, studies are ongoing on KIM-1 usefulness as a diagnostic marker in CKD. Following 3.5-year observation of patients with DKD, it has been shown that the increase of urinary KIM-1 correlates positively with albuminuria and negatively with GFR changes, however, it does not correlate with HbA_{1c} [31]. In turn, Fu *et al.* [32] reported higher urinary KIM-1 in 101 patients with DMt2 observed

for 5 years as compared with the control group, however, they did not confirm the correlations with UACR or eGFR. The available data are insufficient for the assessment of KIM-1 usefulness as a diagnostic tool in DKD, in particular among patients without albuminuria. Further studies are necessary, especially in the Caucasian population.

Cystatin C

CysC is a single chain 120 amino acid polypeptide, belonging to the cysteine proteinases inhibitors. It is freely filtered by glomeruli, then reabsorbed and metabolised in proximal tubules [33]. Its concentrations in serum, unlike creatinine, do not depend on the muscle mass neither the diet [33]. The determination of serum CysC is useful for early detection of the decrease in glomerular filtration rate. A formula utilizing both serum creatinine and serum CysC allows for a precise estimation of GFR [34]. According to the 2012 KDIQO guidelines, serum CysC should be determined in adults with eGFR between 45 and 59 ml/min/1.73 m² and without other signs of renal disease, in order to confirm CKD [35]. In patients with DKD, Woo *et al.* [36] reported higher usefulness of GFR estimation with the use of serum CysC than serum creatinine. Moreover, in Chinese patients with DMt2 [37] and in the study of Triki *et al.* [38] serum CysC predicted cardiovascular complications. The promising report of Garg *et al.* [39] has shown increased CysC concentrations in patients with glucose intolerance and without albuminuria. According to current knowledge, serum CysC should be used for early detection of kidney disease as well as for verification of GFR estimates based on serum creatinine [8].

Uromodulin

UMOD, also known as Tamm-Horsfall's protein, is a glycoprotein synthesized exclusively in kidneys, where it is expressed in a thick ascending limb of the loop of Henle and in the initial segment of the distal tubule. Following its proteolysis, it is released to urine under physiological conditions and is a most abundant urinary protein in healthy people, however, it is also detectable in serum [40]. Urinary UMOD has been reported to prevent urinary tract infections and crystallization of salts [40]. In 1978, pathological localization of UMOD in kidneys has been detected in tubulointerstitial diseases, including medullary cystic disease, chronic pyelonephritis and hydronephrosis [41]. More recently, interesting observations have been provided by the human genome-wide association studies, which identified the associations between UMOD gene mutations and the increased risk of hypertension, kidney stones and CKD [42]. Prajczek *et al.* [43] has shown negative correlations between urinary UMOD concentrations and serum creatinine and positive correlation with eGFR. Also, Zhou *et al.* [44] reported that low urinary UMOD in the initial stages of CKD predicted fast GFR decrease among patients with IgA nephropathy. In the last

study, urinary UMOD correlated with the advancement of tubular atrophy and interstitial tissue fibrosis [44]. The last report is promising, however, more evidence is necessary in order to assess the usefulness of urinary or serum UMOD as the early markers of CKD.

N-acetylo-beta-D-glucosaminidase

NAG is the enzyme that is not filtered in renal glomeruli, and its increased activity in urine appears following toxic injury of renal tubules [45]. In diabetic patients, positive correlations have been observed between increased NAG activity in urine and albuminuria, diabetes duration, poor diabetes control, serum CysC and urine NGAL [46]. In the study of Sheira *et al.* [47] conducted in a group of 50 DMt2 patients, urine NAG positively correlated with UACR, serum creatinine, and HbA_{1c}. Moreover, urine NAG activity was associated with the advancement of kidney disease in DMt2. NAG seems to be the promising DKD marker, increased already in a preclinical DKD, before significant increase in albuminuria. However, there is a need for further research on this marker in Caucasian population.

Liver type fatty acid binding protein

L-FABP is a protein expressed in hepatocytes as well as in the cells of the nephron proximal tubules [48], which appears in urine following tubular injury. Fergusson *et al.* [49] reported the role of L-FABP as a biomarker of AKI. Higher urine concentrations of L-FABP have been observed in DMt2 patients as compared with healthy individuals; moreover, it correlated with increasing albuminuria and decreasing GFR [50, 21]. In the study of Nauta *et al.* [51], among the measured markers of tubular injury, L-FABP was the most strongly correlated with eGFR. L-FABP seems the promising marker of DKD, that may be helpful in detecting the preclinical stages of the disease.

Interleukin 18

IL-18 is a cytokine produced mainly by macrophages. The first known function of IL-18 was a strong stimulation of T lymphocytes and NK cells to the production of interferon γ [52]. The cytokine is also involved in the polarisation of immune response [52]. Apart from the physiological role, IL-18 has been associated with severe inflammatory reactions. Also, its pathophysiological role in DMt2 has been suggested [53]. In a group of 151 patients with DMt2, Moriwaki *et al.* [54] observed significantly increased serum IL-18 concentrations in comparison with the control group. Currently, the research are ongoing aimed at the evaluation of IL-18 as the early marker of DKD. Interesting observations were made by Szeto *et al.* [55] among 220 patients with DMt2: increased IL-18 correlated with the cardiovascular mortality.

New trends in diagnosing DKD

Oxidative stress and novel therapeutic targets in DKD

There are several mechanisms in which hyperglycemia acts in a damaging way on both the endothelial cells and the epithelial cells of renal tubules, including increased polyol pathway flux, increased advanced glycation end-product formation, activation of protein kinase C, and increased hexosamine pathway flux. The common element linking these mechanisms is an increased production of superoxide by the mitochondrial electron-transport chain [56]. The overproduction of reactive oxygen species (ROS) results in the oxidative stress leading among others to renal tubular injury and the interstitial tissue fibrosis in DMt2 [56]. NADPH oxidase plays a key role in the oxidative stress. The enzyme has several isoforms, of which the NOX-4 seems the most important in the mechanism of ROS generation [57]. The animal studies by Thallas-Bonke *et al.* [58], revealed the advantageous effect of NOX-4 gene deletion on the progression of DKD. Further studies are needed to evaluate the associations between NOX isoforms and the DKD progression.

Dysfunction of podocytes in DKD pathogenesis

In the search for early DKD markers, it has been observed that podocyturia as well as urine excretion of proteins associated with podocytes can be a source of early DKD biomarkers. Wang *et al.* [59] has shown the presence of nephrin and synaptopodin in urine of patients with DMt2 with proteinuria and reduced GFR. The results of Zhao *et al.* [60] are also promising. The study suggested a role of the CXCL-16 ligand for C-X-X chemokines, taking part in the LDL metabolism within the podocytes, as the early biomarker of renal injury in DMt2.

MicroRNAs

The microRNAs (miRNAs) seem to be promising as the markers of renal fibrosis in the course of DKD. Xu *et al.* [61] has reported the role of miRNAs: miR-216A, miR-217, miR-192, miR-377, miR-21, miR-29c in DKD prognosis. The increased concentration of miR-21 has been shown in kidney bioplates from patients with DKD [62].

Proteomics as the promising DKD diagnostic and prognostic method

The proteomic methods utilizing mass spectrometry seems to have a great potential in providing data on protein profiles constituting the “finger print” of the diseases [11, 63]. The methods allowed for introducing several biomarkers of AKI and CKD, present in urine, blood or tissues. The proteomics has been termed a „liquid kidney biopsy”, as

a non-invasive tool that could potentially be used in a preclinical phase of kidney diseases [63]. Rossing *et al.* [64] have identified the peptide profiles in urine in the group of patients with DMt1 and DMt2, which seem to have been better predictors of DKD progression than albuminuria. Also, the method identified 12 metabolites of glucose metabolism in the glycolytic pathway, which possess the prognostic value for DKD in DMt2. The proteomics in nephrology has the multidirectional character and constitute the promising method of searching for DKD biomarkers. However, in order to define the diagnostic sensitivity and specificity of the markers, further studies are needed, verifying the results on the larger groups of patients.

Conclusions

Due to the significant diversity of histopathological changes observed in kidneys in the course of DMt2 and the accompanying diverse clinical picture of DKD, additional diagnostic tools are necessary, except for eGFR and albuminuria. The kidney injury in DMt2 is often irreversible, especially in patients with decreased GFR and increasing albuminuria. Early diagnosis and multidirectional treatment constitute the only effective way of stopping the progression of kidney disease and improving prognosis. In clinical practice, the intensive treatment undertaken in the advanced disease may only slow down its progression. Due to the insufficient diagnostic sensitivity of clinically used markers, the research are ongoing to identify the new markers allowing for early diagnosis of the disease and introducing the proper treatment in the preclinical phase of diabetic kidney injury. However, further research is needed before implementing the novel markers in wide clinical practice.

Conflict of interest

None declared.

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