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Utility of Urinary Biomarkers in Kidney Transplant Function Assessment

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1. Introduction

Kidney may undertake normal function immediately after transplantation or even several or over a dozen days delay. Absence of normal renal transplant function may lead to acute kidney injury (AKI), nephrotic syndrome (NS) and chronic kidney disease (CKD). **Acute kidney injury (AKI**) is characterized functionally by a rapid decline in the glomerular filtration rate (GFR), and biochemically by the resultant accumulation of nitrogenous wastes such as blood-urea nitrogen and creatinine (Devarajan, 2010). **Nephrotic syndrome (NS)** is a nonspecific disorder in which the kidneys damage is accompanied by a leak of large amounts of protein (proteinuria at least 3.5 grams per day per $1.73m^2$ body surface area) from the blood into the urine. Nephrotic syndrome is a disorder of the glomerular filtration barrier. The multiprotein complex between adjacent podocyte foot processes the slit diaphragm, is essential to the control of the actin cytoskeleton and cell morphology. Signaling from slit diaphragm proteins to the actin cytoskeleton is mediated via the Rho GTP-ases. These are thought to be involved in the control of podocyte motility, which has been postulated as a focus of proteinuric pathways (Hull & Goldsmith, 2008). It is common belief that nephrotic syndrome after transplantation results mainly from recurrence of renal disease in transplanted kidney and not defective graft function. **Chronic kidney disease (CKD)** – it is kidney damage for ≥3 months, defined by structural or functional abnormalities of the kidney, with or without decreased GFR, manifested by either pathological abnormalities or markers of kidney damage, including abnormalities of blood or urine or abnormalities in imaging tests (Ahmad et al., 2006).

2. Markers of nephrons damage

After kidney transplantation it is particularly important to monitor the biomarkers which allow to detect progress in disease process and determine which functional parts of kidney are going to be damaged, to enable application of a quick appropriate treatment (Lisowska-Myjak, 2010; Alachkar et al., 2010; Metzger et al., 2010). Administration of immunosuppressants for preventing renal graft rejection may lead to progressive damage to the renal tissue (interstitial fibrosis, tubular micro calcifications, atrophy of renal tubules) caused by high toxicity of suppressing drugs. Cyclosporine A(CsA), tacrolimus, mycophenolate mofetil, basiliximab, prednizon and sirolimus (rapamycin) are commonly used in immunosuppressive therapy following kidney transplantation. Cyclosporine A and tacrolimus generate immunosuppressive action by binding to cyclofiline and inhibiting the action of calcineurin 2, which stimulates proliferation and differentiation of lymphocytes T. Cyclosporine A inhibits synthesis of lymphokines by lymphocytes T. Lymphokines synthesized by lymphocytes T stimulate immunological system and have the ability to „kill" inflammatory and neoplastic cells. Mycophenolate mofetil selectively inhibits inosine monophosphate dehydrogenase, a basic enzyme in guanosine synthesis. Mycophenolate mofetil inhibits proliferation of lymphocytes T and B after stimulation with antigenes, cytokines and mitogens. Basiliximab similarly to Daclizumab, blocks receptors for IL-2.

Majority of renal pathological changes concern glomerules, proximal and distal tubules as well as vascular endothelium. At first renal proximal tubular cells (Fig.1.) demonstrating highest metabolic activity, possessing high amounts of mitochondries, lysosomes and peroxysomes are damaged. Remaining sections of nephron such as: Henle's loop, distal tubules and collecting tubules are usually damaged later. There are numerous biomarkers that identify injury the area of the renal nephron, such as the glomerulus, the proximal, and the distal tubule.

2.1. Biomarkers of renal glomeruli

The oldest biomarkers of renal glomeruli injure are serum urea and creatinine as well as clearance of endogenic creatinine, which similarly to inulin (gold standard in GFR determination) is excreted to urine and not absorbed in renal tubules. Clearance of endogenic creatinine is 10-20% higher than clearance of inuline, which is a result trace excretion of creatinine by renal tubules (Finney et al., 2000).

Cystatin C (CYC) is a cysteine protease inhibitor that is stably secreted from all nucleated cells, freely filtered through the glomerulus, and completely reabsorbed by the proximal tubules. During efficient function of proximal renal tubules there are traces of urinary CYC, independent of age and body mass. Given that cystatin C is not normally found in urine in significant amounts, the elevated level of urinary cystatin C may display dysfunction of tubular cells and tubulointerstitial disease. Concentration of CYC in normal urine accounts 0.03-0.3 mg/L (Filler et al., 2005). Increase in serum CYC is proportional to decrease in GFR (Campo et al., 2004). It was reported that serum CYC correlated better with GFR than creatinine (Filler et al., 2005; Schuck et al., 2004). After renal transplantation CYC concentration increased simultaneously to AKI development, because of decreased reabsorption from damaged tubules. Therefore
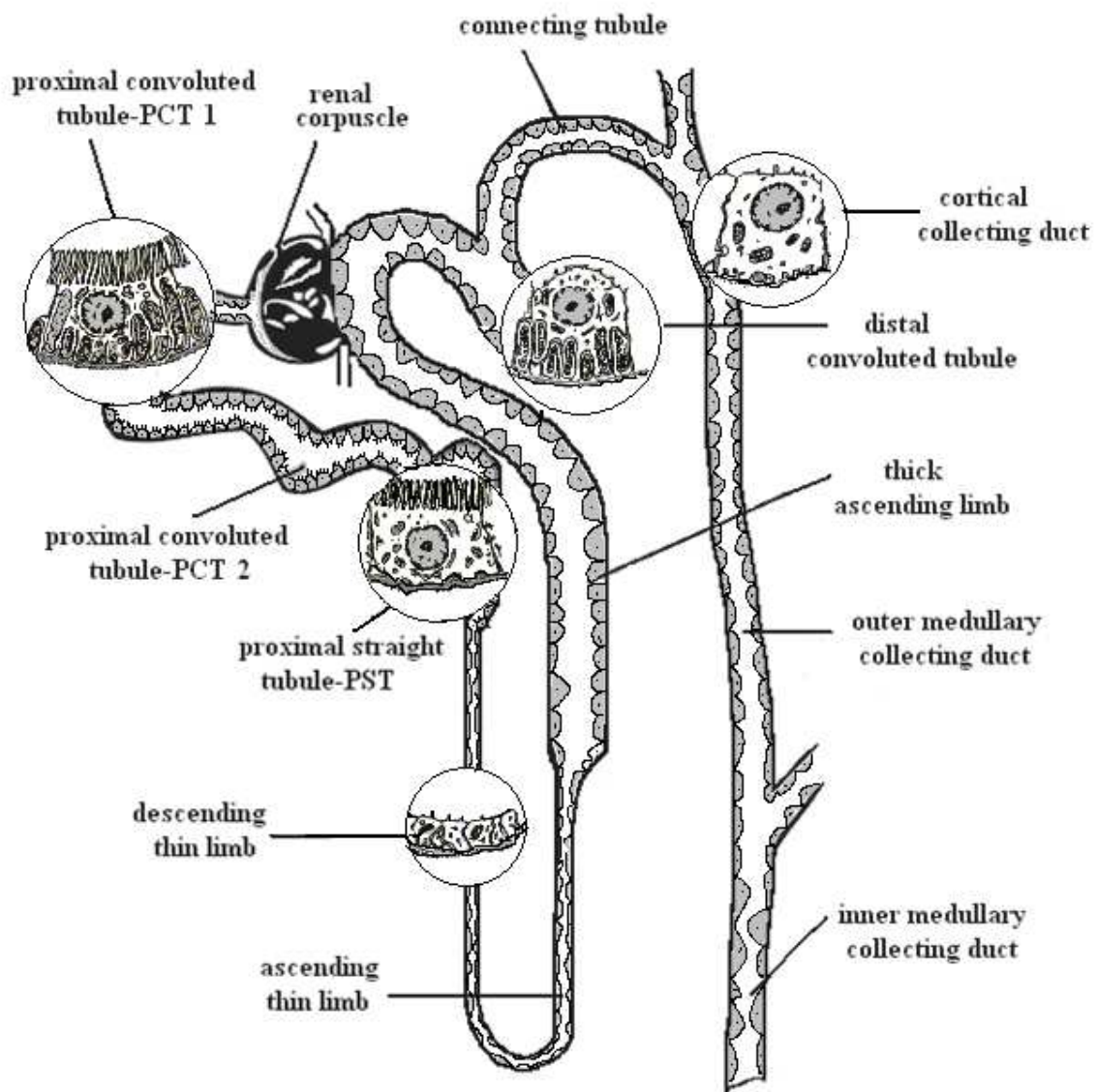
Figure 1. Nephron structure

urinary concentration of CYC may be treated as a good marker of the proximal tubules and effective biomarker of delayed renal graft function due to lack of diurnal changes, and high stability in routine conditions of urinary storage, urinary CYC may be determined in single urinary samples. Urinary CYC / creatinine ratio is a good indicator of renal tubules dysfunction as in disorders of renal tubules, urinary CYC concentration may increase even 200 fold (Uchida & Gotoh, 2002; Lisowska-Myjak, 2010). Two fully automated and quick immunological methods for CYC determination: turbidimetric PETIA (particle enhanced immunoturbidimetric assay) and nephelometric – PENIA (particle enhanced nephelometric immunoassay) were developed in 1994-1997. Presently measurements of urinary CYC are utilized mainly with a

PENIA method designed primary for CYC determination in serum (Herget-Rosenthal et al., 2004). PENIA method (particle enhanced nephelometric immunoassay)allows for a CYC detection at a concentration of 0.05-10.47 mg/L.

Proteinuria reflects increased filtration plasma proteins to tubular fluid and disturbed protein reabsorption by renal proximal tubular cells (Haraldsson & Sörensson, 2004; Halbesma et al., 2006; Giorgio et al., 2004). Proteinuria over 0.5g/24 hours is a marker the severity of the tubular damage, independent risk factor of progressive tubular-interstitial fibrosis and strong predictor of the end-stage renal insufficiency. Evident proteinuria is symptomatic for estab-lished renal damage significantly connected with decreased GFR (Abbate et al., 2006; Eddy, 2004; Tryggvason & Pettersson, 2003; Zoja et al., 2003; Ofstad & Iversen, 2005). Even minimal proteinuria lasting one year after renal transplantation is an indicator of a poor renal graft function and may be a risk factor for renal graft failure (Kang et al., 2009). Urinary protein is a non-invasive and easy to perform parameter. It was reported that proteinuria (<0.5 g/24 h) occurred in half of patients within 3 month after renal transplantation (Sancho Calabuig et al., 2009). Higher than standard doses of everolimus (EVL) resulted in an increase of proteinuria. Therefore the standard doses of EVL are recommended which seems to be suitable for protecting against an acute graft rejection with better prognosis of renal function in longer perspective (Loriga et al., 2010). As chronic allograft nephropathy (CAN) is the most frequent reason for late loss of graft, immunosuppression with mycophenolate mofetil significantly improves graft function and in such circumstances evaluation of proteinuria seems to have prognostic value (Grebe et al., 2004).

Albuminuria as a marker of glomerular filtration is more sensitive than proteinuria. Urinary albumin border value of 200 µg/min differentiates patients with albuminuria and proteinuria. Increase in urinary albumin excretion above 200 µg/min (macroalbuminuria) indicates damage to glomerular filtration membrane, a start of evident proteinuria, progression of kidney disease and cardiovascular changes (Ruggenenti & Remuzzi,2006). After exceeding maximal reab-sorption capacity of proximal tubular cells, protein of primary urine appeared in final urine (Luke, 1999; Remuzzi et al., 2006; Zoja et al., 2003). Excessive accumulation, in proximal tubular cells, plasma proteins excreted to primary urine, induced increase in local expression of cytokines and chemokines, which presence in final urine is a specific indicator of development and extent of renal damage (Lisowska-Myjak, 2010; Alachkar et al., 2010). Renal tubular cells exposed to increased amounts of filtered plasma proteins resulting cell injury. Microalbumi-nuria predicts a loss of renal graft. Determination of urinary albumin and UACR-urine albumin-to-creatinine ratios (UACR) are particularly recommended indicators for detection of changes in transplanted kidney (Erman et al., 2011). Microalbuminuria is considered to be a better indicator of kidney transplant condition than proteinuria (Bandukwala et al., 2009). Albuminuria, the marker of renal glomeruli damage and chronic damage of transplanted kidney, which may also reflect interstitial inflammatory process, is considered a predictor of long-term allograft outcomes in a kidney graft recipient (Nauta et al., 2011).

2.2. Adhesion molecules connected podocytes with basement membrane

Integrin α3 and integrin β3 are particularly recommended biomarkers for monitoring the function of transplanted kidney both at early and remote period after transplantation. The integrin family of cell adhesion proteins promotes the attachment and migration of cells on the surrounding extra cellular matrix (ECM). The signals initiated by integrin binding to ECM proteins are necessary to maintain cell survival, adhesion, migration and invasion. Integrins are transmembrane glycoproteins consisting of two units: α and β. Beta1 family of integrins represents the major class of cell substrate receptors with specificities primarily for collagens, laminins, and fibronectins (Srivastava et al., 2011).

Vascular cell adhesion molecule-1 (VCAM-1), sVCAM-1 (CD106) (soluble vascular cell adhesion molecule 1) and anti-intercellular adhesion molecule-1 (ICAM-1) The ICAM and VCAM – members of the immunoglobulin (Ig) superfamily, are the chief endothelial cell proteins that are recognized by the white cell integrins. Elevated urinary sVCAM-1, IL6, sIL6R and TNFR1 concentrations indicate an acute kidney transplant rejection in the first 2 weeks after transplantation (Reinhold et al., 2012). It was reported that increased urinary concentrations of sICAM-1, determined by ELISA, occured in patients with acute renal graft rejection (Teppo et al., 2001), and in people with proteinuria, high concentrations of sVCAM and sICAM were observed (van Ree et al., 2008). Recently a non-invasive monitoring of the acute renal graft rejection by determination of cell adhesion molecules has been recommended (Gwinner, 2007).

3. Biomarkers of proximal tubules

α_1-**microglobulin (α_1M)** is a 27 kDa glycoprotein related to retinol binding protein synthesized by liver cells, engaged in immunoregulation (binds lymphocytes T and B) and heme catabolism. Determination of α_1M (stable in acid urine) is a sensitive indicator of renal proximal tubules damage (Guder, 2008; Lisowska-Myjak, 2010; Câmara et al., 2009). (Teppo et al., 2004) reported that six month after transplantation, 32% of patients presented microalbuminuria. Evaluation of a damage to renal proximal tubules, on the basis of an increase in urinary α_1M concentration may be a consequence to a deterioration of glomerular filtration. Increase in α_1M /creatinine ratio is an early and sensitive indicator of a poor function of the transplanted kidney, and indicates a poor prognosis of long term survival of renal transplanted patients (Teppo et al., 2004).

Retinol binding protein (RBP), protein of the lipocalin family, synthesized mainly in a liver, supplies retinol to peripheral tissues. RBP removed from plasma by glomerular filtration is subsequently absorbed and catabolized in renal proximal tubules. Increased urinary RBP is caused by a disorder in glomerular filtration and reabsorption in renal proximal tubules (Guder, 2008; Kuźniar et al., 2006; Uchida & Gotoh, 2002; Câmara et al., 2009). It seems that urinary RBP is a better biomarker of proximal tubules damage than β$_2$M, as RBP has greater stability in acid urine than β$_2$M and renal insufficiency is only a clinical situation where an increase in urinary RBP concentration is observed.

Adenosine deaminase binding protein (ABP) is a glycoprotein (120-kDa) present in lungs, liver, placenta and brush border of renal proximal tubules. Increased expression in the urinary ABP is considered an early indicator of acute renal injury (AKI). Increase in urinary ABP was reported in patients with ischemia - without sepsis, after kidney transplantation, after toxic renal tubules damage, and in newborn with sepsis. Recently published opinion suggested that ABP to be the best marker of acute renal damage, better than β2-M or α1-M (Bagshaw, 2007). As ABP excretion was higher among kidney transplants recipients than in people with normal renal function, ABP is considered as a good indicator for detection of renal graft failure (Iglesias & Richard, 1994).

β_2-microglobulin (β_2M) is a membrane protein of major histocompatibility complex HLA. β_2M excretion is used for evaluation of nephrotoxic renal damage (aminoglycoside antibiotics, heavy metal salts) (Guder, 2008). It should be noted that determination β_2M for evaluation function of transplanted kidney may be ambiguous because of coexistence of many factors influencing its plasma and urinary concentration (e.g. toxic drugs action, ischemia reperfusion complications or renal graft rejection). Measurement of urinary β_2M may be helpful in evaluation of the condition of transplanted kidney, however the interpretation of result should be careful because of the plurality of factors influencing β_2M plasma concentration, renal filtration ability and tubular function (Kuźniar et al., 2006).

4. Markers of inflammatory reaction connected with acute renal failure

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein expressed and secreted by immune cells, trachea, stomach, colon, and injured kidney epithelial cells as an monomer (22 kDa), dimer or trimer. NGAL may complex with collagenase type IV of human neutrophils named gelatinase B or metalloproteinase 9 (MMP-9) creating heterodimer (125 kDa) (Flower, 1996). NGAL binds and transports small lipophilic molecules e.g. free fatty acids, retinoids, arachidonic acid, and steroids (Mishra et al., 2003). NGAL is considered as provider iron to proximal renal tubules. Iron stimulates oxygenase synthesis which protects renal tubules cells. NGAL may be applied as a predictor of ischemic or toxic renal damage, before development of a full symptomatic renal insufficiency. Increase in urinary NGAL concentration is early, sensitive and non-invasive marker of renal damage correlating with intensity and time of ischemia and preceding increase in other markers such as hexosaminidase or β2-microglobu-lin. A strict correlation between NGAL concentration and degree of proteinuria was demonstrated (Flower, 1996). NGAL activated formation of nephrons in early step of renal development demonstrates a protective action in kidney (Mori & Nakao, 2007). Low molecular weight, resistant to degradation NGAL is easily excreted by cells of thick ascending arm of Henry loops and collective tubules into urine both free and in complex with MMP-9. Increase in urinary excretion of NGAL is observed several hours after stimuli of nephrotoxic factor. As concentration of urinary NGAL correlates with plasma NGAL concentration, NGAL may be an useful marker in diagnostics of renal diseases (Nickolas et al., 2008). There is an opinion that NGAL is the most promising biomarker for diagnosis of acute renal injury (AKI) in acute renal graft dysfunction (Halawa, 2011; Ting et al., 2012; Hollmen, 2011).

Kidney injury molecule-1 (KIM-1) is a transmembrane glycoprotein receptor (104 kDa) appearing as KIM-1a and KIM 1b. KIM-1 is produced in large quantities in renal proximal tubules after a toxic or ischemic damage. It is assumed that direct cause of KIM-1 induction is an increase of the protein concentration in glomerular ultrafiltration and presence of urinary protein casts favoring tubular obstruction, mechanical stress and an increase in glomerular pressure. An increase in urinary KIM-1 excretion is specific to the ischemic renal damage and is practically independent of chronic renal insufficiency or renal tract infection (Nickolas et al., 2008; Melnikov & Molitoris, 2008). It was reported that KIM-1 extracellular domain (fragment 90 kDa) reaches urine after cleavage by metalloproteinase (Han et al., 2002; Waanders et al., 2010). Urinary KIM-1 is particularly important in the diagnosis of the acute transplanted kidney insufficiency(AKI) (Halawa, 2011). As in renal graft recipients, contrary to urinary NGAL or IL-18, KIM determination gives better possibility for predicting a rate of the transplanted kidney deterioration (Szeto et al., 2010), KIM-1 was proposed as an independent predictor of the long term renal graft survival (Ting et al., 2012).

5. Proteins degrading extracellular matrix (ECM)

Urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR) regulate renal allograft function. Allogenic renal graft uPAR deficiency, strongly attenuates ischemia reperfusion injury and acute kidney allograft rejection. Deficiency of uPAR in renal graft diminished generation of reactive oxygen species and renal cells apoptosis (Gueler et al., 2008). Therefore serum and urinary uPA may be treated as an early marker of the acute kidney transplant rejection (Alachkar, 2012).

Matrix metalloproteinases (MMPs) are extracellular proteases which depend on bound Ca^{2+} and Zn^{2+} for activity. Urinary panel of metalloproteinases was proposed for the early diagnosis of renal allograft rejection (Metzger et al., 2011; Sánchez-Escuredo et al., 2010; Hu et al., 2010).

Tissue inhibitors of metalloproteinases (TIMP) are extracellular inhibitors protease-specific, which bind tightly to the activated protease, blocking its activity. Presently 2% to 4% of renal allografts are rejected one year after from transplantation, because of chronic allograft injury. Mazanowska et al. (Mazanowska et al., 2011) suggest that determination of TIMP in urine may confirm the process of an active rejection of the transplanted kidney.

6. Immunological mediators of inflammatory state and fibrosis of renal tissue

Urinary chemokines CXCL9 and CXCL10 may be treated as noninvasive screening markers of renal graft rejection in patients with interstitial fibrosis and tubular atrophy (IF/TA), leading to shorter life span of renal graft (Jackson et al., 2011; Schaub et al., 2009). Urinary CXCL10 may be a useful noninvasive screening test for tubulitis in renal graft recipients, and urinary

CXCL10 concentration above 1,97 ng /mmol of creatinine is a threshold for consideration of renal biopsy (Ho et al., 2011).

6.1. Immunological markers of renal inflammatory state

Macrophage inflammatory protein 3alpha (MIP-3alpha), chemokine C-C ligand 20 (CCL20) is a major chemokine expressed by epithelial cells that attracts immature dendritic cells (DC). Graft-infiltrating dendritic cells (DC) and alloreactive T lymphocytes play a critical role in renal allograft rejection. Renal proximal tubular epithelial cells (TEC) are considered as an active players in the attraction of leukocytes during renal inflammatory responses. A significant increase in the excretion of major intrinsic protein MIP-3α/CCL20 to urine was observed in renal graft recipients with symptoms of graft rejection (Woltman et al., 2005; Peng et al., 2008).

Growth-related oncogene-alpha (Gro-alpha) is an analog of the keratinocyte-derived chemokine(KC). An increase in serum and urinary analog of Gro-alpha in the experimental renal damage appears the earliest and persists the longest among the 18 chosen cytokines and chemokines (Molls et al., 2006). Serum and urinary Gro-alpha were the highest 3 hours after ischemia, while histological changes were evident after one hour, whereas serum creatinine increased 24 hours after ischemia. Urinary concentration of Gro-α increased significantly in renal graft recipients who required dialysis in comparison to people with normal renal graft function. Urinary Gro-α is considered as an early marker of diagnosis and prognosis of acute kidney injury (AKI) resulted from ischemia (Molls et al., 2006). It was reported that Gro-alfa, significantly increased in patients who received cadaver kidney with poor function and from living donors with minimal ischemia. Therefore determination of KC and Gro-α may be used as biomarkers in the diagnosis of ischemic acute renal failure (ARF) and in the early diagnosis and prognosis of renal ischemia-reperfusion injury (IRI). IRI is the most frequent cause of acute kidney injury (AKI) and acute renal failure in delayed function graft received from a cadaver (Molls et al., 2006).

Interleukin (IL-18) is a proinflammatory cytokine released to urine by epithelium of renal proximal tubules after stimuli of nephrotoxic factor. Urinary IL-18 concentration >100 pg/mg of urinary creatinine is a good diagnostic marker of the acute renal damage and mortality of patients in intensive care units (Parikh et al., 2005) as well as a predictor of the delayed graft function. It seems that urinary IL-18 helps for a detection of the very early stage of kidney damage caused by ischemia or tubular nephrotoxins and plays a role in detecting prerenal nitrogenemia, chronic renal insufficiency and urinary tract infection (Parikh et al., 2005). Furthermore urinary Il-18 is an early predictive biomarker of the acute kidney injury after cardiac surgery (Parikh et al., 2005). IL-18- proinflammatory cytokine caspase-1 dependent (both derived from ischemic renal proximal tubular cells) is a proinflammatory cytokine activated in damaged renal tubules by caspase -1 and released to urine in a case of the acute kidney injury (AKI). A significant increase in urinary concentration of IL –18 and NGAL after transplantation, however before delay in the renal graft function, was found. IL-18 is also a predictor of the AKI severity preceding the increase in serum creatinine (Dinarello, 1999).

Granzymes (*granule-associated enzymes***)** are serine proteases (27-32kDa) of the chymotrypsin family. Granzyme B (GzmB) and Fas-ligand (FAS-L) are cytotoxic molecules involved in the acute renal graft rejection (AR) by the induction of DNA fragmentation of damaged cells (Yannaraki et al., 2006). Granzyme A (GzmA) is a specific noninvasive immunological biomarker for monitoring renal graft condition which facilitate diagnosis and treatment after transplant complications. Granzyme A (GzmA) besides involvement in apoptosis may act as mitogen of B lymphocytes. GzmA is a noninvasive biomarker differentiating patients with subclinical and acute renal graft rejection from patients with renal tubular necrosis or persons with stabile renal graft (van Ham et al., 2010).

6.2. Immunological markers of renal fibrosis

Chemokine regulated upon activation in normal T cells expressed and secreted (RANTES/ CCL5) is a chemokine of the beta subfamily secreted by macrophages and T lymphocytes. RANTES can signal through CCR1, CCR3, CCR5 and US28(cytomegalovirus receptor) receptors. It is chemoattractant towards monocytes, memory T cells(CD4+/CD45RO+), basophils, and neutrophils. RANTES occurs in increased amounts in diseased kidneys and indicate on interstitial inflammatory changes of the tubular cells at the early stages of acute kidney injury, skin or heart graft rejection (Koga et al., 2000; Gwinner, 2007). RANTES expressed in renal different cells (mesanglial cells, endothelium of renal tubules, fibroblasts, lymphocytes) plays an active role in acute and chronic kidney inflammation and development of tubule-interstitial damage. (Baer et al., 2005) reported absence of significant differences in plasma and urinary RANTES in patients with acute renal graft rejection and recipients with normal graft function. Therefore RANTES is not suitable for detection of early kidney graft rejection. However an significant increase in the serum and urinary RANTES was observed immediately after renal transplantation which may reflect an activation of the immunological systems.

Transforming growth factor beta (TGF-β) is responsible for exacerbation of fibrosis, controls growth and differentiation of cells and production of extracellular matrix as well as regulates cellular migration. A participation of TGF-ß1 in lung and kidney fibrosis during chronic allograft rejection was reported by (Awad et al.,1998; Bartnard et al., 1990). Increased excretion of urinary TGF-β was proposed as a marker of the intrarenal production and activity of TGF-ß1 in kidney. An increase in the urinary TGF-ß1 was reported in different nephropathies particularly significant in patients with heavy proteinuria (Schnaper et al., 2003; Böttinger & Bitzer, 2002). The 6-12 month immunosuppression with cyclosporine in renal-transplant recipients caused development of a chronic interstitial nephropathy with decreased GFR. Cyclosporine A (CsA) facilitate the expression of TGF-β in renal tubular cells and cells of renal juxtaglomerular apparatus. Furthermore, CsA stimulates T lymphocytes and endothelial cell to a TGF-β 1 production. Expression of TGF-β 1 is CsA dose dependent. High doses of CsA are risk factors of chronic graft dysfunction, among kidney recipients (Boratyńska et al., 2003).

Vascular endothelial growth factor (VEGF) a dimeric protein containing subunits constituted of 121, 165, 189 or 206 amino acids is a proangiogenic growth factor. In patients with symptoms of acute renal graft rejection high urinary VEGF concentration was found in comparison to

people with normal function of renal graft. Therefore monitoring of urinary VEGF was proposed as a marker of detection acute renal graft rejection and the evaluation of the effectiveness of immunotherapy (Peng et al., 2008; Alachkar, 2012).

Hepatocyte growth factor (HGF) induces angiogenesis by stimulation proliferation, migration and adhesion of endothelial cells. Urinary HGF concentration was highest at the first day after transplantation, decreased quickly within next week and later remained on the same level. Determination of the urinary HGF immediately after kidney transplantation may be a quick, noninvasive marker of long lasting renal graft function (Kwiatkowska et al., 2010).

Endothelin-1 (ET-1) is the strongest vasoconstrictory factor produced by endothelium of blood vessels, glomerular mesangium, renal tubular cells, fibroblasts and macrophages. ET-1 regulates fibrosis by joining interstitial fibroblasts, initiation its proliferation and synthesis of extracellular matrix as well as chemotactic action on macrophages. ET-1 is degraded mostly in lungs and kidneys. Urinary ET-1 excretion reflects its renal production. Increase in ET-1 gene expression and urinary excretion correlates positively with proteinuria and negatively with creatinine clearance (Grenda et al., 2007; Saurina et al., 2007). Plasma and urinary ET-1 concentrations are increased in patients treated with Cyclosporine A and FKJ506. Cyclosporine A and FK506 are calcineurine inhibitors broadly applied for immunosuppression in kidney transplant patients. Cyclosporine A and FK506 significantly improve graft survival. However graft recipients may die because of cardiovascular complications as 80% of renal graft recipients reveal vascular hypertension. Increased ET-1 concentration may reflect activation of the ET-1 system in chronic insufficiency of transplanted kidney (Slowinski et al., 2002).

Monocyte chemotactic peptide-1 (MCP-1/CCL2) mediates recruitment of inflammatory cells: monocytes/macrophages and lymphocytes T, to renal tubules damaged by high concentrations of albumin in tubules. A strict relationship between albuminuria, urinary MCP-1/CCL2 and macrophage infiltration in damaged loci, was demonstrated (Urbschat et al., 2011; Viedt & Orth, 2002). In patients with acute renal graft rejection urinary concentration of MPC-1, determined by ELISA, was ten times higher than in patients with stable graft function (Dubiński et al., 2008). Since chronic damage to renal graft as a result of gradual fibrosis and tubular damage (IF/TA) is the most frequent cause of graft loss, urinary CCL2 may be treated as an independent prognostic marker of development of IF/TA during the next 24 months (Ho et al., 2010).

Fractalkine (CX3CL1) is a chemokine from the CX3C group of complement system, stimulated by CX3CR1 receptor connected to G protein. In experimental renal disease induced by albumin overload and proceeding with proteinuria, increased expression of fractalkine gene correlates with applied albumin dose and time of albumin interaction with the renal tubular cells (Donadelli et al., 2003). Fractalkine urinary concentration is a noninvasive method for detection of acute renal graft rejection (Peng et al., 2008).

Angiotensin II (Ang II) is an important intrarenal factor favoring processes of inflammation and fibrosis by an increase in the expression of the proinflammatory genes (IL-6, TNFα, MCP-1, RANTES). According to latest opinions the urinary concentration of angiotensinogen, reflects amounts of produced Ang II inside the kidney better, than immediate evaluation of Ang II in

the urine. Improvement of the ELISA method for determination of human urinary angioten-sinogen, may allow to disclose influence of Ang II on intrarenal destructive processes (Yamamoto et al., 2007; Katsurada et al., 2007).

Complement is a major mediator system in pathogenesis of various kidney diseases. The presence and localization of complement components in glomerulus and/or the tubule-interstitial area provides diagnostic tools for several human renal diseases (Zoja et al., 2003; Lisowska-Myjak, 2010). Increase in urinary excretion of complement components in patients with proteinuria significantly correlate with urinary excretion of total proteins and decrease in renal function. Therefore increase in urinary C5b-9 in patients with proteinuria may by prognostic marker for the development of kidney insufficiency (Eddy, 2002). Accelerated C3 activation at renal proximal tubules in diseases proceeding with proteinuria are result of increased intratubular protein catabolism, with accompanying increase in ammonia (activator of alternative pathway of complement activation) (Morita et al., 2000; Abbate et al., 2008; Sheerin et al., 2008; Lederer et al., 2003). Everyday urinary determination of C5A and TCC may be a sensitive and reliable marker of the acute insufficiency of the transplanted kidney and predictor of graft rejection (Müller et al., 1997).

Galectin 3 (Gal-3) is a beta-galactoside-binding lectin in diverse fibrotic tissues. Gal 3 plays an important role in fibrosis of transplanted kidney and may be a potential marker of chronic allograft impairment (CAI) (Dang et al., 2012).

7. Tubular enzymes

Currently, in clinical diagnostic practice for renal parenchymal tubular impairment, assess-ment of urinary enzymes is used. Particular advantage of urinary enzymes determination is its localization in appropriate renal cells (glomeruli, tubules) and their organelles (cytoplasm, lysosomes, membranes), which may deliver detailed information concerning nature and dimension of the renal cells damage and an evaluation of their dysfunction or necrosis (Westhuyzen et al., 2003; Trof et al., 2006). Routine, simple, cheap and broadly available spectrophotometric methods are applied for measurement of urinary enzymes activity. An increase in urinary excretion of enzymes reflects damage of particular renal section (D'Amico & Bazzi, 2003; Jung et al., 1986). Determination of urinary FBP-1,6, NAG, glutathione-S-transferase and pyruvate kinase has recently been recommended for the diagnosis of kidney disease and early detection of transplant rejection (Kotanko et al., 1997; Kotanko et al., 1986).

7.1. Enzymes of brush border membranes

Gamma-glutamyltransferase (**GGT**) – is connected with cellular membranes of liver, kidney, pancreas and prostatic gland (Kuźniar et al., 2006). Serial determination of urinary enzymes is a reliable proof for nephrotoxicity resulted from long term cyclosporine A treatment. Lack of enzymuria indicates a recovery of renal tubules to normal function (Tataranni et al., 1992).

Alkaline phosphatase (AP) – is present in cellular membranes of many tissues, mainly bonds, liver and intestine where it participates in metabolism of organic phosphates. Frequent cause

of deterioration to the renal graft function is nephrotoxicity of immunosuppressive drugs (e.g. cyclosporin A) reflected by increase in activity of urinary enzymes : ALP, LDH, GGT, beta-glucuronidase (Refaie et al., 2000; Takahashi et al., 1989; Simić-Ogrizović et al., 1994).

Alanylaminopeptidase (AAP) – proteolytic enzyme degrading oligopeptides. Increases in urinary concentration of hexosaminidase and AAP accompany acute renal tubular necrosis, renal graft rejection or nephrotoxic action of immunosuppresive drugs (e.g. cyclosporin A) administered to patients after kidney transplantat (Kuźniar et al., 2006; Lisowska-Myjak, 2010; Santos et al., 2010). Increases in urinary excretion of tubular enzymes testifies tubular brush border membrane damage with a loss of microvillus structure (Westhuyzen et al., 2003).

7.2. Cytosolic enzymes

Glutathione S-transferase (alpha-GST, pi-GST) is a specific cytoplasmic enzyme of tubular epithelial cells consisting of two isoenzymes: α-GST with alkaline and πi-GST with acidic pH optimum. GST-α appears in epithelium of proximal tubular cells and GST-π in distal tubules (Branten et al., 2000). Determination in urine α-GST and, π-GST is applied to diagnosis acute renal graft rejection with acute tubular necrosis (Kuźniar et al., 2006; Polak, 1999). Differentiated increase in urinary GST- alpha and GST- pi excretion may point to localization of an nephron damage (Westhuyzen et al., 2003; Trof et al., 2006; Herget-Rosenthal et al., 2004; Branten et al., 2000; Gautier et al., 2010).

Fructose-1,6-bisphosphatase (FBP-1,6) is localized mostly in contorted and to less extend in straight part of proximal renal tubules, similarly to hexosaminidase and GST, points to accurate localization of damaged nephron (Trof et al., 2006; Kotanko et al., 1986). Increase in urinary FBP-1,6 was observed in patients after kidney transplantat. Urinary FBP-1,6 excretion was significantly lower in patients with median of cold ischemia below 22 hours, than above 22 hours. Even in lack of graft dysfunction, in situation where it is a long time of cold ischemia, urinary excretion of FBP-ase correlates with a degree of damage to the renal tubules (Kotanko et al., 1997). It was reported that a panel of urinary enzymes activities: FBP-ase, glutathione S-transferase, N-acetyl-beta-D-glucosaminidase and pyruvate kinase is a good marker of the cyclosporin A nephrotoxicity (Kotanko et al., 1986).

7.3. Renal lysosomal enzymes

N-acetyl-β-D-hexosaminidase (HEX) is one of the most frequently determined urinary markers of renal tubules damage, because its activity increased at early steps of the renal tubules damage, before occurrence of disturbances in renal excretory function. Hexosaminidase localized mainly in renal proximal tubular cells, is a specific marker for proximal tubular cells because its high molecular weight (> 130 kDa) excludes its glomerular filtration. In the course of active kidney disease HEX activity is constantly increased. An increase in urinary activity HEX and its isoenzyme B indicate on damage in the renal tubular cells. Therefore urinary HEX and particularly HEX B activity may be treated as a specific marker of damage in the renal proximal tubules of the transplanted kidney (Liangos et al., 2007; Holdt−Lehmann et al., 2000;).

8. Markers of renal ischemia/reperfusion injury

Leukocyte elastase (LE, neutrophil elastase), is a 30-kDa glycoprotein serine protease released from neutrophils as a mediator of ischemia/reperfusion injury after renal transplantation. Urinary LE is a simple noninvasive marker of the neutrophil activation after renal transplantat (Zynek-Litwin et al., 2010).

9. Biomarkers of dystal renal tubules

In the assessment of distal renal tubule dysfunction it is advised to examine urine osmolarity and/or determination Tamm-Horsfall glycoprotein as well as urinary kallikrein (Bhoola et al., 1992).

Renal kallikrein is a serine protease which releases vasodilatatory peptides: bradykinine and calidine, from kininogen. Renal kallikrein is present in renal collecting tubules and is released to tubular fluid by terminal section of dystal segment of nephron (Manucha & Vallés, 1999; Thongboonkerd & Malasit, 2005). An increase in activity of urinary kallikrein was observed in insufficiency and loss of the renal graft function (Krimkevich, 1990).

AnnexinA11 (**ANXA11**). Annexins are calcium-binding proteins which binds to acidic phospholipid and F-actin. Depending on calcium concentration Annexin A 11 participate in signal transduction, cell proliferation, regulation of vesicular transport and interaction with the cell membranes. Annexin occurs in high quantities in renal distal tubular cells and epithelium of renal glomeruli. Annexin physiologic role seems to be related to cell apoptosis (Rodrigues-Garcia et al., 1996). Significant correlation between urinary Annexin V and other proteins and lack of correlation with urinary urea and creatinine concentration suggests that Annexin V is not an indicator of kidney function, but rather reflects local kidney damage (Matsuda et al., 2000). Annexin A11 may act as an atypical calcium channel and useful marker of acute and chronic renal graft rejection (Srivastava et al., 2011)

Renal papillary antigen-1 (RPA-1) a renal papillary antigen-1, sensitive and specific antigen of renal papillary cells is a sensitive and specific urinary marker of damage renal collecting tubules (Gautier et al., 2010).

Prominin-2 (PROM-2) analog of **CD133 (prominin-1)** is an membrane glycoprotein (112kD) with the highest expression in epithelial cells of matured kidney. Prominin-2 is a cholesterol-binding protein associated with apical and basolateral plasmalemmal protrusions in polarized epithelial cells and released into urine (Florek et al., 2007) and a novel marker of distal tubules and collecting ducts of the human and murine kidney (Jászai et al., 2010).

µ-glutathione-S-transferase (µ-GST) is a conjugating glutathione with electrophilic compounds that occurs in epithelial cells of ascending part of Henle's loop (Gautier et al., 2010; Holmquist & Torffvit, 2008). After nephrotoxic drugs treatment (e.g. cisplatin) µ-GST quickly appear in urine. µ-GST is a more specific marker of nephrotoxicity (AUC 1.000) than α-GST

(AUC 0.984) or albuminuria (AUC 0.984). μ-GST is an early biomarker for Henle's loop and distal tubules damage (Tonomura et al., 2010).

10. The future of biomarkers

Development of new technologies involved in molecular biology, analysis of m-RNA expression, proteomics and metabolomics create a possibility of discovery of new markers for early diagnosis of AKI and IF/TA. Relatively new method of microarrays (microarrays of cDNA and oligonuclotides- DNA chips) are sets of molecular probes attached to solid background in strictly determined order constituting two dimensional system of microscopic areas with defined sequences of nucleic acid. Microarray technology allow for detection of thousands of molecules of nucleic acids due to possibility of performing simultaneously many hybridization experiments (Dean et al., 2012). DNA microarrays technology permit for simultaneous monitoring expression of many genes (Scian et al., 2011). Identification of these genes constitute further step in earlier diagnosis and better prognosis of TA/IF(tubular atrophy/interstitial fibrosis).

Proteomic techniques Recently broadly applied proteomic techniques facilitate discovery of new biomarkers useful in evaluation of transplanted kidney function. Proteomics apply protein analysis using techniques such as MS e.g. (MALDI-TOF-Matrix Assisted Laser Desorption Ionisation - Time of Flight; SELDI-TOF-Surface Enhanced Laser Desorption Ionisation - Time of Flight; ES multielementary I - LTQ−FTICR-Electrospray Ionisation - Linear Trap Quadrupole - Fourier Transform Ion Cyclotron Resonance). Proteomics combine series of techniques for simultaneous analysis of hundreds or thousands of cells proteins. Proteomics objective is not only creation of the list of important proteins, but first of all exploration of differences in protein profiles of healthy and diseased people. Proteomic identification of urinary protein profiles is an noninvasive method for detection of renal proximal tubules dysfunction of transplanted kidney (Srivastava et al., 2011; Gwinner, 2007). Proteomic techniques are alternative for diagnostics based on single markers, because it allows for simultaneous analysis of large numbers of protein and peptide markers creating specific „finger print" of disease. Proteomics determines pattern of expression or secretion taking into account qualitative and quantitative relations between peptides and proteins produced in defined pathophysiological conditions.

Metabolomics based on analysis sets of metabolites connected with proteins, lipids, carbohydrates, hormones, etc. evaluate qualitative and quantitative relations between particular metabolites. Due to metabolomics it is possible to determine definite metabolites characteristic for specific groups of diseases and changes occurring under influence of genetic and pathophysiological stimuli (Wishart, 2006).

New technologies and bioinformatics tools offer tremendous research possibilities which should make possible now and in the future precise monitoring of kidney graft, allow early detection and treatment of renal graft rejection and allow both for preventing and treatment of renal transplant complications as well as to improve number of long term patients survival.

Markers	Acute kidney injury (AKI); Acute graft rejection (AGR); Acute tubular necrosis (ATN)	Chronic allograft nephropathy (CAN/ IFTA); Delayed graft function (DGF)	References
β2M,α1M	+	+	Johnston et al., 2011; Du et al., 2011; Câmara et al., 2009;Kuźniar et al., 2006
Netrin-1	+		Ramesh et al., 2010; Urbschat et al., 2011
NGAL	+		Ramesh et al., 2010; Nauta et al., 2011; Przybyłowski et al., 2011; Halawa, 2011; Devarajan, 2011; Hall&Parikh, 2010; Du et al., 2011;Ting et al., 2012
IL-16,IL-2,IL-6,IL-18,TNF	+	+	Alachkar er al., 2010; Halawa, 2011; Devarajan, 2011; Reinhold et al., 2012; Urbschat et al., 2011
KIM-1	+	+	Nauta et al., 2011; Halawa, 2011; Devarajan,2011; Hall &Parikh, 2010; Du et al., 2011; Ting et al., 2012; Urbschat et al., 2011
NAG		+	Nauta et al. 2011; Câmara et al., 2009; Ting et al., 2012; Kuźniar et al., 2006; Alachkar et al., 2010
H-FABP, L-FABP	+	+	Nauta et al., 2011; Przybylowskiet al., 2011
Cystatin C	+		Przybylowski et al., 2011; Hall &Parikh, 2010
CXCL9,CXCL10	+	+	Ho et al., 2011; Schaub et al., 2009; Jackson et al., 2011; Ting et al., 2012
alpha-GST, pi-GST	+	+	Câmara et al., 2009;Hall &Parikh, 2010; Ting et al., 2012; Kuźniar et al., 2006; Oberbauer , 2008
GzmA,GzmB (granzyme)	+		van Ham et al., 2010; Peng et al., 2008; Oberbauer, 2008
Galectin-3(Gal-3)		+	Dang et al., 2012
Integrin α3, integrinβ2	+	+	Srivastava et al., 2011
ANXA11	+	+	Srivastavaet al., 2011
sVCAM	+		Reinhold et al., 2012
MMP7, MMP-8	+		Metzger et al., 2011; Ling et al., 2010
LDH, ALP, γ-GT, AAP	+		Refaie et al., 2000;Kuźniar et al., 2006

Markers	Acute kidney injury (AKI); Acute graft rejection (AGR); Acute tubular necrosis (ATN)	Chronic allograft nephropathy (CAN/IFTA); Delayed graft function (DGF)	References
OX40,OX40L,PD-1	+		Afaneh et al., 2010
HLA-DR	+		Ting et al., 2010
CTGF		+	Yue et al., 2010; Bao et al., 2008
uPA	+		Alachkar , 2012
Leukocyte elastase (LE)	+	+	Zynek-Litwin et al., 2010
SERPING1	+		Ling et al., 2010
TIMP1	+		Ling et al., 2010
MIP-1delta,	+	+	Hu et al., 2009
Osteoprotegerin	+	+	Hu et al., 2009
VEGF	+		Peng et al., 2008
fractalkine	+		Peng et al., 2008
MCP-1	+		Dubiński et al., 2008; Urbschat et al., 2011
RBP	+		Kuźniar et al., 2006; Câmara et al., 2009
Perforin	+		Oberbauer, 2008
FOXP3	+		Oberbauer, 2008

Table 1. Urinary biomarkers for the early detection of acute and chronic allograft dysfunction.

11. Conclusion

In this chapter we presented traditional and new biomarkers for diagnostics and monitoring condition of transplant kidneys. Urine is practical, easy to obtain, noninvasive material for diagnosis of kidney diseases. Numerous reports from molecular biology, genetics, proteomics and metabolomics disclosed an array of new markers specifically connected with damage of specific nephron segments in the course of successive steps of disease. Particular expectations are connected with proteins represented particular nephron section, or produced locally in the place of nephron damage. Presence of cytokines and chemokines in urine is an early sign of renal inflammatory state, due to influx of granulocytes to the damaged nephron area. Majority of traditional biomarkers, particularly enzymuria retains diagnostic value in an evaluation of the renal tubules function. Multitude of presented biomarkers suggest their limited diagnostic value. Discovering universal marker seems to be very difficult. However, it is potentially more fruitful to identity the putative biomarker proteins useful in diagnostics of kidney disease.

Scientiscs are still looking for the "kidney troponin". Actually, more than ten promising biomarkers for kidney damage have been identified. The most relevant and the best studied substances are neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, kidney injury molecule-1 (KIM-1), beta-2 microglobulin (β2M), and interleukin-18 (IL- 18). In kidney allograft recipients, urinary KIM-1 expression provides prognostic information in relation to the rate of renal function decline, irrespective of the kidney pathology (Ting et al., 2012; Han et al., 2002; Szeto et al., 2010).

Validation of those kidney markers in various pathologic conditions is actually ongoing. However, the majority of publications reviewed are small cross-sectional studies, and there are only a handful of longitudinal studies. Another important point is that biomarkers only have clinical value if the results are reproducible. However none of the biomarkers reviewed here have been studied in more than 2 longitudinal trials so their clinical applicability needs to be confirmed in good quality, long-term, large longitudinal trials.

Among enzymes which retain high diagnostic value in diagnostics of renal diseases are: hexosaminidase and its isoenzyme B as a marker of the proximal tubular damage as well as AAP or GST as a marker of the tubular brush border membrane. Cytosolic FBP-1,6 is of great diagnostic value for assessment of graft function. It is commonly believed that appropriate panel of urinary proteins and enzymes may by a practical marker for evaluation of the nephron function of transplant kidney and prognosis of the renal allograft fate. In the future, discovery of new biomarkers and research techniques may change practical approach to treating patients with renal grafts. In summary we feel it is necessary for an international body to develop a renal marker utility grading system, to evaluate the usefulness of particular markers of nephron function and to make recommendations for the use of renal transplant markers, similar to those instilled for tumor markers (Hayes et al., 1996; Locker et al., 2006).

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