Published in final edited form as:

Endocrine. 2013 June ; 43(3): 494–503. doi:10.1007/s12020-012-9820-y.

Review and Discussion of Tubular Biomarkers in the Diagnosis and Management of Diabetic Nephropathy

Gianfranco Tramonti¹ and Yashpal S. Kanwar²

¹Dipartimento di Medicina Interna – Sezione di Nefrologia, Università di Pisa, Italy

²Department of Pathology, Feinberg School of Medicine, Northwestern University, 303 E. Chicago Avenue, Chicago, Illinois 60611, USA

Abstract

The prevalence of diabetic nephropathy has tremendously increased with the relentless rise in the incidence of diabetes over the last couple decades. Diabetic nephropathy is a leading cause of morbidity and mortality, and it invariably leads to an end stage renal disease (ESRD). In an effort to delay the onset of ESRD systematic screening and appropriate management are needed to evaluate the progression of renal damage in diabetic nephropathy. The reliability of current tests in predicting the onset, progression and response to various regimens for diabetic nephropathy is still under debate; and it has engendered a search for more sensitive and specific urinary biomarkers, especially those reflective of tubular dysfunctions. It is well known that there is a good correlation between the degree of damage to the tubulo-interstitial compartment and the deterioration of renal functions. In view of this, the utility of urinary biomarkers, reflective of tubular injury, reported in the literature is discussed in this brief review.

Keywords

Diabetic Nephropathy; biomarkers; N-GAL; KIM-1; L-FABP; TNF- α

Introduction

In 2010, worldwide adult population with diabetes mellitus was estimated to be about 285 millions, and by 2030 it is predicated to have an increase by 54% totaling to about 439 millions. With this remarkable increase in the population of diabetics, it is likely that diabetic nephropathy, a common targeted reno-vascular complication of diabetes, would become the leading cause of end-stage renal disease (ESRD) [1].

Diabetic nephropathy (DN) ensues in both type 1 and type 2 diabetes, and it is characterized by proteinuria and progressive loss of renal functions ultimately leading to ESRD [2]. The DN affects all the renal cellular elements, that is, glomerular endothelia, mesangial cells, podocytes and tubular epithelia. Glomerular damage usually results in proteinuria, due to both increased permeability of plasma proteins, such as, albumin and transferrin, that are normally not freely filtered through the glomerulus and increased synthesis of extracellular matrix (ECM) proteins. Three major renal morphologic changes observed in the glomerular compartment in patients with diabetic nephropathy include, mesangial expansion, basement membrane thickening and formation of Kimmelstiel-Wilson nodules, the latter eventually progress to glomerulosclerosis [3]. More recently, it has been increasingly reported that the

Address correspondence to: Yashpal S. Kanwar, M.D., Ph.D., Department of Pathology, Northwestern University Medical School, 303 E. Chicago Ave, Chicago, Illinois 60611, ☎ (312) 503-0004, ✉ (312) 503-0627, ✉ y-kanwar@northwestern.edu.

renal tubulo-interstitium plays an integral role in the pathogenesis of diabetic nephropathy. The proximal tubules are particularly susceptible to injury associated with diabetes since they are subjected to a prolonged exposure to various metabolic and hemodynamic perturbations. This invariably is followed by setting in of tubulo-interstitial fibrosis that is fostered by the action of inflammatory and pro-fibrogenic cytokines with a consequential accumulation of extracellular matrix (ECM). In DN, progressive tubulo-interstitial fibrosis represents a final common pathway of chronic renal failure, and it is as a result of increased production or altered degradation of ECM components. In diabetes, hyperglycemia is a major driving force in initiating metabolic and signaling events of various pathways that inevitably leads to amassing of ECM accompanied with fibrosis. With the decline in the number of nephrons, that parallels an increasing degree of fibrosis, interstitial scarring replaces the spaces left by nephrons lost in this pathological process. The net result would be impaired renal function [4]. With respect to the interplay between the tubulo-interstitial and glomerular compartment, as the diabetic nephropathy progresses, damage to the glomeruli induce changes in their microcirculation that result in hyperfiltration in the remaining previously unaffected glomeruli with an inevitable increase in the intra-glomerular pressure. The hyperfiltration with intra-glomerular hypertension insidiously damages the remaining glomeruli and tubuli, thus leading to further glomerulosclerosis and tubulo-interstitial fibrosis with first expected progressive and then later precipitous decline in the GFR [5]. Incidentally, a role of the tubules in promoting glomerular hyperfiltration has also been proposed [6]. This dogma of vicious cycle of intra-glomerular hypertension and glomerulosclerosis is supported by the fact that therapeutic measures in reducing initial hyperfiltration have a tremendous beneficial effect in the maintenance of normal GFR [7].

Clinically, microalbuminuria is considered the earliest manifestation for the onset of diabetic nephropathy [8]. However, pathologic abnormalities have been reported to precede much before the onset of microalbuminuria. When the GFR decreases, 20 - 40% of type 2 diabetic patients with microalbuminuria progress to overt nephropathy. Incidence wise at least 20% of patients with diabetic nephropathy progress to ESRD, usually 15-20 years following the onset of nephropathy [2]. Thus, assessing renal functions, *i.e.*, GFR, earlier during the course of disease and dampening the progression to ESRD in diabetic nephropathy would be crucial to reduce the cost of dialysis and transplantation. Usually, the prognosis in such patients with diabetes and ESRD is grim, with <50% of patients surviving beyond 5 years following the established diagnosis of diabetic nephropathy. At times, some patients have a decrease in GFR and may progress to ESRD “without” having significant albuminuria. However, in most of the patients, especially those with newly diagnosed diabetics, GFR begins to decrease prior to the appearance of microalbuminuria. Thus, in view of the weak correlation between proteinuria/albuminuria and GFR it would suggest that the urinary albumin lacks both sensitivity and specificity to detect “early” stages of diabetic nephropathy [9]. Interestingly, in chronic cases of diabetic nephropathy renal function correlate better with the degree of tubulo-interstitial injury than that of the glomerular lesions [10], suggesting perhaps one should search for tubular markers to gauge renal functions in the management of patients with diabetic nephropathy.

Interestingly, proteinuria may be one of the common pathologic functional links between the tubulo-interstitial and glomerular compartments of the kidney [11]. Intriguingly, during proteinuric phase in a given glomerular disease process the proximal tubular epithelial cells (PTEC) may assume a pro-inflammatory and pro-fibrotic role in which they begin to expresses a variety of chemokines and thus initiate certain phlogistic signals that culminate into progressive tubular injury and interstitial inflammation and fibrosis [12]. The extent of tubular damage depends upon factors, such as, high glucose and advanced glycation end-products (AGEs), besides the excessive amount of albumin filtered across the glomerular capillary barrier [11, 13]. These set of events would be difficult to dissect out if one has to

assess precisely the tubular functions given the sole dependence on micro-albuminuria as a predicative marker for the progression of diabetic nephropathy. Since in some of the literature reports tubular involvement has been described even in early stages of diabetic nephropathy, thus an accurate assessment for the progression of renal damage markers other than microalbuminuria would be warranted [11]. Furthermore, some patients with microalbuminuria have advanced renal pathological changes for which therapy is less effective than earlier stages of the disease. Therefore, novel biomarkers for earlier diagnosis of diabetic nephropathy are crucially required. Moreover, the incidence rate of diabetes-induced ESRD remains high despite good control of blood glucose levels and adequate treatment with appropriate regimens, indicating the need of new therapeutic targets for better management outcome and successful prevention of DN in diabetic patients.

In recent years there has been an active growing interest in alternative biomarkers that might provide a more sensitive and rapid means of detecting progression of diabetic nephropathy. In this regard, biomarkers that reflect tubular damage have been proposed by various investigators [14 -17]. The association between markers of glomerular damage like albuminuria and markers of tubular involvement should allow to better unravel the severity of glomerular damage. Often one observes that the tubulo-interstitial damage better predicts the progression of renal insufficiency. Among the tubular urinary biomarkers the three most employed and studied are neutrophil-gelatinase associated lipocalin (N-gal), kidney injury molecule 1 (KIM-1) and liver-fatty acid-binding protein (L-FABP) [18]. The other more recently proposed urinary biomarkers are also discussed in this review.

Neutrophil Gelatinase Associated Lipocalin (Ngal)

Neutrophil-gelatinase associated lipocalin (Ngal), also known as oncogene 24p3, uterocalin, siderocalin or lipocalin 2, is a 24 kDa secreted glycoprotein, which was originally purified from a culture of mouse kidney cells transfected with simian virus 40 (SV-40). Follow-up studies revealed that it is a member of lipocalin family of proteins that transport small hydrophobic ligands. Since then, Ngal expression has been reported in several normal tissues where it serves to provide protection against bacterial infection and modulate oxidative stress. Its expression is also dysregulated in several benign and malignant neoplasms. Its small size, secreted nature and relative stability have led to it being explored as a diagnostic as well as prognostic biomarker in numerous diseases [19 - 21]. Ngal is expressed in renal tubular epithelium, and a rise in urinary concentrations may provide an indication of acute renal injury that is detectable before the rise in serum creatinine concentration [21].

In healthy adults, Ngal and other lipocalins (M_r range: 17 - 43 kDa), including retinol-binding protein 4, α 1-microglobulin and possibly also liver-type fatty acid-binding protein 1 and prostaglandin D synthase are freely filtered across the glomerular capillary. Following which, they bind to multi-ligand scavenger-receptor megalin (expressed abundantly and specifically on the brush borders of proximal tubules), and they are taken up efficiently by endocytosis along with albumin and β 2-microglobulin. Impaired tubular reabsorption due to specific saturation of the endocytic pathway in the setting of heavy proteinuria or dysfunction of proximal tubules should contribute in part to increased urinary Ngal excretion [22]. In the case of kidney injury, an early and rapid Ngal induction is known to occur. Its biological role relates to the preservation of cellular functions, attenuation of apoptosis and an induction of proliferation in response to cellular loss [23, 24]. As expected, gene expression studies in AKI have demonstrated a rapid and massive up-regulation of Ngal mRNA in the distal nephron segments, specifically in the thick ascending limb of Henle's loop and the collecting ducts [25]. The resultant synthesis of Ngal protein in the distal nephron and secretion into the urine comprises the major fraction of urinary Ngal.

Animal models of AKI-to-CKD transition have identified Ngal as one of the most up-regulated genes and thereby increased expression of Ngal protein in the kidney, thus implicating its possible role to serve as a potential biomarker of chronically injured kidney [26]. In fact, uNgal excretion has been well-correlated with serum creatinine levels, and inversely with residual glomerular filtration rate. The latter's correlation was found to be much closer than with serum creatinine. Multivariate models validate these correlations, thus confirming that in these patients uNgal is a better predictor of GFR than serum creatinine [19]. Urine Ngal may also represent an early biomarker for the degree of chronic injury in patients with IgA nephropathy and lupus nephritis, but it may also be increased in urinary tract infections [27]. Similarly, in a study in patients with type 2 diabetic nephropathy, uNgal concentrations at baseline correlated with follow-up levels of eGFR, serum creatinine and cystatin C [28]. In the largest study reported to date, 96 subjects with CKD stages 2 to 4 resulting from a variety of causes were followed up for a median time of 18.5 months; during which 32% of the subjects reached the composite end point for CKD progression (doubling of baseline serum creatinine and/or onset of ESRD) [19]. Both urine and plasma Ngal levels at baseline predicted CKD progression that was independent of other factors, such as, eGFR and age. High values at baseline for both serum and uNgal were associated with a significantly faster evolution to the composite end point. Another study carried out in patients with diabetic nephropathy elevated uNgal was reported to be associated with the progressive course of the disease leading to ESRD [28]. However, this correlation did not hold true following the adjustment for other known disease progression parameters, namely proteinuria [18]. Probably the increased urinary excretion of Ngal in the setting of proteinuria reflects an impaired tubular reabsorption similarly to other small proteins like RBP and the α -2-u-globulin lipocalin that enters the cell by megalin-mediated pathway and is readily trafficked to lysosomes for subsequent degradation [29]. Higher uNgal levels have also been found associated with high incidence of CKD stage 3. However adjustment for urinary creatinine and albumin concentration negated this association. Additional studies are needed to confirm these findings and to assess the utility of uNgal as a marker of CKD [30]. Finally, due to the strong link with proteinuria, its use in routine assessment of renal functions in patients with CKD with background of diabetic nephropathy may not be advisable at present.

Kidney Injury Molecule 1 (KIM-1)

KIM-1 is a type 1 transmembrane protein with extracellular immunoglobulin and highly O-glycosylated mucin subdomains. The extracellular ectodomain of KIM-1 is detached by metalloproteinases and is excreted into urine. This ectodomain protein segment has been suggested to be a quantitative marker of AKI. The increased expression and enhanced synthesis of KIM-1 occur mainly in the areas of tubulo-interstitial lesions with inflammation and fibrosis. Interestingly, the induction of KIM-1 in tubules also occurs in acute proteinuria. On the other hand, due to the ability of KIM-1 to interact with other proteins, it may actively modulate the processes related to damage or repair [31]. KIM-1 is primarily found in regions with influx of interstitial macrophages and pre-fibrotic lesions, and KIM-1 positive tubular cells show a dedifferentiated phenotype, which suggest a role of KIM-1 in tubular fibrosis [32]. KIM-1 is also expressed in the proximal tubules and is up-regulated following acute renal ischemia. Therefore, Kim-1 is considered a potential novel urinary biomarker in the early detection of AKI. It might be especially beneficial in the diagnosis of ischemic ATN [33], in view of the fact that urinary KIM-1 levels become detectable within 24 hrs following acute tubular necrosis [34]. Urinary KIM-1 (uKIM-1) levels may also be elevated after exposure to a variety of nephrotoxic agents, even when serum creatinine concentration is normal [20]. Based on its early and time-dependent increase, its remarkably elevated levels and its high accuracy and sensitivity of detection, uKIM-1 is considered to be the best biomarker for gauging drug-induced proximal tubular injury. Conceivably, KIM-1

facilitates in the repair of the damaged tubulo-interstitial compartment by removing cellular debris and apoptotic bodies. In spite of the reported protective functions of KIM-1 in AKI, there is ample evidence describing its role in chronic injury in CKD. In a kidney biopsy study of 74 patients with CKD having various etiologies, KIM-1 was primarily expressed at the luminal side of dedifferentiated proximal tubules in areas with fibrosis and inflammation [35]. Kim-1 was expressed in dedifferentiated tubular epithelium in CKD similar to seen in AKI, which also suggested a role for KIM-1 in tubular fibrosis for some of the chronic renal diseases. As a consequence, KIM-1 can also be regarded as a marker of the more chronic fibrotic disease processes. The magnitude of KIM-1 expression in tubular epithelial cells correlates well with the tissue levels of osteopontin and α -smooth muscle actin expression, and it also co-localizes with these 2 markers in tubulo-interstitial damage [36]. However, it remains to be seen whether or not stimulation of KIM-1 is beneficial in boosting the processes that restore tubular functions. Interestingly, rats with protein overload nephropathy have increased urinary KIM-1 excretion, as do patients with proteinuria in the CKD state, who may be at risk of progression to renal failure [37]. This biomarker therefore seems to be a promising candidate for noninvasive monitoring of the chronic renal disease states including diabetic nephropathy. Indeed, uKIM-1 is reduced by anti-proteinuric therapy, irrespectively of the conventional treatment regimen or reduction in blood pressure [17]. It is likely that the decrease in uKIM-1 by anti-proteinuric therapy may be related to the amelioration of the tubulo-interstitial injury. The available data, however, do not conclusively establish that KIM-1 has selective prognostic value for monitoring the disease progression in diabetic nephropathy [18, 38, 39]. Future long-term studies are needed to investigate the independent prognostic impact of KIM-1 and proteinuria. If so, it might be worthwhile to test whether targeting KIM-1, in addition to anti-proteinuric therapy, can improve the outcome of progressive loss of renal function in diabetic nephropathy. Finally, it is worth mentioning here that the limitations referred to above for Ngal as a marker of chronic kidney disease progression may also apply to KIM-1.

Liver-Fatty Acid-Binding Protein (L-FABP)

Liver-fatty acid-binding protein (L-FABP) is an intracellular carrier protein that is expressed in proximal tubules of the kidney and liver. Although its precise function is unknown it is believed to be endowed with protective functions [40]. L-FABP in the kidney has been postulated to represent an endogenous antioxidant capable of suppressing tubulo-interstitial damage [41]. Its urinary excretion is also increased in the setting of CKD. By immuno-histochemical staining of renal biopsy specimens, it has been shown that urinary LFABP (u-LFABP) excretion is associated with structural and functional tubular damage [42]. This was confirmed in a number of disease processes with significant proteinuria leading to tubular damage and chronic kidney disease (CKD), including minimal-change nephrotic syndrome, lupus nephritis and diabetic nephropathy [43].

The increased filtered protein across the glomerular barrier imposes a tremendous stress on proximal tubules and causes accelerated excretion of L-FABP from the tubular compartment into urine. Thus, the level of urinary L-FABP for the most part reflects the extent of tubulo-interstitial damage. Furthermore, uL-FABP correlates well with the progression of chronic kidney disease [43]. Thus, uL-FABP can probably serve as a useful marker for tubulo-interstitial damage. To add further insult to the injury an overload of free fatty acids (FFA) bound to albumin may also induce oxidative stress in proximal tubules in various proteinuric states. Incidentally, L-FABP expressed in human proximal tubules participates in intracellular FFA metabolism, and conceivably exerts anti-oxidative effects on the progression of tubulo-interstitial damage [44]. Overall, although urinary L-FABP may serve as a useful biomarker of tubular damage since it is selectively expressed in the renal tubules, however, injury to the glomerular compartment needs to be excluded [40]. In recent years,

the clinical usefulness of uL-FABP as a prognostic biomarker in diabetic nephropathy has also been evaluated [45]. Urinary L-FABP accurately reflected the severity of diabetic nephropathy in type 2 diabetes, and its levels were high even in patients with normal trace amounts of excretion of albumin [45]. Moreover, higher excretion of urinary L-FABP was regarded as a risk factor for progression of diabetic nephropathy. Incidentally, L-FABP levels in serum do not affect the u-LFABP excretion, which suggests that the LFABP measured in urine is primarily derived from the tubular cells. The uL-FABP excretion increases with the deterioration of renal functions, with no relation to urinary protein/albumin excretion. However, uL-FABP was found to be increased in patients with hypertension or diabetes even in the absence of kidney damage. Furthermore, in diabetic nephropathy uL-FABP did not show an independent relationship with decline in renal functions when adjusted for proteinuria [18]. Therefore, data regarding uL-FABP as a reliable clinical biomarker for monitoring the progression of diabetic nephropathy awaits further evaluation [38, 39].

Biomarkers and Glomerular Hyperfiltration

More recently tubular biomarkers were studied also in patients affected by DN during the stage of glomerular hyperfiltration (GHF) [5]. The results of this study demonstrated that GHF group had higher urinary Ngal and KIM-1 levels, but serum Ngal levels were lower compared to diabetic patients with normal GFR and the control groups. Also, slightly lower serum N-GAL and increased urinary Ngal levels were seen in the group with normal GFR compared with control cohorts; however, no statistical differences in the urinary Ngal values were noted among these three groups. Further analyses indicated that eGFR was positively correlated with urinary Ngal and KIM-1, but negatively with serum Ngal in the GHF group. The authors thus concluded that the higher urinary excretion of biomarkers seen in diabetic patients with GHF compared to normal GFR and control groups supports the contention that GHF is deleterious to the overall biology of tubules in diabetic nephropathy [5]. These data raise the possibility that Ngal may become a useful noninvasive tool for the early detection of incipient nephropathy and for estimating the severity of kidney involvement. However, until further results are available, periodic measurements of microalbuminuria and serum creatinine (for estimated GFR calculations) remain the standard of care for screening of patients suspected having diabetic nephropathy. Nevertheless, Ngal represents a novel and independent risk predictor for progression and severity of the renal disease. Furthermore, a recent study reported that decreased urinary levels of KIM-1 and Ngal were associated with regression of microalbuminuria in patients with diabetic nephropathy [17]. Taken together, Ngal, KIM-1 and L-FABP do not seem to unquestionably represent useful in gauging the disease progression in patients with previously established diagnosis of diabetic nephropathy [38, 39].

Inflammatory Cytokines as Biomarkers in Diabetic Nephropathy

Literature reports during the past decade link pathogenesis of diabetic nephropathy to inflammatory processes characterized by leukocyte infiltration with elaboration of cytokines at various stages of evolution of its lesions in the glomerular compartment [46]. The inflammatory cytokines act as pleiotropic polypeptides that regulate inflammatory and immune responses, thus providing important signals in various pathologic and physiologic processes, including diabetic nephropathy [47]. In recent years, inflammation and activation of the immune system are believed to be involved in the pathogenesis of diabetes and its microvascular complications. Pro-inflammatory, Th1, Th2, and Th17 cytokines, as well as transforming growth factor-beta (TGF- β) have been reported to take part in the development and progression of diabetic nephropathy [46]. In this regard, gene polymorphism of cytokines and their receptors may exhibit functional variations, the delineation of which may

be useful to predict the susceptibility and progression of diabetic nephropathy [48]. Certainly, improved knowledge of recognizing cytokines as significant pathogenic mediators in diabetic nephropathy would open new avenues for development of novel potential therapeutic agents in the future. In patients with Type 2 diabetes, urinary excretion of tumor necrosis factor- α (TNF- α) is elevated and correlates with the severity of renal damage confined to both glomerular and tubulo-interstitial compartments, suggesting a significant role for TNF- α in the pathogenesis and progression of renal injury in diabetes mellitus [49, 50]. Up-regulation of TNF receptors 1 and 2 (TNFR1 and TNFR2) have been described to be associated with the increased risk for ESRD [51]. Although, these two markers display significant correlations, it is the TNFR1 which exhibit much higher correlation for the progression of diabetic nephropathy to ESRD. Having elevated concentrations of circulating TNFRs in patients with type 2 diabetes at baseline or at the onset are very strong predictors of the development of subsequent progression to ESRD in patients with or without proteinuria [51].

In terms of excretion of inflammatory cytokines, urinary concentrations of 27 cytokines in type 2 diabetic patients with normo- and micro-albuminuria by multiplex bead immunoassay have been evaluated. Urinary levels of IL-8, IP-10, MCP-1, G-CSF, EOTAXIN, RANTES, and TNF- α in micro-albuminuric patients were significantly increased compared to those in normo-albuminuric patients or controls. GM-CSF, MIP-1 α , and MIP-1 β levels were more elevated in micro-albuminuric patients than in controls. IP-10 and MCP-1 levels were correlated with urinary albumin excretion rate ($r = 0.668$ and 0.544), and estimated glomerular filtration rate ($r = -0.454$ and -0.418). EOTAXIN, GM-CSF, IP-10, MCP-1 and RANTES levels were correlated with HbA1c ($r = 0.457, 0.466, 0.678, 0.567, 0.542$, respectively). These results indicate that urinary cytokine determination might be useful for the early diagnosis and management of patients with diabetic nephropathy [52].

Exploration of Newer Biomarkers

Urine proteome analysis is rapidly emerging as a novel tool for the diagnosis and prognosis in various disease states [53, 54]. A great interest has been devoted to the proteomic approach for identification of diabetic nephropathy related biomarkers in urine [54, 55]. In this regard, Alkhalaf *et al.* provided the data for the first independent confirmation that profiling of the urinary proteome/peptideome by capillary electrophoresis-mass spectrometry (CE-MS) can adequately identify subjects with DN [55]. Their results although indicated several false positive upregulation/downregulation of peptides; however, dysregulation of collagen fragments was consistently observed, and it is further discussed below (*vide infra*).

Orosomucoids

Jiang *et al.* also utilized proteomic approach, and their study suggested that urinary orosomucoid could be another diabetic nephropathy (DN) related biomarker, which is associated with the development and progression of DN [56]. Furthermore, increased urinary orosomucoid excretion rate (UOER) has been regarded by some investigators as an independent risk factor for DN [56]. In another study, the clinical significance of the urinary excretion of two peptides was evaluated [57]. These peptides were: SGSVIDQSRVLNLGPITR (uromodulin precursor, m/z 1912) and IGP^{Hyp}GP HypGLMGPP (present in the collagen- α -5(IV) chain precursor, m/z 1219). They were measured by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI/MS) in microalbuminuric or nephropathic diabetic patients and in non-diabetic nephropathic patients. A progressive increase in the abundance of the ion at m/z 1219 and a decrease in the abundance of the ion at m/z 1912 were noted in diabetic microalbuminuric, diabetic-nephropathic and nephropathic patients. Linear correlations were noted between the serum

creatinine values and the abundance of ions at m/z 1219 and m/z 1912. Correlations between the MALDI data and the estimated glomerular filtration rate (eGFR) were also found, while relationship with urinary albumin excretion was noted only in a fraction of subsets of these patients. Analysis of “receiver operating characteristic curves” revealed a sensitivity of ~96% and a specificity of ~84% for these two ionic species, thus enabling to distinguish diabetic patients with different degrees of nephropathy from healthy subjects. These data would suggest that the urinary abundance of these two peptides at m/z 1219 and m/z 1912, as determined with MALDI/MS, may be considered as a possible diagnostic tool for monitoring kidney functions and for the determination of progression toward renal failure in diabetic patients [55, 58 - 61].

Extracellular Matrix (ECM) related proteins/peptides

One pathological response to tissue injury is the development of fibrosis, which involves notable accumulation of extracellular matrix (ECM) in both glomerular and tubulo-interstitial compartments. The major factors that regulate ECM in diabetes are thought to be related to an imbalance in the expression/activity of pro-fibrogenic cytokines, *e.g.*, TGF- β , and protease/anti-protease systems, *i.e.*, matrix metallo-proteinases (MMPs) [62]. The key markers and regulators of tissue fibrosis have been investigated extensively in the pathogenesis of diabetic nephropathy [54], but whether their levels in biological fluids have any clinical utility remains to be established. Their expression is modulated by various signaling pathways, including oxidant stress. In any instance, the assessment of type IV collagen and connective tissue growth factor (CTGF) in plasma and urine may hold promise in diabetic renal disease [63, 64]. In this regard, there are literature reports which suggest that the increase in urinary type IV collagen correlates well with the amount of urinary albumin, but also with the changes in the GFR [65, 66]. Such notions were confirmed by the CE-MS proteomic studies of Alkhalaf *et al.* (*vide supra*), which indicated that the urinary collagen fragments can serve as specific biomarkers for diabetes-induced renal damage.

Oxidative Stress Markers

With respect to oxidative stress, it is known to contribute to the progression of diabetes itself as well as its complications, *i.e.*, diabetic nephropathy [67]. Interestingly, a significant correlation between the content of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a product of oxidative DNA damage, in urine or leukocytes and the severity of diabetic nephropathy and retinopathy has been reported [68]. Here, the authors investigated whether 8-oxodG in urine or leukocytes is associated with the progression of diabetic nephropathy. Their findings provided the evidence that increased oxidative stress has a primary role in the pathogenesis of diabetic nephropathy. It is likely that a “local tissue” enhancement of oxidative stress in a diabetic kidney might explain the possible linkage between the increased urinary excretion of 8-oxodG and the development of nephropathy. Consensus at the moment is that 8-oxodG in the urine may be a useful clinical biomarker to predict the development and progression of diabetic nephropathy in diabetic patients.

Enzymes

Other studies evaluated glomerular vs tubular involvement in diabetic nephropathy using urinary biomarkers of glomerular fibrosis (collagen IV), proximal tubular damage (α -glutathione-S-transferase, GST) and distal tubular damage (π GST). The most common abnormality noted was elevated urinary collagen IV (glomerular, 35%) compared to α GST (proximal tubule, 18%) or π GST (distal tubule, 15%). The proportion of patients with abnormal biomarkers increased across the normo-, micro- and macro-albuminuric groups. For collagen IV excretion the respective increase was: 26%, 58% and 65%, and for π GST it was 11%, 25%, 35%, while for α GST increase was minimal. In patients with diabetes, these urinary biomarkers appear to delineate renal damage that is related to, but distinct from,

urine albumin/creatinine ratios. The markers of glomerular fibrosis and distal tubular damage correlated most closely to the degree of albuminuria. Thus, it seems that these biomarkers can detect early renal disease with greater specificity and sensitivity than the albumin/creatinine ratio [63].

Advanced Glycation End-products (AGEs)

Another crucial pathogenetic aspect of diabetes relates to the accentuated generation of advanced glycation end products (AGEs), which accumulate in the kidney and are excreted in the urine. The usefulness of the urinary excretion of proteins modified by advanced glycation as biomarkers for diabetic nephropathy in individuals with type 1 or type 2 diabetes has been evaluated recently [69]. In patients with both type 1 and 2 diabetes, a highly significant association between the degree of albuminuria and urinary AGE-modified proteins was found. Exclusive to patients with type 1 diabetes, urinary excretion of the AGE carboxymethyllysine correlated with the albumin excretion rate (AER); whereas patients with type 2 diabetes and macro-albuminuria had an increase in urinary methylglyoxal, an intermediary compound of AGE. These changes were independent of radioisotopic measurements of glomerular filtration rate (GFR). Thus it seems that urinary excretion of proteins modified by AGEs may be useful biomarkers that are correlative of albuminuria in patients with type 1 and 2 diabetes, but not that of glomerular renal functions, *i.e.*, GFR [69].

Podocyte markers

Glomerular podocytes detachment following injury and their subsequent spillage into the urine conceivably play a crucial role in the pathogenesis and progression of diabetic nephropathy (DN). Quantification of messenger RNA (mRNA) expression in urinary sediment by real-time PCR is emerging as a noninvasive method of screening DN-associated biomarkers related to podocyte injury. Zheng *et al.* evaluated if the urinary mRNA profile of podocyte-associated molecules may provide important clinical insights into the different stages of diabetic nephropathy [70]. The urinary mRNA levels of all genes studied were significantly higher in the DN group compared with controls, and mRNA levels increased with the progression of diabetic nephropathy. Urinary mRNA levels of all target genes positively correlated with both UAE and BUN. The expression of podocalyxin, CD2-AP, α -actin4, and podocin mRNA correlated with serum levels of creatinine. Furthermore, podocalyxin mRNA was found to negatively correlate with eGFR. The urinary mRNA profiles of synaptopodin, podocalyxin, CD2-AP, α -actin4 and podocin were found to increase with the progression of DN, which suggested that quantification of podocyte-associated molecules will be useful biomarkers of progression of renal damage in diabetic nephropathy [70].

Pigment Epithelium-Derived Factor (PEDF)

Another biomarker of DN recently investigated is PEDF, a serine protease inhibitor which regulates extracellular matrix production in the kidney [71]. The association between urinary PEDF (uPEDF) and development of nephropathy among patients with type 2 diabetes has been reported. Compared to controls, uPEDF was significantly higher in patients with diabetic nephropathy. Irbesartan significantly decreased the PEDF expression in the kidneys of diabetic rats as well as of uPEDF. It is conceivable that uPEDF may serve as a novel marker for screening for nephropathy among patients with DN and monitoring the response to therapy [71].

Cystatin C: The Old Story

The still unresolved question that lingers on is how one can assess the progression of renal damage in diabetic nephropathy. Creatinine and its derivative formulae, and creatinine

clearance, may not be the best markers to measure renal function, especially in the presence of glomerular proteinuria/albuminuria [72]. The use of low-molecular weight proteins, namely cystatin C, has been proposed. Blood values of cystatin C (molecular weight 13 kDa) do not add much value to the assessment of renal functions [73]. Cystatin C is easily filtered by the glomeruli, and is reabsorbed and catabolized by renal tubules. As a consequence, cystatin C is measured in urine in trace amounts and is not affected by nonrenal factors, such as, age or body mass. As it is not subjected to the circadian variations, cystatin C can be reliably determined in a single random urine sample. The urinary cystatin C:creatinine ratio is a good indicator of renal tubular dysfunction. With impaired renal tubular function, urine cystatin C concentrations may increase up to 200-fold. Increased excretion of cystatin C in the urine of patients with AKI may predict unfavorable AKI development and need for kidney replacement therapy in the initial phases of the disease, prior to oliguric phase, so that progression of the disease can be prevented. A high stability of cystatin C has been described under routine storage conditions [31]. Even when blood levels of cystatin C and creatinine together were taken into more complex equations, they were found to be insensitive in detecting low-level reduction in the nephron mass, particularly in patients with well-preserved renal functions or in presence of proteinuria [74, 75]. Finally, decades of clinical experience indicates that the “direct” measurement of glomerular filtration rate using exogenous markers seems preferable in the assessment of renal functions. However, the use of exogenous substances, *e.g.*, inulin, makes the matter more complex when the procedural logistics are taken into account. Simplified radioisotopic methods have been proposed, which may be of real practical use in bigger nephrology centers where a substantial volume of patients are seen in nephrology clinics [76]. Thus, it seems that routine estimation of renal functions can be gauged by measurement of plasma creatinine or cystatin C and their derivative formulae, but periodic checking of the true glomerular filtration rate (not eGFR) is advisable. The use of tubular markers to assess the progression of diabetic nephropathy needs further evaluation at present while taking into account the sensitivity and specificity of the biomarkers, and above all their ready feasibility at smaller medical centers globally, which could be easily accessible to all the patients with diabetic nephropathy.

Concluding Remarks

Diabetic nephropathy is the leading cause of chronic kidney disease (CKD), which makes estimation of renal functions a crucial task in the management of patients with diabetes. Formulae have been devised to estimate GFR from serum creatinine and cystatin C but the uncertainty of these estimations is of a particular concern for patients with diabetes. Also, lack of good correlation of albuminuria with renal functions raises uncertainty of AER usefulness as a marker to gauge progression of diabetic nephropathy. In order to improve the assessment of renal damage progression various other urinary biomarkers have been proposed, and the status of most widely employed markers at major medical centers are discussed in this review. The number of new biomarkers is increasing progressively, however, most studies are limited either by their small sample size or their cross-sectional nature, as a result the ability to assess the progression and severity of DN is lacking. Certainly, proteomic approach seems to be promising in order to arrange a panel of parameters related to the decline of renal functions. They would be useful to ascertain the rate of progression of renal damage that slowly occurs rather than to assess the function at single event. For the latter statistical analysis in terms of sensitivity or specificity would be difficult to carry out at single time interval or incidence. Also, no exhaustive data demonstrating the accuracy in detecting the efficacy of treatment to relent the progression of renal failure are available. The assessment of urinary biomarkers is still a subject matter of ongoing investigations and discussion. Their usefulness in predicting the decline of renal functions in DN perhaps should be evaluated by means of prospective, multi-center trials

with a large number of diabetic patients with and without nephropathy for another at least two decades before firm conclusions can be drawn and “concrete” recommendations made.

Acknowledgments

Supported by NIH grant DK60635. We thank Dr. Elisabeth I. Wallner for proof-reading the manuscript.

References

1. Collins AJ, Foley RN, Chavers B, Gilbertson D, Herzog C, Johansen K. United States Renal Data System 2011 Annual Data Report: Atlas of chronic kidney disease & end-stage renal disease in the United States. *Am J Kidney Dis.* 2012; 59(1 Suppl 1):A7, e1–420. [PubMed: 22177944]
2. Kanwar YS, Sun L, Xie P, Liu FY, Chen S. A glimpse of various pathogenetic mechanisms of diabetic nephropathy. *Annu Rev Pathol.* 2011; 6:395–423. [PubMed: 21261520]
3. Tisher CC, McCoy RC. Diabetes mellitus and the kidney. *Perspect Nephrol Hypertens.* 1976; 3:105–128. [PubMed: 55997]
4. Hills CE, Squires PE. The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy. *Cytokine Growth Factor Rev.* 2011; 22:131–139. [PubMed: 21757394]
5. Fu WJ, Li BL, Wang SB, Chen ML, Deng RT, Ye CQ. Changes of the tubular markers in type 2 diabetes mellitus with glomerular hyperfiltration. *Diabetes Res Clin Pract.* 2012; 95:105–109. [PubMed: 22015481]
6. Vallon V, Thomson SC. Renal function in diabetic disease models: the tubular system in the pathophysiology of the diabetic kidney. *Annu Rev Physiol.* 2012; 74:351–375. [PubMed: 22335797]
7. Fabris B, Candido R, Armini L, Fischetti F, Calci M, Bardelli M. Control of glomerular hyperfiltration and renal hypertrophy by an angiotensin converting enzyme inhibitor prevents the progression of renal damage in hypertensive diabetic rats. *J Hypertens.* 1999; 17:1925–1931. [PubMed: 10703891]
8. Mogensen CE, Chachati A, Christensen CK, Close CF, Deckert T, Hommel E. Microalbuminuria: an early marker of renal involvement in diabetes. *Uremia Invest.* 1985; 9:85–95. [PubMed: 3915933]
9. Halimi JM. The emerging concept of chronic kidney disease without clinical proteinuria in diabetic patients. *Diabetes Metab.* 2012 May 21. Epub ahead of print.
10. Thomas MC, Burns WC, Cooper ME. Tubular changes in early diabetic nephropathy. *Adv Chronic Kidney Dis.* 2005; 12:177–186. [PubMed: 15822053]
11. Tang SC, Leung JC, Lai KN. Diabetic tubulopathy: An emerging entity. *Contrib Nephrol.* 2011; 170:124–134. [PubMed: 21659765]
12. Baines RJ, Brunskill NJ. Tubular toxicity of proteinuria. *Nat Rev Nephrol.* 2011; 7:177–80. [PubMed: 21151210]
13. Liao YC, Lee YH, Chuang LY, Guh JY, Shi MD, Huang JS. Advanced glycation end products-mediated hypertrophy is negatively regulated by tetrahydrobiopterin in renal tubular cells. *Mol Cell Endocrinol.* 2012; 355:71–77. [PubMed: 22326994]
14. Matheson A, Willcox MD, Flanagan J, Walsh BJ. Urinary biomarkers involved in type 2 diabetes: a review. *Diabetes Metab Res Rev.* 2010; 26:150–171. [PubMed: 20222150]
15. Fu WJ, Xiong SL, Fang YG, Wen S, Chen ML, Deng RT. Urinary tubular biomarkers in short-term type 2 diabetes mellitus patients: a cross-sectional study. *Endocrine.* 2012; 41:82–88. [PubMed: 21779943]
16. Waanders F, Navis G, van Goor H. Urinary tubular biomarkers of kidney damage: potential value in clinical practice. *Am J Kidney Dis.* 2010; 55:813–6.
17. Vaidya VS, Niewczas MA, Ficociello LH, Johnson AC, Collings FB, Warram JH. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-beta-D-glucosaminidase. *Kidney Int.* 2011; 79:464–470. [PubMed: 20980978]

18. Nielsen SE, Andersen S, Zdunek D, Hess G, Parving HH, Rossing P. Tubular markers do not predict the decline in glomerular filtration rate in type 1 diabetic patients with overt nephropathy. *Kidney Int.* 2011; 79:1113–1118. [PubMed: 21270761]
19. Bolignano D, Lacquaniti A, Coppolino G, Campo S, Arena A, Buemi M. Neutrophil gelatinase-associated lipocalin reflects the severity of renal impairment in subjects affected by chronic kidney disease. *Kidney Blood Press Res.* 2008; 31:255–258. [PubMed: 18600028]
20. Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int.* 2009; 75:285–294. [PubMed: 19148153]
21. Waring WS, Moonie A. Earlier recognition of nephrotoxicity using novel biomarkers of acute kidney injury. *Clin Toxicol (Phila).* 2011; 49:720–728. [PubMed: 21970770]
22. Mori K, Nakao K. Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. *Kidney Int.* 2007; 71:967–970. [PubMed: 17342180]
23. Mishra J, Mori K, Ma Q, Kelly C, Yang J, Mitsnefes M. Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol.* 2004; 15:3073–3082. [PubMed: 15579510]
24. Devarajan P. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease. *Scand J Clin Lab Invest Suppl.* 2008; 241:89–94. [PubMed: 18569973]
25. Schmidt-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P. Dual action of neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol.* 2007; 18:407–413. [PubMed: 17229907]
26. Ko GJ, Grigoryev DN, Linfert D, Jang HR, Watkins T, Cheadle C. Transcriptional analysis of kidneys during repair from AKI reveals possible roles for NGAL and KIM-1 as biomarkers of AKI-to-CKD transition. *Am J Physiol Renal Physiol.* 2010; 298:F1472–83. [PubMed: 20181666]
27. Devarajan P. Biomarkers for the early detection of acute kidney injury. *Curr Opin Pediatr.* 2011; 23:194–200. [PubMed: 21252674]
28. Yang YH, He XJ, Chen SR, Wang L, Li EM, Xu LY. Changes of serum and urine neutrophil gelatinase-associated lipocalin in type-2 diabetic patients with nephropathy: one year observational follow-up study. *Endocrine.* 2009; 36:45–51. [PubMed: 19390997]
29. Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest.* 2005; 115:610–621. [PubMed: 15711640]
30. Bhavsar NA, Kottgen A, Coresh J, Astor BC. Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule 1 (KIM-1) as Predictors of Incident CKD Stage 3: The Atherosclerosis Risk in Communities (ARIC) Study. *Am J Kidney Dis.* 2012; 60:233–240. [PubMed: 22542304]
31. Lisowska-Myjak B. Serum and urinary biomarkers of acute kidney injury. *Blood Purif.* 2010; 29:357–365. [PubMed: 20389065]
32. van Timmeren MM, Bakker SJ, Vaidya VS, Bailly V, Schuur TA, Damman J. Tubular kidney injury molecule-1 in protein-overload nephropathy. *Am J Physiol Renal Physiol.* 2006; 291:F456–64. [PubMed: 16467126]
33. Huang Y, Don-Wauchope AC. The clinical utility of kidney injury molecule 1 in the prediction, diagnosis and prognosis of acute kidney injury: a systematic review. *Inflamm Allergy Drug Targets.* 2011; 10:260–271. [PubMed: 21539514]
34. Bailly V, Zhang Z, Meier W, Cate R, Sanicola M, Bonventre JV. Shedding of kidney injury molecule-1, a putative adhesion protein involved in renal regeneration. *J Biol Chem.* 2002; 277:39739–39748. [PubMed: 12138159]
35. van Timmeren MM, van den Heuvel MC, Bailly V, Bakker SJ, van Goor H, Stegeman CA. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. *J Pathol.* 2007; 212:209–217. [PubMed: 17471468]
36. Huo W, Zhang K, Nie Z, Li Q, Jin F. Kidney injury molecule-1 (KIM-1): a novel kidney-specific injury molecule playing potential double-edged functions in kidney injury. *Transplant Rev (Orlando).* 2010; 24:143–146. [PubMed: 20447817]

37. Zhao X, Zhang Y, Li L, Mann D, Imig D, Emmett N. Glomerular expression of kidney injury molecule-1 and podocytopenia in diabetic glomerulopathy. *Am J Nephrol.* 2011; 34:268–280. [PubMed: 21822010]
38. Tramonti G, Kanwar YS. Tubular biomarkers to assess progression of diabetic nephropathy. *Kidney Int.* 2011; 79:1042–1044. [PubMed: 21527942]
39. Devarajan P. The use of targeted biomarkers for chronic kidney disease. *Adv Chronic Kidney Dis.* 2010; 17:469–479. [PubMed: 21044769]
40. Kamijo-Ikemori A, Sugaya T, Kimura K. Urinary fatty acid binding protein in renal disease. *Clin Chim Acta.* 2006; 374:1–7. [PubMed: 16860300]
41. Kamijo-Ikemori A, Sugaya T, Obama A, Hiroi J, Miura H, Watanabe M. Liver-type fatty acid-binding protein attenuates renal injury induced by unilateral ureteral obstruction. *Am J Pathol.* 2006; 169:1107–1117. [PubMed: 17003471]
42. Yokoyama T, Kamijo-Ikemori A, Sugaya T, Hoshino S, Yasuda T, Kimura K. Urinary excretion of liver type fatty acid binding protein accurately reflects the degree of tubulointerstitial damage. *Am J Pathol.* 2009; 174:2096–2106. [PubMed: 19435794]
43. Kamijo A, Kimura K, Sugaya T, Yamanouchi M, Hikawa A, Hirano N. Urinary fatty acid-binding protein as a new clinical marker of the progression of chronic renal disease. *J Lab Clin Med.* 2004; 143:23–30. [PubMed: 14749682]
44. Kanaguchi Y, Suzuki Y, Osaki K, Sugaya T, Horikoshi S, Tomino Y. Protective effects of L-type fatty acid-binding protein (L-FABP) in proximal tubular cells against glomerular injury in anti-GBM antibody-mediated glomerulonephritis. *Nephrol Dial Transplant.* 2011; 26:3465–3473. [PubMed: 21525165]
45. Kamijo-Ikemori A, Sugaya T, Yasuda T, Kawata T, Ota A, Tatsunami S. Clinical significance of urinary liver-type fatty acid-binding protein in diabetic nephropathy of type 2 diabetic patients. *Diabetes Care.* 2011; 34:691–696. [PubMed: 21273494]
46. Wu CC, Sytwu HK, Lin YF. Cytokines in diabetic nephropathy. *Adv Clin Chem.* 2012; 56:55–74. [PubMed: 22397028]
47. Chen L, Zhang J, Zhang Y, Wang Y, Wang B. Improvement of inflammatory responses associated with NF-kappa B pathway in kidneys from diabetic rats. *Inflamm Res.* 2008; 57:199–204. [PubMed: 18465086]
48. Buraczynska M, Zukowski P, Wacinski P, Berger-Smyka B, Dragan M, Mozul S. Chemotactic cytokine receptor 5 gene polymorphism: relevance to microvascular complications in type 2 diabetes. *Cytokine.* 2012; 58:213–217. [PubMed: 22325341]
49. Kalantarinia K, Awad AS, Siragy HM. Urinary and renal interstitial concentrations of TNF-alpha increase prior to the rise in albuminuria in diabetic rats. *Kidney Int.* 2003; 64:1208–1213. [PubMed: 12969138]
50. Moriwaki Y, Inokuchi T, Yamamoto A, Ka T, Tsutsumi Z, Takahashi S. Effect of TNF-alpha inhibition on urinary albumin excretion in experimental diabetic rats. *Acta Diabetol.* 2007; 44:215–218. [PubMed: 17767370]
51. Niewczas MA, Gohda T, Skupien J, Smiles AM, Walker WH, Rosetti F. Circulating TNF receptors 1 and 2 predict ESRD in type 2 diabetes. *J Am Soc Nephrol.* 2012; 23:507–515. [PubMed: 22266663]
52. Liu J, Zhao Z, Willcox MD, Xu B, Shi B. Multiplex bead analysis of urinary cytokines of type 2 diabetic patients with normo- and microalbuminuria. *J Immunoassay Immunochem.* 2010; 31:279–289. [PubMed: 21113841]
53. Jain S, Rajput A, Kumar Y, Uppuluri N, Arvind AS, Tatu U. Proteomic analysis of urinary protein markers for accurate prediction of diabetic kidney disorder. *J Assoc Physicians India.* 2005; 53:513–520. [PubMed: 16121805]
54. Ben Ameer R, Molina L, Bolvin C, Kifagi C, Jarraya F, Ayadi H. Proteomic approaches for discovering biomarkers of diabetic nephropathy. *Nephrol Dial Transplant.* 2010; 25:2866–2875. [PubMed: 20472580]
55. Alkhalaf A, Zurbig P, Bakker SJ, Bilo HJ, Cerna M, Fischer C. Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy. *PLoS One.* 2010; 5:e13421. [PubMed: 20975990]

56. Jiang H, Guan G, Zhang R, Liu G, Liu H, Hou X. Increased urinary excretion of orosomucoid is a risk predictor of diabetic nephropathy. *Nephrology (Carlton)*. 2009; 14:332–337. [PubMed: 19143942]
57. Lapolla A, Molin L, Seraglia R, Sechi A, Cosma C, Bonfantel L. Urinary peptides as a diagnostic tool for renal failure detected by matrix-assisted laser desorption/ionisation mass spectrometry: an evaluation of their clinical significance. *Eur J Mass Spectrom (Chichester, Eng)*. 2011; 17:245–253.
58. Dihazi H, Muller GA, Lindner S, Meyer M, Asif AR, Oellerich M. Characterization of diabetic nephropathy by urinary proteomic analysis: identification of a processed ubiquitin form as a differentially excreted protein in diabetic nephropathy patients. *Clin Chem* 2007. 2007; 53:1636–1645.
59. Fisher WG, Lucas JE, Mehdi UF, Qunibi DW, Garner HR HR, Rosenblatt KP. A method for isolation and identification of urinary biomarkers in patients with diabetic nephropathy. *Proteomics Clin Appl*. 2011; 5:603–612. [PubMed: 21956890]
60. Jiang H, Guan G, Zhang R, Liu G, Cheng J, Hou X. Identification of urinary soluble E-cadherin as a novel biomarker for diabetic nephropathy. *Diabetes Metab Res Rev*. 2009; 25:232–41. [PubMed: 19177462]
61. Lim SC, Liying DQ, Toy WC, Wong M, Yeoh LY, Tan C. Adipocytokine zinc alpha(2) glycoprotein (ZAG) as a novel urinary biomarker for normo-albuminuric diabetic nephropathy. *Diabet Med*. 2012; 29:945–949. [PubMed: 22211921]
62. Chen S, Jim B, Ziyadeh FN. Diabetic nephropathy and transforming growth factor-beta: transforming our view of glomerulosclerosis and fibrosis build-up. *Semin Nephrol*. 2003; 23:532–543. [PubMed: 14631561]
63. Cawood TJ, Bashir M, Brady J, Murray B, Murray PT, O'Shea D. Urinary collagen IV and piGST: potential biomarkers for detecting localized kidney injury in diabetes--a pilot study. *Am J Nephrol*. 2010; 32:219–225. [PubMed: 20664197]
64. Ban CR, Twigg SM. Fibrosis in diabetes complications: pathogenic mechanisms and circulating and urinary markers. *Vasc Health Risk Manag*. 2008; 4:575–596. [PubMed: 18827908]
65. Sthaneshwar P, Chan SP. Urinary type IV collagen levels in diabetes mellitus. *Malays J Pathol*. 2010; 32:43–47. [PubMed: 20614725]
66. Katavetin P, Susantitaphong, Townamchai N, Tiranathanagul K, Tungsanga K, Eiam-Ong S. Urinary type IV collagen excretion predicts subsequent declining renal function in type 2 diabetic patients with proteinuria. *Diabetes Res Clin Pract*. 2010; 89:e33–e35. [PubMed: 20542586]
67. Kashihara N, Haruna Y, Kondeti VK, Kanwar YS. Oxidative stress in diabetic nephropathy. *Curr Med Chem*. 2010; 17:4256–4269. [PubMed: 20939814]
68. Hinokio Y, Suzuki S, Hirai M, Suzuki C, Suzuki M, Toyota T. Urinary excretion of 8-oxo-7, 8-dihydro-2'-deoxyguanosine as a predictor of the development of diabetic nephropathy. *Diabetologia*. 2002; 45:877–882. [PubMed: 12107732]
69. Coughlan MT, Patel SK, Jerums G, Penfold SA, Nguyen TV, Sourris KC KC. Advanced glycation urinary protein-bound biomarkers and severity of diabetic nephropathy in man. *Am J Nephrol*. 2011; 34:347–355. [PubMed: 21876347]
70. Zheng M, Lv LL, Ni J, Ni HF, Li Q, Ma KL. Urinary podocyte-associated mRNA profile in various stages of diabetic nephropathy. *PLoS One*. 2011; 6:e20431. [PubMed: 21655212]
71. Chen H, Zheng Z, Li R, Lu J, Bao Y, Ying X. Urinary pigment epithelium-derived factor as a marker of diabetic nephropathy. *Am J Nephrol*. 2010; 32:47–56. [PubMed: 20523035]
72. Branten AJ, Vervoort G, Wetzels JF. Serum creatinine is a poor marker of GFR in nephrotic syndrome. *Nephrol Dial Transplant*. 2005; 20:707–711. [PubMed: 15713698]
73. Laterza OF, Price CP, Scott MG. Cystatin C: an improved estimator of glomerular filtration rate? *Clin Chem*. 2002; 48:699–707. [PubMed: 11978596]
74. Stevens LA, Schmid CH, Greene T, Li L, Beck GJ, Joffe MM. Factors other than glomerular filtration rate affect serum cystatin C levels. *Kidney Int*. 2009; 75:652–660. [PubMed: 19119287]
75. Tramonti G, Cipollini I, Annichiarico C, Lorusso P, Panicucci E, Mariani G G. Creatinine clearance, cystatin C, beta2-microglobulin and TATI as markers of renal function in patients with proteinuria. *J Nephrol*. 2012 Jan 30. Epub ahead of print.

76. Bianchi C, Donadio C, Tramonti G. Noninvasive methods for the measurement of total renal function. *Nephron*. 1981; 28:53–57. [PubMed: 7027061]