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Urinary proteomics using capillary electrophoresis coupled to mass spectrometry for diagnosis and prognosis in kidney diseases

Pedro Magalhães[a,b], **Harald Mischak**[a,c], **Petra Zürbig**[a]

[a]Mosaiques Diagnostics GmbH, Hannover, Germany

[b]Hannover Medical School, Hannover, Germany

[c]Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK

Abstract

Purpose of review—Urine is the most useful of body fluids for biomarker research. Therefore, we have focused on urinary proteomics, using capillary electrophoresis coupled to mass spectrometry (CE-MS), to investigate kidney disease in recent years.

Recent Findings—Several urinary proteomics studies for the detection of various kidney diseases have indicated the potential of this approach aimed at diagnostic and prognostic assessment. Urinary protein biomarkers such as collagen fragments, serum albumin, alpha-1-antitrypsin, and uromodulin can help to explain the processes involved during disease progression.

Summary—Urinary proteomics has been used in several studies, in order to identify and validate biomarkers associated with different kidney diseases. These biomarkers, with improved sensitivity and specificity when compared to the current gold standards, provide a significant alternative for diagnosis and prognosis, as well as improving clinic decision making.

Keywords

urinary proteomics; kidney diseases; capillary electrophoresis-mass spectrometry; biomarkers

Introduction

Renal function is essential for maintaining body homeostasis by contributing to the control of blood composition, pressure, volume and pH (1). Proteomics, the analysis of the total protein content of a sample, is a field of research constantly evolving and during the last decade several techniques have been developed to analyze and characterize the proteome of samples from very different origins (2). Urinary proteomics has become an essential tool for the discovery of novel biomarkers in kidney disease. It has been shown to contribute to early

Correspondence to Harald Mischak, Mosaiques diagnostics GmbH, Rotenburger Strasse 20, 30659 Hannover, Germany, Tel: +49 511 554744 13; fax: +49 511 554744 31; mischak@mosaiques-diagnostics.com.

Conflict of interest

H.M. is cofounder and a shareholder of mosaiques diagnostics GmbH. P.M. and P.Z. are employees of mosaiques diagnostics GmbH.

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diagnosis and clinical assessment, based on better insight into kidney disease and its development (3)*.

Current situation of clinical proteomics

In the last two decades, mass spectrometry (MS) has become one of the most prevalent techniques in the detection and characterization of proteins and peptides (4). Techniques such as two-dimensional gel electrophoresis (2-DE), liquid chromatography (LC), surface enhanced laser desorption/ionization (SELDI), and capillary electrophoresis (CE) coupled with MS have had a significant impact in biology and medicine. The advantages and disadvantages of these different proteomics approaches have been described in a number of reviews in detail (5–7). Although a large number of studies have been published on the use of proteomics for the identification of biomarkers for diseases, they often had significant shortcomings, e.g. absence of appropriate statistical assessment, insufficient power, or lack of validation in an independent cohort. Sparked by these observations, recommendations for biomarker research in the field of clinical proteomics were elaborated to improve the validity of biomarkers identified in these studies (8–10).

Recent urinary proteomic biomarker studies

Several clinical urinary proteomic studies in kidney diseases conforming to the above recommendations were recently (last five years) published. However, most of them are based on capillary electrophoresis coupled to mass spectrometry (CE-MS). In the next sections, these studies will be discussed in detail and are listed in Table 1, as well as depicted in Figure 1.

Chronic Kidney Diseases

Chronic kidney disease (CKD) is defined as the progressive loss of kidney function and a reduction in the glomerular filtration rate (GFR) which can result in end-stage renal disease (ESRD). At this stage, the patients require renal replacement therapies like dialysis or kidney transplantation (29). The most common causes of CKD are diabetic nephropathy (DN), hypertension, and glomerulonephritis (30). DN is caused by diabetes mellitus, a chronic metabolic disease, associated with cardiovascular and renal complications. CKD diagnosis is currently obtained by the detection of alterations in estimated glomerular filtration rate (eGFR) and/or albuminuria as indicators of renal dysfunction (29). However, eGFR has limited value in predicting risk of CKD progression unless substantially reduced. Instead (or together with eGFR) albuminuria is often used even though, in a non-negligible number of patients, renal disease progresses despite the absence of albuminuria (31).

Over the last five years CE-MS has been a frequently used proteomics approach to discover urinary biomarkers for the diagnosis and prognosis of CKD (see Figure 2). The basis for all these validation studies was a publication in 2010 (11). Using CE-MS, Good *et al.* were able to define 273 urinary peptide markers for CKD (named the “CKD273-classifier”) in a cohort of 379 healthy controls and 230 patients with CKD derived from different etiologies. These peptide markers were mostly different fragments of various collagens, blood proteins (e.g.

serum albumin, α-1-antitrypsin) and specific kidney-derived proteins (e.g. uromodulin). In order to validate the defined biomarkers, Good *et al.* applied the CKD273-classifier to a set of 144 samples consisting of 34 controls and 110 patients with CKD, showing a sensitivity of 85% and specificity of 100% (AUC=0.96).

This CKD273-classifier was further validated in several independent studies with patients with CKD derived from different origins: The first study was to validate the diagnostic performance in patients suffering from type 2 diabetes. Molin *et al.* compared 137 urine samples (62 patients and 75 controls), based on CKD273-classifier (12). The validation resulted in a similar accuracy (AUC=0.96) as in the initial discovery study (11). Argilés *et al.* examined the CKD273-classifier based on a cohort of 53 patients with CKD at different stages, where the CKD273-classifier enabled prediction of ESRD or death (13). Gu *et al.* also confirmed the ability of the CDK273-classifier to predict progression of CKD in a cohort of 797 individuals. With an average follow-up time of 4.8 years, this validation effectively predicted development of renal dysfunction and cardiovascular complications (14). Zürbig *et al.* studied the prognosis of DN with the use of the CKD273-classifier in a longitudinal study of normoalbuminuric diabetic individuals (15). The authors were able to predict progression to macroalbuminuria over 5 years with an AUC of 0.93 superior to baseline albuminuria (AUC=0.67). Furthermore, the CKD273-classifier identified the progressors in 65% of the case subjects earlier than urinary albumin, the standard clinical test. On average the CKD273-classifier detected progression 1.5 years earlier than microalbuminuria. In a similar study with a follow-up over 3 years, Roscioni *et al.* evaluated the prediction of the transition from normo- to microalbuminuria and from micro- to macroalbuminuria (16). In a cohort of 44 patients with type 2 diabetes, the CKD273-classifier allowed assessment of early renal risk in diabetic patients. Recently, the CKD273-classifier was also validated in a cohort of 18 patients with CKD stage 4-5 (of whom six had hypertensive nephropathy) and 17 healthy controls, in order to compare the prevalence of CKD and progression beyond albuminuria (17)*. Special intention was paid to the diagnosis of hypertensive nephropathy, because it is surprisingly understudied, and proteomic analyses has never been performed (32). The results showed that the classifier performed equally well in patients with hypertensive nephropathy as in patients with other CKD causes.

A further validation of the classifier was obtained in assessing treatment. Using the CKD273-classifier, Andersen *et al.* analysed the effects of Irbesartan, an angiotensin receptor blocker, in a cohort of patients with type 2 diabetes with microalbuminuria (33). The scores of the classifier changed significantly after treatment with Irbesartan to values of more healthy individuals. Furthermore, the authors identified several peptides that showed significant change after 2 years with Irbesartan treatment in contrast to placebo. After Irbesartan treatment, an increase of collagen fragments showed the most significant association with DN. These results have led to the initiation of the PRIORITY study (34)* in order to evaluate the value of urinary proteomics in patient stratification for the presence of early signs of DN in an interventional trial. In this context, 165 type 2 diabetes patients were analysed with the CKD273-classifier assessing the benefits of stratifying patients for intervention with urinary proteomics (18).

Recent validation studies used much larger cohorts of individuals with different stages of CKD for the assessment of the prognostic potential of the CKD273-classifier: In a large cross-sectional multicenter cohort of 1990 individuals, including 522 with follow-up data, Schanstra *et al.* correlated the CKD273-classifier score with the estimated glomerular filtration rate (eGFR) and its decline (19)**. They validated that the CKD273-classifier performed significantly better in detecting and predicting progression of CKD than the current clinical standard, urinary albumin. The classifier was also more sensitive for identifying patients with rapidly progressing CKD. Compared with the combination of baseline eGFR and albuminuria (AUC=0.76), the addition of the CKD273-classifier significantly improved CKD risk prediction (AUC=0.83). In addition, when applying the Oxford Evidence-Based Medicine (EBM) and Strength of Recommendation Taxonomy (SORT) guidelines, additional evidence was obtained which supports the CKD273-classifier's value in predicting CKD progression (35). In a recent study, the classifier and urinary albumin excretion was compared to predict the progression of CKD, involving 2672 patients at different CKD stages. The findings confirmed that the CKD273-classifier has a better performance at early stages of disease. On the other hand urinary albumin was a better predictor for progression at a late stage. In moderately advanced disease, these two tests had similar predictive abilities (20)**.

In spite of its invasive nature and risks, kidney biopsy is currently required for precise diagnosis of many chronic kidney diseases (CKDs). Therefore, specific biomarkers for different types of CKD, such as ANCA-associated vasculitis, IgAN, and DN, were defined using urinary proteome analysis. The value of CE-MS-based proteomics to differentiate types of CKD etiologies was demonstrated, but mostly in small patient populations (36–39). A recent study from Siwy *et al.* (21)** was designed with the aim of identifying specific urinary peptide markers for main types of CKD using datasets from a large cohort of 1180 individuals with CKD. For seven different types of CKD (primary focal segmental glomerulosclerosis, IgA nephropathy, minimal-change disease, membranous nephropathy, diabetic and hypertensive nephropathy, lupus nephritis, and vasculitis-induced kidney disease), several potential urinary biomarker peptides (ranging from 116 to 619 peptides, see Table 1) were defined and combined into classifiers specific for each CKD. These classifiers were validated in an independent cohort and showed good to excellent accuracy for discrimination of one CKD etiology from the other (AUCs ranged from 0.77 to 0.95).

Polycystic kidney disease

The most common genetic disorder in kidney disease (1:400 and 1:1000 individuals) is autosomal dominant polycystic kidney disease (ADPKD), a mutation in PKD1 (85% of cases) and PKD2 (15% of cases), results in cyst formation and loss of renal function (40;41). Kistler *et al.* (22) performed CE-MS analysis to identify peptide markers for ADPKD. Urine samples from 41 ADPKD patients and 189 healthy controls were analyzed leading to the identification of 142 consistent peptide biomarkers associated with ADPKD. Combined in a panel, these markers classified an independent validation cohort of 251 ADPKD patients from five different centres and 86 healthy controls with 84.5% of sensitivity and 94.2% of specificity (22). As in the CKD273 classifier, most of the urinary biomarkers were fragments of collagen, which may indicate changes in extracellular matrix during cyst formation. In a

recent study involving 221 ADPKD patients aged 15-46 years and followed-up for 10-13 years, urinary proteome analysis using CE-MS generated a 20 peptide-based classifier allowing prediction of progression risk in ADPKD patients to ESRD (23)**. Prediction of the proteases involved in generation of these peptides, was performed suggesting modification of matrix metalloproteinases and cathepsin activity in ADPKD patients progressing to ESRD.

Fabry induced kidney disease

Fabry disease is a rare X-linked lysosomal inherited disorder characterized by deficient enzymatic activity of α-galactosidase A (GLA) (42), which can cause a wide range of systemic symptoms (like pain, kidney involvement, and cardiac manifestations). Fabry induced kidney disease (FIKD) is a glomerular disease, which was investigated by Kistler *et al.* (24). Urine samples from 35 treatment-naive female Fabry patients and 89 age-matched healthy controls were collected and analysed by CE-MS. A classifier was established based on 64 urinary peptides for FIKD diagnosis, with high specificity (97.8%) and sensitivity (88.2%) in an independent cohort composed by 17 treatment-naive Fabry patients and 45 controls. The most common of the sequenced peptides were collagen and uromodulin fragments. Interestingly, the up-regulated collagen fragments exhibited one of two characteristic C-terminal motivs, PPG or PGP. Furthermore, the uromodulin fragments (all were C-terminal fragments) were also up-regulated as in another recent urinary proteomic study of Fabry disease (43).

Congenital anomalies of the kidney and urinary tract

Congenital anomalies of the kidney and urinary tract (CAKUT) constitute approximately 20-30% of all anomalies identified in the prenatal period (44). Defects can be bilateral or unilateral, and different defects often coexist. Because CAKUT plays a causative role in 30-50% of cases of ESRD in children (45), it is important to diagnose these anomalies and initiate therapy to minimize renal damage, preventing or delaying the onset of ESRD, and providing supportive care to avoid complications of ESRD. CAKUT manifest as structural abnormalities of the kidney, including obstructive uropathy, renal dysplasia and urinary tract malformations (46). CAKUT displays an extensive spectrum of prenatal and postnatal outcomes alternating from death *in utero* to normal postnatal renal function (47).

Posterior urethral valves (PUV), the prototypic bilateral CAKUT is an obstructing membrane in the posterior male urethra. Klein *et al.* (25) studied the fetal urinary peptidome with CE-MS in a cohort of 28 patients with PUV. They identified 26 fetal urinary peptides associated with fetuses with PUV displaying early ESRD. Twelve of these peptides were combined in a model (12PUV-classifier). This classifier allowed correct prediction of postnatal renal function with 88% sensitivity and 95% specificity, in an independent blinded cohort of 38 PUV patients. Collagen fragments were the main constituents of the classifier, and in contrast to the collagen fragments associated with CKD in postnatal urine in adults, the abundance of collagen fragments, was increased in fetal urine of patients with PUV displaying severe ESRD (47).

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Ureteropelvic junction obstruction (UPJO) is a frequent cause of congenital obstructive nephropathy and is characterized by a stenosis between the ureter and the kidney, inducing accumulation of urine in renal pelvis and calyces, called hydronephrosis (48;49). In severe cases this condition is treated surgically. However, in the milder UPJO cases (often) invasive surveillance is necessary during the first years of life to determine whether surgery is necessary. Urinary proteome analysis was employed by Decramer *et al.* in 2006 to determine the presence of urinary markers that could predict the progression of UPJO at an early stage (26). They identified 51 markers that combined in a classifier predicting progression of UPJO with the need of surgical intervention several months in advance. Drube *et al.* (27) validated this classifier in an independent study with 27 pediatric patients. In 19 children <1 year old, the model for UPJO showed 83% sensitivity and 92% specificity. On the other hand, in older patients, the analysis yielded a sensitivity and specificity of 20% and 66%, respectively. This suggests that classifiers should be validated within their context of use, in this case detection of severe UPJO before the age of 1 year. Bandin *et al.* (50) used urinary proteome analysis to suggest that early surgery in UPJO might be beneficial compared to classical conservative clinical surveillance of the disease. At 5-year follow-up urinary proteomes were similar between patients with early surgical correction of UPJO and age matched controls. In contrast, urinary proteomes differed significantly between conservatively followed patients and controls. Analyses of the proteome differences suggested ongoing renal or ureteral remodeling in the conservatively followed patients that was not clinically visible.

High-grade vesicoureteral reflux (VUR) is described by an abnormal condition of flow of the urine from the bladder to the kidney during micturition. This condition is a risk factor for impaired renal function, renal scarring and arterial hypertension, although is not a frequent condition in children. Current diagnosis requires an invasive and highly uncomfortable method - voiding cystourethrography (VCUG). Drube *et al.* studied a cohort of 73 children with the use of CE-MS analysis (28). In this case-control study, a VUR-classifier was established in 18 patients with primary VUR grade IV or V, distinguishing these from 19 patients without VUR. This VUR-classifier was independently validated in a blinded cohort of 17 patients with VUR grade IV or V and 19 patients without VUR with a sensitivity of 88% and a specificity of 79%. Five of the urinary peptides of the classifier were sequenced. Three were fragments of collagen alpha-1 (I) chain and the other two were fragments of sodium/ potassium-transporting ATPase and of CD99 antigen.

Conclusion

Urinary proteomics using CE-MS has identified a number of peptide classifiers that can become an important alternative for diagnosis and prognosis of kidney disease compared to the current, often poorly performing, invasive clinical tests. Several urinary biomarkers were identified including fragments from different collagens, serum albumin, alpha-1-antitrypsin and uromodulin which could also provide more information regarding the patho-physiology of kidney disease and ongoing disease progression. In the near future these different biomarker classifiers could be applied in clinical trials as well as in clinical practice, with the aim of improving the benefits for the patients with respect to early intervention or stratification.

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** of outstanding interest:

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Key points

- The urinary proteomic classifier, CKD273, was validated in several independent studies to be significantly associated with chronic kidney disease (CKD) and to enable detection of CKD at very early stages of the disease superior to albuminuria.
- First multicentre interventional trial with the use of urinary proteomic biomarkers is used to target a preventive and therapeutic approach in clinical practice (stratified medicine).
- Differential diagnosis of CKD subtypes with the use of proteome analysis, in contrast to biopsy, offers the possibility of being applied early in the course of the disease when the benefit of intervention is optimal and of being repeated without any risk for the patient.
- Urinary proteome analysis can also be applied for the diagnosis and prognosis of other kidney diseases, like autosomal dominant polycystic kidney disease or ureteropelvic junction obstruction.

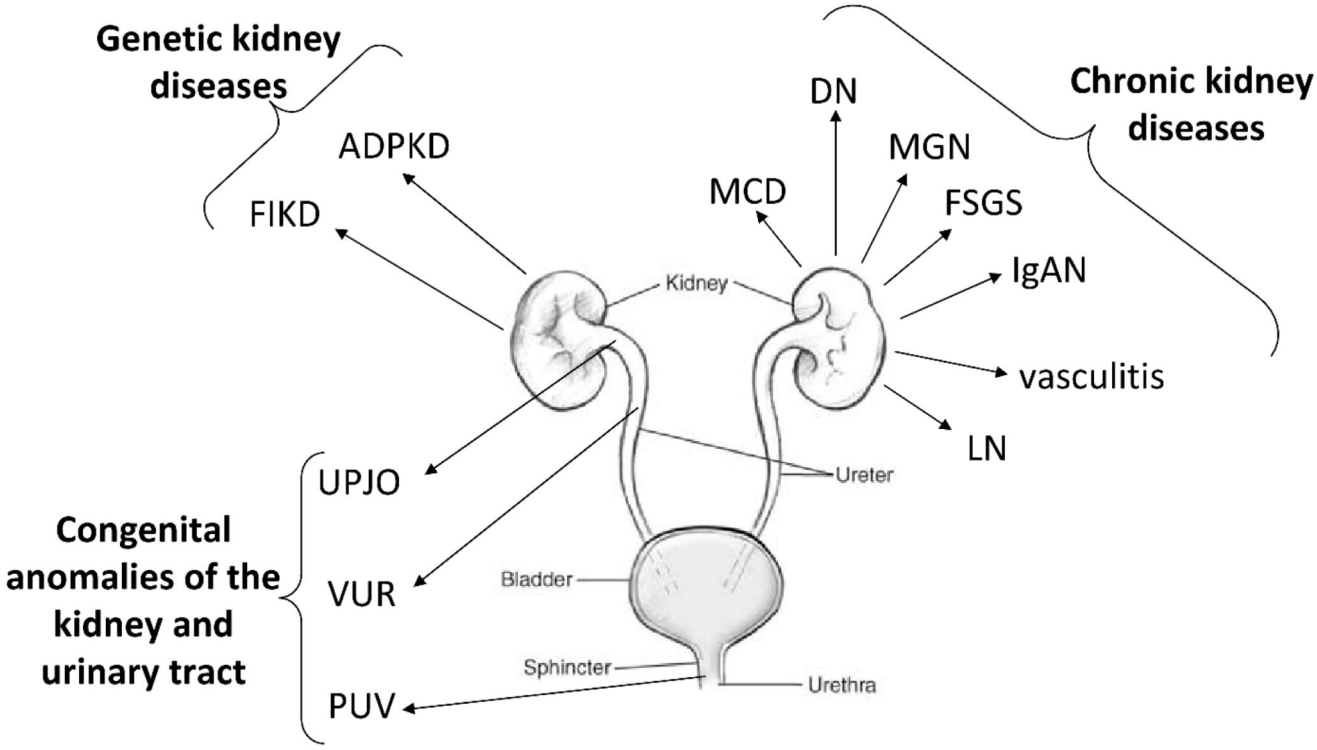

Figure 1.

Overview of the different kidney diseases, which were successfully investigated in the context of clinical urinary proteome analysis. The arrows pointed out in which area of the renal system the disease appears. Abbreviations: ADPKD - autosomal dominant polycystic kidney disease; DN – diabetic nephropathy; FIKD – Fabry induced kidney disease; FSGS - focal segmental glomerulosclerosis; IgAN – IgA nephropathy; LN – lupus nephrithis; MCD – minimal changed disease; MGN - membranous glomerulonephritis; PUV - posterior urethral valves; UPJO - ureteropelvic junction obstruction; vasculitis - ANCA-associated vasculitis.

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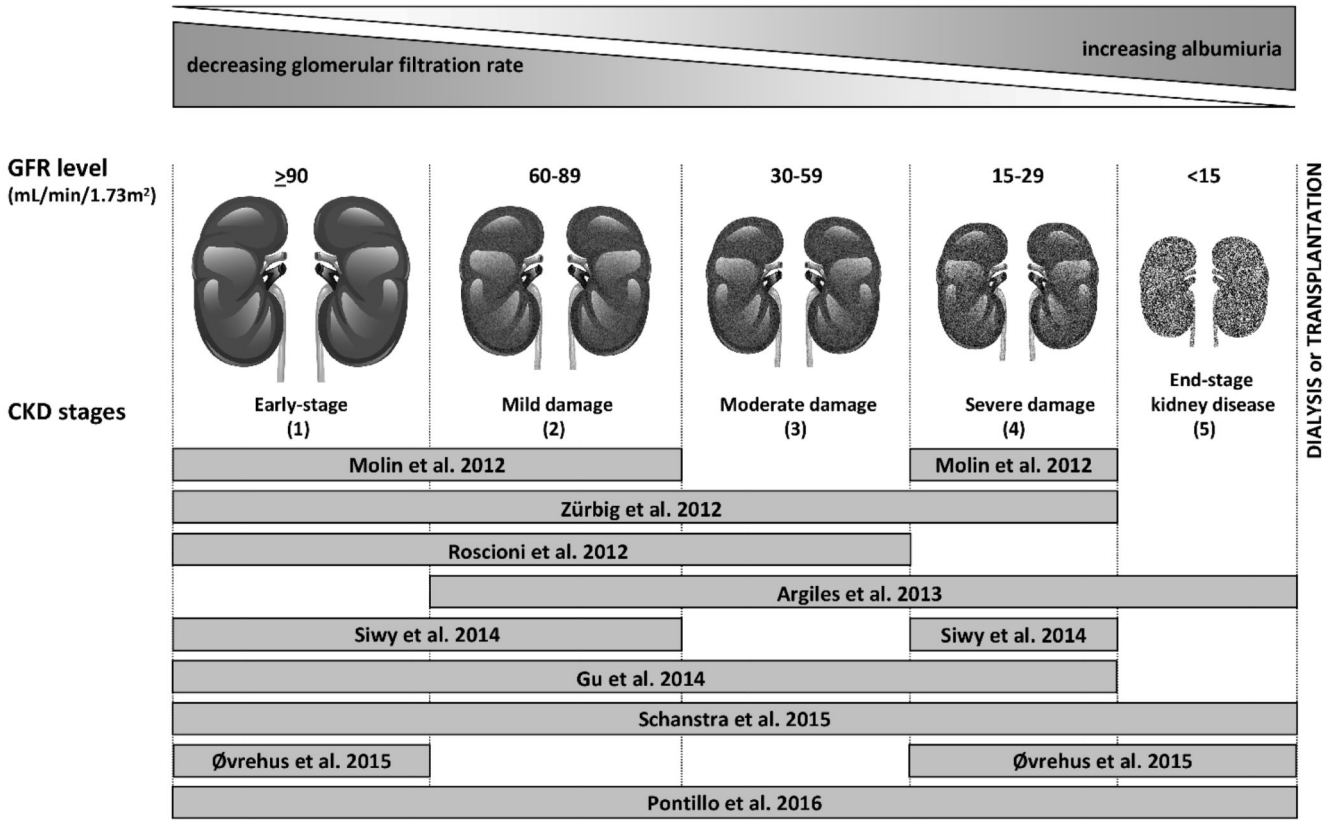

Figure 2.

Schematic depiction of the reviewed studies evaluating the performance of the CKD273-classifier in diagnosis and prognosis of CKD according to disease stage. The bars shows the CKD stages of the in the study included patients. Figure adapted from Critselis *et al.* (35).

Table 1

Summary of recent publications for the diagnosis and prognosis of kidney diseases based on CE-MS.

In some studies sensitivity, specificity, or AUCs are not reported. *added value of proteome analysis with respect to the currently used parameters

Kidney disease	Number of peptides	Performance	Accuracy	References
CKD	273	Diagnosis	Sensitivity: 86% Specificity: 100% AUC: 0.96	(11)
DN		Diagnosis	Sensitivity: 95% Specificity: 89% AUC: 0.96	(12)
CKD		Prognosis	---	(13)
CKD		Prognosis	---	(14)
DN		Prognosis	AUC: 0.92	(15)
DN		Prognosis	---	(16)
CKD		Diagnosis (Prognosis)	Sensitivity: 95% Specificity: 100% AUC: 0.98 (AUC: 0.91*)	(17)
DN		Diagnosis	AUC: 0.95	(18)
CKD		Prognosis	AUC: 0.83*	(19)
CKD		Prognosis	---	(20)
MGN	311	Diagnosis	AUC: 0.87	(21)
FSGS	287	Diagnosis	AUC: 0.88	
MCD	291	Diagnosis	AUC: 0.77	
DN/HN	619	Diagnosis	AUC: 0.92	
IgAN	116	Diagnosis	AUC: 0.82	
Vasculitis	509	Diagnosis	AUC: 0.95	
LN	172	Diagnosis	AUC: 0.82	
ADPKD	142	Diagnosis	Sensitivity: 85% Specificity: 94%	(22)
ADPKD	20	Prognosis	AUC: 0.83	(23)
FIKD	64	Diagnosis	Sensitivity: 88% Specificity: 98% AUC: 0.97	(24)
PUV	12	Prognosis	Sensitivity: 88% Specificity: 95%	(25)
UPJO	51	Prognosis	Sensitivity: 100% Specificity: 85% AUC: 0.92	(26)
		Prognosis (age<1)	Sensitivity: 83% Specificity: 92% AUC: 0.88	(27)
VUR	9	Diagnosis	Sensitivity: 88% Specificity: 79%	(28)