

REVIEW

Capillary zone electrophoresis on-line coupled to mass spectrometry: A perspective application for clinical proteomics

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Clinical proteomics, a rapidly growing field, intends to use specific diagnostic proteomic/peptidomic markers for initial diagnosis or prognosis of the progression of various diseases. Analyses of disease-associated markers in defined biological samples can provide valuable molecular diagnostic information for these diseases. This approach relies on sensitive and highly standardized modern analytical techniques. In the recent years, one of these technologies, CZE online coupled to MS (CZE-MS), has been increasingly used for the detection of peptide biomarkers (<20 kDa) in body fluids such as urine. This review presents the most relevant urinary proteomic studies addressing the application of CZE-MS in clinically relevant biomarker research between the years 2006 and 2014.

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Abbreviations: **AAV**, ANCA-associated vasculitis; **ADPKD**, autosomal-dominant polycystic kidney disease; **aGvHD**, acute graft-versus-host disease; **AKI**, acute kidney injury; **allo-HSCT**, allogeneic hematopoietic stem cell transplantation; **ANCA**, antineutrophil cytoplasmic autoantibody; **BBD**, benign biliary disorder; **BCa**, bladder cancer; **CAD**, coronary artery disease; **CC**, cholangiocarcinoma; **CKD**, chronic kidney disease; **CVD**, cardiovascular disease; **DN**, diabetic nephropathy; **ECM**, extracellular matrix; **ESRD**, end-stage renal disease; **HF**, heart failure; **IgAN**, IgA nephropathy; **IPP**, informative peptide panel; **LV**, left ventricular; **LVDD**, LV diastolic dysfunction; **No-OP**, nonoperated individuals with UPJ obstruction; **NRI**, net reclassification improvement; **OP**, individuals with severe UPJ obstruction; **PCa**, prostate cancer; **PSA**, prostate-specific antigen; **PSC**, primary sclerosing cholangitis; **PUV**, posterior urethral valves; **RCC**, renal cell carcinoma; **ROC**, receiver operating characteristic; **SF**, single factor; **SVM**, support vector machine; **T2D**, type 2 diabetes; **TCMR**, T-cell-mediated tubulointerstitial rejection; **UPJ**, uretero-pelvic junction obstruction

1 Introduction

During the last decade, CZE online coupled to MS (CZE-MS) has proven its value and is now increasingly used for proteomic as well as for metabolomic analysis, in diagnosis, therapeutic treatment, and drug development [1–7].

CZE-MS as analytical tool is complementary and may provide advantages over other technologies in analyzing urinary proteomic biomarkers of various diseases [8–10]. The main advantage of modern proteomic tools such as CZE-MS for routine diagnostic analysis is the simultaneous detection of multiple markers [11]. CZE-MS is currently the method with the shortest route from the analytical laboratory to clinical application as it can be used for discovery, validation, and clinical implementation, missing out on the costly and time-consuming process of changing technology platforms with the risk of losing biomarkers [12]. Technically, CZE-MS can provide fast analysis, typically resolving 1000–4000 peptides per sample within approximately 45 min. Some of the

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further advantages such as robustness (toward interfering compounds, participates, etc.) and high comparability of the datasets established this technique as a basis for biomarker discoveries [13, 14]. The raise of CZE-MS-based biomarker discovery in the recent years coincided with intensification of proteome/peptidome analysis of the human urine, which can be collected in large quantities in noninvasive and convenient way. Thirty percent of urine proteome is of the systemic origin [15]. It may therefore provide biomarkers for urogenital as well as systemic diseases. In fact, based on the analysis by CZE-MS more than 100 000 different peptides (<20 kDa) could be detected in a total of >20 000 urine samples. At least 5600 of these peptides were detected with high frequency (>30%) and can hence be considered as typical components of urine proteome [13]. However, alterations in peptide distinction and concentration (detection limits, and/or multiple detections of the peptides) limit the number of peptides typically detected in a sample to about 900–2500. The concentration of proteins and peptides in the urine is changing on a daily basis, depending on food and fluid intake [16]. These variations can be fairly well compensated by an adjustment of the concentration relative to urinary creatinine levels or other so-called “housekeeping peptides” that are typically present in urine [17].

In this article, we aim to comprehensively review the CZE-MS-based proteomic biomedical studies performed between the years 2006 and 2014, focusing on the detection of human urinary peptide biomarkers.

2 Technical aspect of CZE-MS

CZE is a fast high-resolution separation technology based on differential mobility of ions through a conductive medium in a high-voltage electric field. Mobility of the analytes is influenced by their charge, the viscosity of the medium, and molecule's radius. Known for its compatibility with most buffers and analytes [12], CZE can provide stable flow and

constant voltage conditions [18]. This stability is advantageous when connecting to MS and establishing a robust CZE-MS system [11, 19] that demonstrates high resolution, short analysis time, and good repeatability, all of which are important factors for clinical application. Including preanalytical steps, CZE-ESI-TOF-MS analysis can be used for reasonably fast, stable, and reproducible analysis of body fluids in a clinical laboratory setting (Fig. 1) [13]. Applying suitable software solutions to process the CE-MS raw data, including peak detection, charge assignment, calibration, and database deposition [13, 14, 19, 20], allowed development of an urine proteome database with currently >20 000 comparable datasets [14]. While in the past direct interfacing CE with MS/MS was challenging [21] and hence the correlation of basic amino acids with migration time was often used for sequence assignment [22], recent developments have enabled sequencing using CE-MS/MS, with similar efficiency like LC-MS/MS [23].

CZE-ESI-TOF-MS-based analysis has been utilized for the initial discovery and validation of proteomic biomarkers (Fig. 2) [24]. However, regardless of all advantages associated with a CZE-MS approach, it is not yet routinely used for clinical standard diagnostics for reasons such as the momentary unavailability of the techniques as a ready-to-use local on-site solution due to, e.g., high technical demands, and the lack of approval of diagnostic disease-specific peptide/protein patterns by regulatory agencies [25].

3 Clinical applications for CZE-MS

Nonetheless in the recent years, a number of articles reported on clinical CZE-MS applications in proteomics, focusing on proteomic biomarker discovery, disease diagnosis, and assessment of therapeutic treatment. Mosaiques group has developed CZE-MS proteomic platform and own the intellectual property rights of this technology. Therefore, most prominent examples of urinary biomarker discovery using CZE-MS since 2006 are given in Table 1.

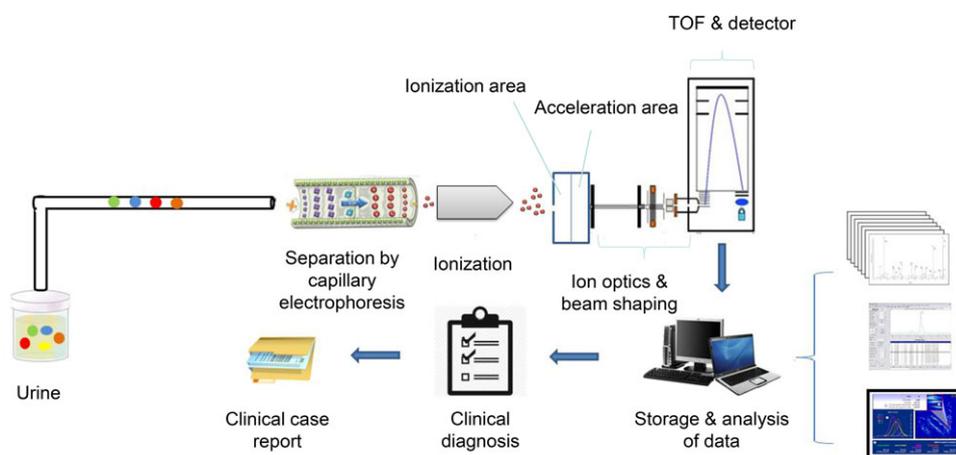


Figure 1. Schematic overview of the CZE-MS biomarker analysis. This figure illustrates the workflow of CZE-MS-based biomarker analysis.

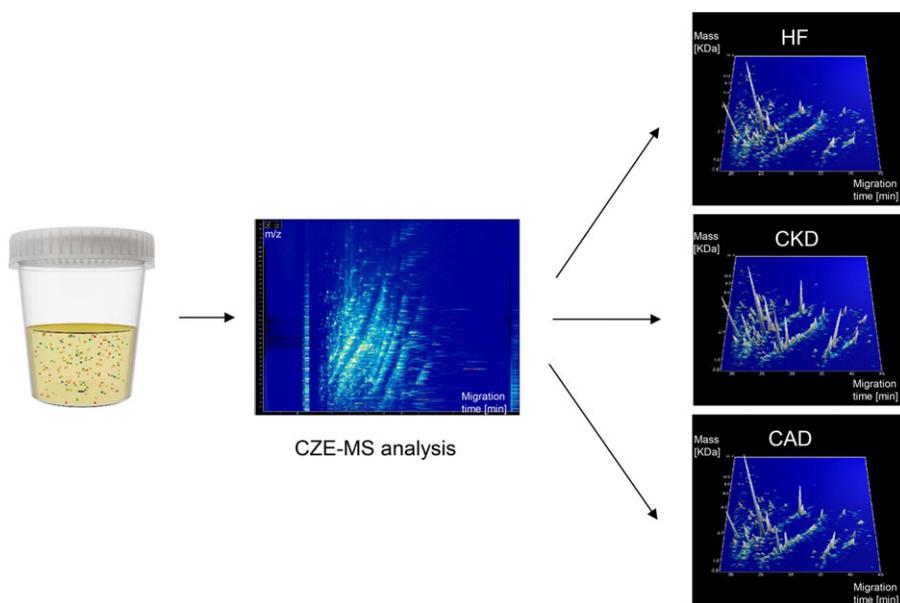


Figure 2. Diagnostic potential of CZE-MS-based proteome analysis. One full urinary peptide pattern resulting from CZE-MS-based proteomic analysis may be classified by multiple disease-specific classifiers allowing for the diagnosis of various different pathological conditions from one proteomic analysis. In the figure, 3D counterplots generated by CAD, HF, and CKD classifiers are shown. Displayed in three dimensions: x-axis, migration time in minutes; y-axis: logarithmic mass in Daltons; z-axis: signal intensity. CAD: cardiovascular disease. HF: heart failure with preserved ejection fraction. CKD: chronic kidney disease.

3.1 Kidney and urological diseases

According to the information provided by the American Kidney Fund, kidney diseases are the eighth leading cause of deaths in the United States, which might be representative for most developed countries. It is estimated that 31 million people, 10% of USA population, have chronic kidney disease (CKD) (<http://www.kidneyfund.org/about-us/assets/pdfs/akf-kid-neydiseasestatistics-2012.pdf>).

3.1.1 Diabetic nephropathy

Diabetic nephropathy (DN) is a progressive disease often complicating long-standing diabetes and the most frequent reason for dialysis in Western countries. Standard clinical diagnoses is based on the presence of proteinuria as well as changes in serum creatinine level connected to decrease in the glomerular filtration rate [26].

In a first CZE-MS-based study, 102 DN-specific urinary peptide biomarkers were reported in 2008 [27]. Sixty five of these biomarkers were selected for a DN-specific classifier through support vector machine (SVM) modeling to differentiate diabetes type 1 patients with macroalbuminuria and normoalbuminuria. In a blinded cohort of 35 patients with diabetes and macroalbuminuria and 35 healthy individuals, the classifier identified DN with 97% sensitivity and healthy individuals with specificity (see Table 1). This classifier was further applied to 30 patients with diabetes and microalbuminuria. Seventeen scored positive, eight of whom showed a progression toward macroalbuminuria. The classifier was

further validated in a case–control study reported by Alkhalaf et al. in 2010 [28] in 148 patients with type 2 diabetes (T2D), of whom 64 presented with DN (macroalbuminuria > 300 mg/day). The performance of the classifier with regard to the discrimination between type 2 diabetics with and without DN was confirmed with a sensitivity and specificity of 94 and 91%, respectively, as well as an AUC of 0.95. In this study, the number of sequenced peptides increased to 34 of the 65 peptide biomarkers. Most of them were identified as collagen fragments that were downregulated in the urine of DN patients. Even though the DN classifier performed well and shares 24 peptides with the classifier CKD273, in later studies, it apparently was replaced by the classifier CKD273 [29], which proved to be a more stable general diagnostic modality for renal diseases without sacrificing sensitivity toward DN and provided a much better depiction of pathological processes as it is based on 273 sequenced peptides.

3.1.2 CKD

CKD is characterized by a slow, progressive loss of renal function and glomerular filtration that may ultimately result in end-stage renal disease (ESRD). Patients with ESRD require dialysis and in the end kidney transplantation. Good et al. successfully showed that CZE-MS-based proteomic analysis can be utilized for diagnosis of CKD [29]. Examining 379 healthy controls and 230 patients with different stages of CKD, the authors identified a urinary biomarker pattern of 273 peptides and established the disease classifier CKD273 through SVM modeling based on this set of peptide marker.

Table 1. Latest reports on peptide biomarkers discovered with CZE-MS for prognosis/diagnosis of various diseases

Disease	Biofluid	Number of biomarkers employed	Biomarker discovery phase (<i>n</i> cases/ <i>n</i> controls)	Validation phase (<i>n</i> cases/ <i>n</i> controls)	Sensitivity % (test set)	Specificity % (test set)	References
1 Diabetic nephropathy (DN)	Urine	65	60/30	35/35	97	97	Rossing et al. [27]
2 Chronic kidney disease (CKD)	Urine	273	230/379	110/34	86	100	Good et al. [29]
3 ANCA-associated Vasculitis	Urine	18	18/425	10/30	90	90	Haubitz et al. [42]
4 IgA nephropathy	Urine	25	402/207	22/27	90	90	Julien et al [44]
5 Autosomal polycystic kidney disease (ADPKD)	Urine	142	17/86	24/35	88	98	Kistler et al. [47]
6 Acute kidney injury (AKI)	Urine	20	16/14	9/11	89	82	Metzger et al. [52]
7 Posterior urethral valves obstruction (PUV)	Urine	12	13/15	16/22	88	95	Klein et al. [55]
8 Ureteropelvic junction obstruction (UPJ)	Urine	53	13 controls/19 OP	23/13	94	80–100	Decramer et al. [56]
9 Coronary artery disease	Urine	15	30/20	47/12	98	83	Zimmerli et al. [68]
	Urine	17	15/14	26/12	81	92	von Zur Muhlen et al. [69]
10 Left ventricular dysfunction	Urine	238	212/196	71/67	79	88	Delles et al. [39]
11	Urine	35	65/41	32/15	56	93	Dwanson et al. [79]
	Urine	85	19/19	16/16	69	94	Kuznetsova et al. [73]
	Urine	85	96/98	32/15	56	93	Zhang et al. [75]
12 Acute graft versus host disease (aGvHD) grade III and IV	Urine	31	13/50	119/480	83	77	Weissinger et al. [84]
13 Acute renal allograft rejection	Urine	14	16/23	28/36	93	78	Metzger et al. [87]
14 Bladder cancer (BCa)	Urine	22	46/33	31/11/138	100	100	Theodorescu et al. [96]
15 Renal cell carcinoma (RCC)	Urine	86	40/68	70/6	80	87	Frantzi et al. [106]
16 Prostate cancer	Urine	12	51/35	264	91	69	Theodorescu et al. [111]
17 Cholangiocellular carcinoma	Urine	42	14/27	42/81	83	79	Metzger et al. [115]

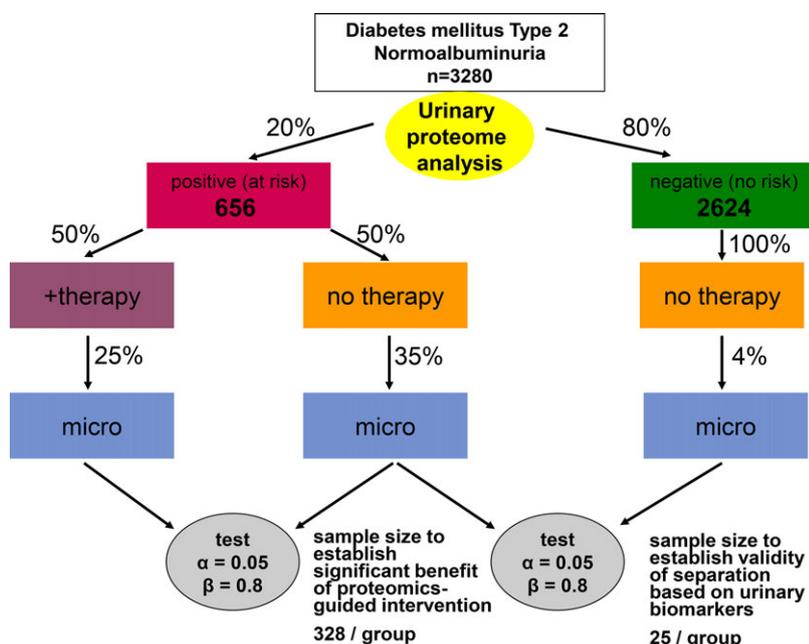


Figure 3. Design of the proteomics-driven intervention trial to interfere with development of DN, PRIORITY. Underlying assumptions are that 20% of normoalbuminuric patients (diabetes duration: 5–10 years) will show pathophysiological changes indicative for early stages of DN. Targeted therapeutic intervention will reduce the development of microalbuminuria during a period of 3 years in this selected cohort from 35 to 25%. To demonstrate significant benefit ($\alpha = 0.05$, $\beta = 0.8$), a total of 3280 patients have to be screened, among them 656 who are at risk will be randomized. Reprinted with permission [124].

The performance of the classifier was assessed using a blinded cohort of 110 patients with various stages of CKD and 34 healthy controls, revealing a sensitivity of 85.5% and specificity of 100% (see Table 1). Argilés et al. examined the value of the CKD273 in predicting ESRD or death in 53 patients [30]. During follow-up of 3.6 years, none of the patients with CKD273 score <0.55 required dialysis or died compared to 15 patients who reached an endpoint having CKD273 score >0.55 . These results confirmed a prognostic value of CKD273 classifier, unrelated to serum creatinine levels and eGFR in disease progression. The correlation of individual peptides to either different CKD-stages (advanced-stage CKD, $\text{eGFR} \leq 5 \text{ mL/min/1.73 m}^2$, $n = 321$; moderate-stage CKD patients, $\text{eGFR} > 45 \text{ mL/min/1.73 m}^2$, $n = 1669$) showed higher association in CKD prognosis and diagnosis than albuminuria. Correlating peptides in both studies, regardless of CKD-stage or CKD-progression, were either fragments of major circulating proteins (beta-2-microglobulin, apolipoprotein A-I, alpha-1-antitrypsin, serum albumin) suggesting failure of the glomerular sieving properties, or fragments of extracellular matrix (ECM) (collagen type I and III) suggesting changes in intrarenal ECM turnover [31–35].

Gu et al. [36] investigated the net reclassification improvement and integrated discrimination improvement in assessing renal function when the classifier CKD273 was combined with classifiers for left ventricular (LV) dysfunction into a single factor (SF) through principal component analysis. A higher SF classifier level correlated ($p \leq 0.010$) with worse renal function. During follow-up over 4.8 years, SF predicted progression of CKD from stage 2 or ≤ 2 to stage ≥ 3 with significant net reclassification improvement and integrated discrimination improvement.

Nkuiou-Kenfack et al. [37] explored the potential combination of plasma and urine proteomics and metabolomics to assess mild and advanced CKD patients. In total, 49 patients were studied: 26 patients with DN and 23 with other etiologies. From 43 patients, follow-up data (2.8 ± 0.8 years) were available. Peptides or metabolites showing significant alternation between the two patient groups were combined into SVM-driven classifiers. Three classifiers were developed: one plasma metabolite based (MetaboP), one urinary metabolite based (MetaboU), and one urinary peptide based (Pept). Scores generated with all three classifiers correlated well with eGFR. The Pept model performed best, and no added value could be detected by combining the proteomics and metabolomics biomarkers into unified classifier. The latter is likely owed to the excellent performance of each of these classifiers on its own, however, in a yet small cohort.

CZE-MS-based urine proteome profile analysis combined with diagnostic scoring by the CKD273 classifier was also utilized to evaluate the effects of Irbesartan, an angiotensin receptor blocker, in T2D patients with microalbuminuria [38]. In this study, 22 patients treated daily with either Irbesartan ($n = 11$) or placebo ($n = 11$) over a period of 2 years were analyzed with this classifier. For patients treated with Irbesartan, this classification indicated an improvement of the kidney physiology depicted by the significant decline in the median of the classification factor. This effect could not be observed in the placebo group. Similar effects of Irbesartan were observed in patients with coronary artery disease (CAD) after 2-year treatment [39]. These studies illustrated the potential of CZE-MS-based urine proteome analysis in monitoring therapeutic treatments.

Zürbig et al. [26] investigated the applicability of the CKD273 classifier for early prognosis of DN in a longitudinal cohort of normoalbuminuric subjects, 16 patients with type 1 diabetes and 19 patients with T2D. In a total of 316 baseline and follow-up urine samples, CKD273 successfully predicted macroalbuminuria 3–5 years before the clinical onset during follow-up with an AUC of 0.93 compared to microalbuminuria based on urinary albumin secretion rate with an AUC of 0.67. In a prospective study by Roscioni et al. with an average follow-up time of 3 years, the classifier CKD273 allowed prediction of transformation from normo- to micro- and micro- to macroalbuminuria [40]. The proteomic classifier predicted the progression of albuminuria with AUC of 0.94. This study showed, even though on a small test cohort, that urinary biomarker enables early renal risk assessment in patients with diabetes.

The results led to the initiation of the PRIORITY trial, where 3260 diabetic patients at risk of developing DN will be examined using the CZE-MS-based urine proteome analysis and the CKD273 classifier. An outline of the study is presented in Fig. 3. This is the first application of clinical proteomics in a large multicentric interventional trial. If successful, this study will provide the first evidence for a benefit of clinical proteomics in a patient-relevant outcome. Before starting this longitudinal prospective trial, the stability of CKD273 classifier was confirmed in set of prospectively collected urine from 165 T2D-patients in proceeding PRE-PRIORITY study [41]. High consistency of the CKD273-based classification across the different centers was observed in receiver operating characteristic (ROC) curve analysis based AUCs ranging from 0.95 to 1.00. This assured that CKD273 fulfills the initial requirements to stratify patients for intervention.

3.1.3 ANCA-associated vasculitis

Antineutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) represent a group of systematic disorders characterized with inflammation and damage of the blood vessel walls. These disorders often restrict accurate diagnosis and monitoring of the disease activity [42]. Availability of new and more sensitive diagnostic test could provide better prognosis of the patients affected by AAV.

By using urine samples from patients with AAV, Haubitz et al. [42] applied CZE-MS to identify biomarkers that allow for the diagnosis of AAV, and especially assessment of disease activity. Renal activity is difficult to assess in AAV, but it is highly relevant to perform appropriate therapeutic intervention. Comparing the CZE-MS-based proteome from 18 patients with active AAV to 425 controls (200 healthy individuals and 225 patients with CKD of different etiology) enabled identification of 113 peptide markers that differed significantly between active renal AAV and controls. For 58 of the 113 AAV-specific peptides, the amino acid sequence was

shown. As the biomarker identification was only based on 18 patients, the authors subselected 18 out of the 58 sequenced peptide biomarkers by removing peptides that did not affect accuracy, sensitivity, and specificity through the take-one-out method. The subselected pattern of 18 peptide biomarkers have been used to establish to classifiers based on SVM and linear modeling for the differentiation of patients with vasculitis from healthy controls as well as those with other renal diseases. Both classifiers were validated in a blinded cohort of 10 patients with active AAV, 29 patients with other glomerulus diseases (9 membranous glomerulonephritis, 6 focal segmental glomerulosclerosis, 4 IgA nephropathy (IgAN), 4 proliferative lupus nephritis, 2 minimal change disease, 2 membranoproliferative glomerulonephritis, 2 glomerular sclerosis), and 1 normal control. Both classifiers gave similar results in regard of accuracy showing AUCs of 0.893 (linear) and 0.888 (SVM), respectively. Discrimination of AAV from other renal diseases was possible with 90% sensitivity and 86.7–90% specificity depending on the classifier. The authors further established both linear and SVM modeling based classifiers utilizing 47 sequenced biomarkers to address the response to therapy/activity of disease. The scores generated by these classifiers changed with the progression of immunosuppressive treatment. The authors concluded that proteome analysis represents an early and accurate tool for noninvasive diagnosis of AAV.

3.1.4 IgA nephropathy (IgAN)

IgAN represents one of the most common types of primary glomerulonephritis. It is characterized by deposition of polymeric IgA antibody in the glomerular mesangium of the kidneys, provoking inflammation and renal damage. The current diagnosis of IgAN is by renal biopsy [43]. Thus, discovering noninvasive biomarkers would contribute to predict and diagnose IgAN patients prior to renal biopsy.

Julien et al. [44] evaluated the value of CZE-MS in detecting urinary biomarkers for IgA-associated glomerulonephritis. In a cohort of 402 patients with various renal disorders and 207 healthy controls, the authors defined 95 biomarkers for renal damage in general and 25 biomarkers specific for IgAN in particular. SVM modeling based classifiers for general renal damage (95 biomarkers) and IgAN (25 biomarkers) were tested in a blinded cohort including patients with IgAN ($n = 10$), Henoch-Schoenlein purpura with nephritis ($n = 10$), and IgA-associated glomerulonephritis due to hepatitis C virus induced cirrhosis ($n = 9$) as well as healthy controls ($n = 12$). The classifier for general renal damage indicated a renal damage pattern in 80, 80, and 100% of patients, respectively, and in 17% of healthy controls. The more specific IgAN classifier however did so in 90, 90, and 1% of the patients, respectively, and in none of the healthy controls. The authors concluded that, if these finding can be further validated in prospective study with renal biopsy and urinary testing in the near

future, then it will be possible to adapt CZE-MS methodology to develop novel tests for detection of renal injuries at early stages, assess clinical manifestation, and monitor responses to therapy in IgA-associated renal diseases.

3.1.5 Autosomal dominant polycystic kidney disease

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent hereditary kidney disease, affecting between 1 per 400 and 1 per 1000 individuals in the general population. ADPKD is the result of mutations in PKD1 or PKD2 (85 and 15% occurrence, respectively), resulting in cyst formation and loss of renal function [45, 46]. In two consecutive studies by Kistler et al. [47, 48], 38 ADPKD-specific urinary peptide biomarkers were identified to distinguish ADPKD patients from healthy volunteers and patients with other renal diseases. Based on these biomarkers, a disease classifier was established and validated in an independent test set of 24 ADPKD patients and 35 healthy subjects showing a sensitivity of 87.5% and specificity of 97.5% [49]. The second study was specifically designed to assess if the proteome analysis is able to predict the severity of ADPKD. A comparison of the proteomic profiles of 41 ADPKD patients and 189 healthy controls resulted in the development of a 142 polypeptide biomarker disease classifier, demonstrating a sensitivity of 84.4% and specificity of 94.2% in an independent validation cohort of 251 ADPKD patients and 86 healthy controls [48]. The majority of identified peptides were collagen fragments, which may indicate changes in ECM structural organization during cyst formation.

3.1.6 Acute kidney injuries

Acute kidney injury (AKI) is characterized by rapid decline of glomerular filtration and/or urine output [50, 51]. Early and accurate prediction is important to take intervention measure against its progression and life-treating complications (e.g. metabolic acidosis, uremia, and death). Currently, AKI is detected by serum creatinine. However, there are several limitations to the usage of creatinine as diagnostic parameter, most notably since it overestimates GFR and raise relative to kidney injuries [52]. Metzger et al. [53] performed CZE-MS-based analysis of urine samples from of 30 patients, 16 of which presented with AKI. They defined a pattern of 20 AKI-specific peptide biomarkers and established an SVM-based classifier. Validation of these findings was performed on a blinded cohort including 9 patients with AKI and 11 patients without AKI in the intensive care unit. ROC curve analysis showed 89% sensitivity and 82% specificity for the classification of AKI (see Table 1). In a further validation, this classifier characterized 16 patients with AKI and 22 patients without AKI after allogeneic hematopoietic stem cell transplantation (allo-HSCT) as different underlying

etiology of AKI. The analysis yielded a sensitivity and specificity of 94 and 82%, respectively. Basically, fragments of collagen type I and α_1 -antitrypsin were reported, which play a role in altered ECM turnover and renal ischemia/reperfusion injuries, respectively. An increased excretion of beta-2-microglobulin was attributed to impaired megalin and cubilin expression [54].

3.1.7 Posterior urethral valves obstruction

Posterior urethral valves (PUV) consist of a thin membrane of tissue and represent the most common cause of lower urinary tract obstruction in male infants. PUVs affect both the upper and lower urinary tracts, causing abnormalities such as renal dysplasia, changes in tubular function, and changes in bladder function [55]. Klein et al. utilized CZE-MS to analyze the urinary proteome of fetuses with PUV, searching for biomarkers predicting postnatal renal function [56]. A PUV classifier based on 12 urinary peptide biomarkers correctly predicted postnatal renal function with 88% sensitivity and 95% specificity in an independent blinded cohort of 38 PUV patients (see Table 1).

3.1.8 Ureteropelvic junction obstruction

Ureteropelvic junction obstruction (UPJ) is the most frequently found cause of congenital obstructive nephropathy as a result of hydronephrosis induced by accumulation of urine in renal pelvis or calyces [56]. Fifty-three UPJ-specific urinary peptide biomarkers were identified by Decramer et al. in neonates using CZE-MS [57, 58]. According to the degree of hydronephrosis and the gestational age upon its detection, three groups were defined: nonoperated individuals with UPJ obstruction (no-OP), individuals who might possibly undergo operation, and individuals with severe UPJ obstruction (OP). [57]. Validation in a blinded independent cohort of individuals with OP and no-OP resulted in 94% sensitivity in regard of OP and 80% specificity in regard of no-OP (see Table 1). After 9 months, urinary prediction based on proteome profiling was found to be accurate for 34 of 36 patients [58]. Moreover, after 15 months, the prediction was accurate for 35 of 36 patients. Drube et al. highlighted that the classifier established by Decramer et al. was able to predict the need for surgery in infants but not in older children with UPJ [59]. To investigate the long-term consequences of UPJ, Bandin et al. studied the urinary proteome of 42 patients with UPJ obstruction 5 years postoperatively or 5 years following spontaneous resolution [60]. They found no significant differences in urinary proteomes of patients with early surgical correction of UPJ obstruction and age-matched controls. In contrast, urinary proteomes differed significantly between conservatively followed patients and controls.

3.2 Cardiovascular diseases

According to the European Society of Cardiology, cardiovascular diseases (CVDs) account for over 4 million deaths each year in Europe, nearly half (47%) of all deaths (<http://www.escardio.org/about/what/advocacy/EuroHeart/Pages/2012-CVD-statistics.aspx>). Common manifestations of CVDs are chronic condition such as arteriosclerosis, CAD characterized by arteriosclerosis in the cardiac vasculature, and heart failure (HF) as well as acute event such as myocardial infarction and stroke. CAD is the most common cause of death before the age of 65 in Europe accounting for over 330 000 deaths/year [61].

CVDs may affect large blood vessels of the macrocirculation (arteries, veins), small blood vessels of the microcirculation (arterioles, venule, capillaries), as well as tissue of the heart, brain, lungs, and other organs. The diagnosis of CVD is based on various clinical and biochemical parameters. In recent years, the diagnostic and prognostic accuracy in regard of chronic CVDs like HF and acute CV events have considerably improved, in part due to the discovery of different specific proteomic biomarkers such as high-sensitivity troponins and natriuretic peptides indicative of pathological processes [62–64]. Some of these molecular indicators for distinct pathophysiological mechanisms hold the potential for early diagnosis and risk stratification. Ongoing progress in the identification of new biomarkers for different CVDs could therefore play a significant role in diagnosis, prognosis, prediction of recurrences, and monitoring of therapies [65, 66]. These biomarkers may be detectable in blood and/or urine. Urine may be an especially valuable source for biomarkers of cardiovascular complications of CKD. CKD has been recognized as an independent risk factor for CAD with a pathology that differs from the one found in the general population [67, 68], e.g. by a pronounced and fast-progressing arterial calcification [69] and proinflammatory, prooxidant, and procoagulant effects of uremic toxins [70]. Up to now, CZE-MS-based biomarker research for CVDs has been mostly focused on urine.

3.2.1 CAD

During recent years several studies assessing the urinary proteome of CAD patients by CZE-MS were published. Starting in 2008, Zimmerli et al. had shown that urinary proteomics can identify CAD patients with high confidence and might be useful in monitoring the effects of therapeutic treatments [71]. Fifteen CAD-specific peptides were identified using discovery phase of 30 with severe CAD, 18 patients before and after Ramipril treatment, and 252 healthy controls. These peptides then were utilized to establish an SVM-based CAD classifier. Validation of this classifier in an independent test set of 47 patients with coronary artery bypass graft surgery and acute coronary syndrome demonstrated a sensitivity of 98% and a specificity of 83% in 12 healthy controls, respectively. In another study by von zur Muhlen et al.

[72], patients either diagnosed CAD positive by coronary angiography or with well-established history of unstable angina pectoris were included. In the initial discovery phase, a pattern of 17 peptides was identified in urine but not in plasma by comparing 15 patients with CAD and 14 without CAD. The resulting classifier identified 26 CAD patients and 12 non-CAD patients in independent test set of with sensitivity of 81% and specificity 92%, respectively [72]. The classifier was further applied to a randomly selected independent set of 120 urine samples patients with malignancies and renal failure. Eighty percent of patients with malignancies were negative whereas fifty percent with renal failure were positive to this CAD test. In 2010, Delles et al. [39] validated the CAD specific biomarker panel established by Zimmerli et al [71] and von zur Muhlen et al. [72]. Applied on 138 urine samples from patients with and without CAD, classification by the 15 biomarker classifier showed 81.4% sensitivity and 48.5% specificity while the 17 biomarker classifier showed 51.4% sensitivity and 87.9% specificity. Due to this limited performance, Delles et al. [39] used the larger number of patients originating from these and other cohorts to develop a more accurate CAD classifier. By comparing urine samples from 204 CAD patients and 382 controls, 238 CAD-specific peptide markers were identified and used to establish CAD238 through SVM modeling. The validation was performed on 71 CAD patients and 67 controls showing a sensitivity of 79% and a specificity of 88%. In all three studies, mainly peptides belonged to fibrillar components of the ECM, originated especially from collagen type I and III, which are also present in atherosclerotic plaques [73]. In the most recent study, additional peptides derived, e.g., from α 1-antitrypsin.

In 2012, von zur Muhlen et al. also discovered urinary peptides that reflected atherosclerosis and its progression in an ApoE-deficient mouse model [74]. Interestingly, these peptides comprised fragments of α 1-antitrypsin, EGF, collagen type I, and kidney androgen-regulated protein in line with previously reported studies on human subjects [29, 39]. In addition, the match of these urinary protein fragments with the histological evaluation of atherosclerotic plaques indicated the biological relevance of the identified proteins in atherogenesis associated with CAD.

3.2.2 HF

Left ventricular (LV) HF manifests clinically either as HF with reduced ejection fraction (HFrEF; systolic) or HF with preserved ejection fraction HFpEF; diastolic) [75]. Early diagnosis is crucial but challenging and would benefit from easily applicable screening techniques. In 2013, Kuznetsova et al. used CZE-MS to identify peptide biomarkers specific for subclinical asymptomatic LV diastolic dysfunction (LVDD) by analyzing urine samples from 19 hypertensive patients with subclinical LVDD and 19 healthy volunteers [76]. This resulted in the discovery of 85 discriminating peptide biomarkers. The classifier for this preclinical stage of HF established by SVM modeling was validated in a test set of

16 hypertensive patients with mild to moderate symptomatic HF (New York Heart Association class II–III) and 16 healthy controls with a sensitivity of 64% and specificity of 94%. The panel consisted of downregulated fragments of collagens type I and V as well as upregulated fragments of collagen type III. The authors also observed reduced levels of WW domain binding protein 11 (WBP11) pointing toward a potentially increased activity of protein phosphatase-1 (PP-1), which is involved in calcium handling and relaxation via dephosphorylation of phospholamban [77]. Recently, Zhang et al. [78] validated this classifier (HF1) and investigated an additional one (HF2) based on 671 peptide biomarkers in a large population study including patients with LV filling pressure, impaired LV relaxation HF patients at any stage. This resulted in development of classifier with 671 polypeptide biomarkers. Both classifiers are mainly based on up- or downregulated collagen fragments as biomarkers and discriminated individuals with subclinical LVDD from healthy individuals and individuals with uncomplicated hypertension. By optimization of the thresholds for HF1 and HF2, these classifiers showed sensitivity ranging from 65.6 to 93.8% toward LVDD/HFpEF and specificity from 31.1 to 66.3% in regard of controls without pathological pulmonary conditions. The classifiers thereby correlated with physiological tissue Doppler echocardiography based parameters, especially early diastolic mitral annulus velocity (e') and the E/e' -ratio (E = peak mitral inflow velocity of the early rapid filling E-wave), which indicates LV filling pressure. This holds great promises for clinical diagnostics as high E/e' values predict cardiac mortality and rehospitalization in HF patients [79] and e' predicts fatal and nonfatal cardiovascular events in a general population [80].

3.2.3 Stroke

Even though clinicians are excellent in assessing stroke, biomarkers supporting clinical diagnosis, identifying patients at risk of stroke, and/or providing prognosis of outcome may allow for risk stratification and help to decrease morbidity and mortality [81]. Dawson et al. [82] assessed the urine proteome of 20 patients with transient cerebral ischemic attack, 10 with acute cerebral infarction and 35 with cerebrovascular disease. They discovered two patterns of peptide biomarkers consisting of 14 and 35 peptides, respectively. Based on these patterns, they established two classifiers through SVM modeling. The 35 biomarker classifier performed better when tested in an independent blinded test cohort of 32 cases with acute stroke or transient cerebral ischemic attack and 15 controls with cardiovascular risk, showing a sensitivity of 56%, and specificity of 93%. While these results indicated association of urinary proteomic biomarkers with stroke, their value in diagnosis appears moderate, so far. However, the identified peptide biomarkers may help to broaden our knowledge in regard of the pathology of acute ischemic stroke. This especially refers to inter-alpha-trypsin inhibitor heavy chain H4, which has also been shown by other groups to be underexpressed

in acute ischemic stroke [83]. Another protein identified is FXD4 (CHIF), a regulator of Na-K-ATPase and therefore a key feature of the cytotoxic edema that occurs in acute ischemic stroke [84].

Overall, based on the evidence presented, CZE-MS-based proteomic analysis holds the potential to help in early diagnosis of CVDs and allows new insights into the underlying pathology. Further clinical trials and prospective studies may extend current CVD biomarker patterns.

3.3 Transplantation-associated complications

3.3.1 Graft-versus-host disease after hematopoietic stem cell transplantation

allo-HSCT is the most common immunotherapy to treat hematological malignancies (i.e. leukemia) and certain nonautoimmune disorders (i.e. thalassemia) [85]. Although allo-HSCT provides rapid and potent antitumor immunity, it is associated with major complications, such as severe acute graft-versus-host disease (aGvHD) and infections.

Currently, diagnosis of aGvHD is mainly based on clinical features such as skin lesions and tissue biopsies [86], since a noninvasive laboratory test is as currently not available. CZE-MS has been used to identify aGvHD-related peptide markers in urine of leukemic patients after HSCT. Weissinger et al. [87] compared 13 samples from patients with aGvHD of grade II or higher with 50 samples from control subjects. The resulting peptide biomarker pattern comprised about 170 GvHD-related peptides of which 31 were used in an aGvHD classifier. In fact, validation of this classifier with 599 urine samples (119 aGvHD and 480 controls without aGvHD) collected at day +2 up to day +365 from 141 patients with hematologic malignancies ($n = 132$) and hematopoietic failure syndrome ($n = 9$; e.g. aplastic anemia) undergoing allo-HSCT resulted in diagnosis of aGvHD grade I or II with a sensitivity of 83.8% and specificity of 75.6%. More recently, a CZE-MS analysis was proposed to stratify patients undergoing allo-HSCT for the risk for aGvHD [88]. A set of patient samples was used, namely 37 patients with biopsy-proven aGvHD > grade II as case and: 76 time-matched samples of patients without aGvHD or aGvHD grade I without infection or relapse at the time of sampling as controls. The original aGvHD classifier was adapted to stratify patients at risk for progression to severe aGvHD stages (grades III and IV) at least 14 days before the onset of clinical signs and to differentiate them from non-aGvHD and aGvHD stages I–II. The aGvHD-specific classifier was further validated in 1106 prospectively collected samples of 423 leukemic patients in the range of 7–100 days after HSCT. This classifier allowed the distinction of patients with severe aGvHD (grades III and IV) from those who never developed aGvHD, patients with low or moderate aGvHD (grades I and II), and patients with chronic GvHD > 100 days after allo-HSCT with 82.4% sensitivity for aGvHD and 77.3% specificity.

3.3.2 Acute T-cell-mediated tubulointerstitial kidney allograft rejection

Reliable and timely diagnosis of acute T-cell-mediated tubulointerstitial rejection (TCMR) is important in the first year of posttransplant surveillance after kidney allograft transplantation [89]. Currently, posttransplant surveillance of acute allograft rejection is based on regular monitoring of serum creatinine levels together with tissue biopsy upon signs of functional renal impairment is part of posttransplant surveillance. Unfortunately, these diagnostic approaches identify rejection only in an already advanced stage while earlier subclinical stages of renal episodes remain uncharacterized [90]. Employing CZE-MS-based urinary proteome analysis, Metzger et al. [90] developed a noninvasive test to detect both clinical and subclinical forms of acute TCMR. The study included 39 patients for biomarker discovery (16 cases with subclinical acute TCMR and 23 nonrejection controls). Based on the comparison of these subjects, 14 urinary peptide biomarkers were combined in SVM modeling based classifier of TCMR. The performance of the classifier was tested in a validation cohort of 28 patients with TCMR (including 18 subclinical and 10 clinical rejection episodes) and 36 patients without rejection. Major constituents of the sequenced peptides were collagen type I and III fragments, which implicated alterations in the ECM as further supported by the presence of MMP-8-positive cell in glomerular and peritubular capillaries in the interstitium in immunohistological stainings of areas of interstitial infiltrates. The clinical value of this classifier is currently investigated in multicenter diagnose phase III trial (ClinicalTrials.gov identifier. NCT01315067) and as a part in a multicenter diagnostic phase III trial (European FP7- BIOMARGIN project; <http://www.biomargin.eu/>).

3.4 Cancerogenesis/cancer

According to the Cancer Research UK, in 2012 worldwide an estimated 8.2 million deaths were caused by cancer: 4.7 million (57%) in males and 3.5 million (43%) in females. The age-standardized mortality rate shows that there are 126 cancer deaths for every 100 000 men in the world and 83 for every 100 000 women (<http://www.cancerresearchuk.org/cancerinfo/cancerstats/world/mortality/>). This implies the necessity for an effective stratification of patients in the earliest stages of cancer that can potentially being met by cancer-specific biomarker patterns.

3.4.1 Bladder cancer

Bladder cancer (BCa) is the most common tumor of the genitourinary system, leading to approximately 145 000 deaths per year worldwide [91]. Transitional cell carcinoma (TCCs) is the most common subtype of BCa in Western countries

[92]. Based on different stages and prognosis, it is categorized into two distinct groups: none muscle invasive cancer (pTa, pT1, pTis) and muscle invasive cancer (pT2, pT3, pT4) [93]. The current standard diagnosis and surveillance procedure for BCa is based mainly on invasive cystoscopy [94]. Recently, different reviews emphasized the need to identify biomarkers for BCa [95–97]. Currently, two published studies applied CZE-MS to urothelial carcinoma biomarker discovery [98,99]. Theodorescu et al. established an urothelial carcinoma pattern consisting of 22 peptide biomarkers by comparing 46 patients with urothelial carcinoma and 33 healthy subjects [99]. The diagnostic accuracy of the classifier based on this pattern was validated in 31 patients with urothelial carcinoma compared to 11 healthy subjects and 138 patients with nonmalignant genitourinary diseases showing 100% sensitivity and specificity. The peptide biomarker pattern contains fibrinopeptide A, as a most prominent biomarker linked to pathophysiology. Fibrinopeptide A is also described as biomarker of ovarian and gastric cancer [99]. It is formed by thrombin-catalyzed hydrolytic cleavage from fibrinogen during blood coagulation. A connection between the coagulant pathway and cancer incidence was described previously [100]. Moreover, according to Theodorescu et al., the level of fibrinopeptide A might be an indicator of chemotherapeutic resistance in urothelial carcinoma [99]. More recently, Schiffer et al. defined a urinary biomarker pattern of four peptides associated with muscle-invasive BCa by comparing 71 patients with noninvasive BCa (pTis-1) and 56 patients with invasive BCa (pT2–4) [98]. The classifier based on these four biomarkers was validated in an independent test set of 90 samples from patients with noninvasive and 40 samples with invasive tumors. In this validation trail, the classifier showed 90% sensitivity toward invasive tumors and 52% specificity (noninvasive tumors). All four peptides were downregulated in invasive compared to noninvasive BCa, which were identified as fragments of collagen type I, collagen type III, membrane-associated progesterone receptor component 1 (PGRMC1), and uromodulin. Interestingly, PGRMC1 is reported to be involved in tumor progression and drug binding [101–103]. Dysregulation of matrix metalloproteinase activity and, therefore, collagen homeostasis has also been shown for different cancers including BCa [104]. To enable implementation of these promising candidates, appropriately powered clinical trials for the specific context-of-use have been advocated [105], and recently initiated in the TransBioBC programme (www.transbiobc.org).

3.4.2 Renal cell carcinoma

Renal cell carcinoma (RCC), the most common malignancy of the kidney, accounts for approximately 3% of adult cancers [106]. It is rapidly progressing and highly metastasizing tumor. For that reason, the mortality rate is high, resulting in over 100 000 annually deaths worldwide [107]. The prognosis in RCC is closely correlated with disease stage; 5-year

relapse-free survival rates range from 80 to 20% for stage I and stage IV, respectively. The clinical outcome is not only influenced by tumor grade, but also by the presence of either histological necrosis or small renal masses [108]. Thus, biomarkers suitable for early diagnosis of RCC would be highly desirable and reduce the need for invasive biopsies. Frantzi et al. identified 86 urinary peptide biomarkers that could be specifically associated to RCC by comparing 40 RCC patients and 68 age-matched healthy subjects [109]. The classifier based on these 86 discriminatory peptides was evaluated in 30 RCC patients and 46 nondiseased controls showing a sensitivity of 80% and a specificity of 87%. A RCC-specific differential excretion was observed for fibrinogen α - and β -chains, immunoglobulin Fc regions, hemoglobin subunits, Na/K-transporting ATPase subunit γ , retinitis pigmentosa GTPase regulator, VPS10 domain containing receptor SorCS3, and the endothelial adhesion molecule CD99 antigen like protein 2. The clinical utility of the RCC marker panel would be to differentiate radiological indeterminate lesions, to obtain further information before ablative treatments, and to select patients with small renal masses for surveillance protocols. Additionally, peptide marker sequence information could provide useful information on the decision on targeted pharmacologic therapy in the setting of metastatic disease with the ability to monitor response.

3.4.3 Prostate cancer

Early detection of prostate cancer (PCa) is mainly based on combinations of digital-rectal examinations, transurethral resections of the prostate, and measurements of serum prostate-specific antigen (PSA) levels. Clinical screening for PSA led to a significant increase of diagnosed cases [110–112]. However, present limitations of PSA screening in discrimination of benign and malignant prostatic condition results in 76% false positives and unnecessary prostate biopsies [113, 114]. Based on these facts, Theodorescu et al. [115] applied CZE-MS-based urinary proteome analysis on first void urine samples. First the authors showed that the first 10 mL of the urine void (first void) contains most of the seminal/prostatic fluid, a wash out from the prostatic urethra, which included the material necessary for the identification of PCa-specific biomarkers. To evaluate the quality of first void urine samples in regard of prostatic fluid content, a so-called “informative” peptide panel (IPP) was developed by comparing first void urine from 86 patients with and without PCa and midstream urine of 138 male controls as reference control. Further 46 female urine samples were evaluated to exclude any nonseminal/prostatic fluid specific peptides. This resulted in an SVM-based classifier derived from a pattern of eight IPP biomarkers indicative for prostatic fluid containing first void urine. The IPP classifier identified 79 of 86 first void urine samples to be “informative,” whereas 135 of 138 midstream urine samples were qualified to be “non-informative.” The remaining 47 (from 51) patients with biopsy-proven PCa

and 32 (from 35) controls with negative prostatic biopsy were compared for PCa-specific biomarker discovery. This resulted in the discovery of 12 biomarkers that were used to establish a PCa-specific classifier through SVM modeling. The classifier was then validated in “informative” urine samples from an independent cohort of 213 subjects, 118 with PCa and 95 without. Patients with PCa were detected with 73% sensitivity and individuals without PCa with 60% specificity. Adjustment of the observed ROC curve according to a false-negative rate of prostate biopsy resulted in improved test characteristics. Integrating age and percent-free PSA to the proteomic signatures resulted in 91% sensitivity and 61% specificity. Schiffer et al. [116] further validated this PCa classifier in an independent cohort of 184 subjects with 49 confirmed PCa cases. The PCa classifier achieved a sensitivity of 86% and specificity of 59%. Cost-effectiveness analysis showed that CZE-MS-based urine proteomic analysis outperformed the biopsy approach as well as PSA tests.

3.4.4 Cholangiocellular carcinoma

Cholangiocarcinoma (CC) represents a rare tumor that arises from cholangiocytes of the intrahepatic and extrahepatic biliary tract, with incidence in United Kingdom of approximately one to two cases in 100 000 population [117]. The prognosis of CC remains poor, with surgical dissection or orthotopic liver transplantation as the only curative treatment option, which however only can be performed at an early tumor stage. Unfortunately, CC is often detected at an advanced stage due to the lack of accurate diagnostic tests. This most particularly is true for patients with primary sclerosing cholangitis (PSC) who are at >160-fold increased risk for CC development [117]. PSC is a rare cholestatic liver disease of unknown etiology, characterized by chronic inflammation, and obliterative fibrosis of the intra- and/or extrahepatic bile ducts. In patients with PSC, the differentiation between benign and malignant strictures is particularly difficult even for specialists in the field, because CC as well as chronic or acute inflammation frequently result in similar cholangiographic findings. After first establishing CZE-MS-based bile proteomic analysis for the detection of local changes in the biliary tract caused by CC depending on bile collection during invasive endoscopic retrograde cholangiography [118], Metzger et al. [119] established a noninvasive CZE-MS-based urine proteomic test to detect systemic changes caused by CC progression. This was done by comparing proteome profiles of 14 CC patients with those of 13 patients with PSC and 14 patients with other benign biliary disorders (BBD) thereby identifying a CC-specific pattern composed of 42 CC-specific peptide biomarkers. The SVM-based classifier derived from this biomarker pattern was validated in a cohort of 123 patients including 42 CC patients, 45 PSC patients, and 36 BBD patients. It achieved 83% sensitivity (CC) and 79% specificity (PSC and BBD) and was therefore of equal diagnostic accuracy as bile proteome analysis (84% sensitivity, 78% specificity). Recently, a logistic

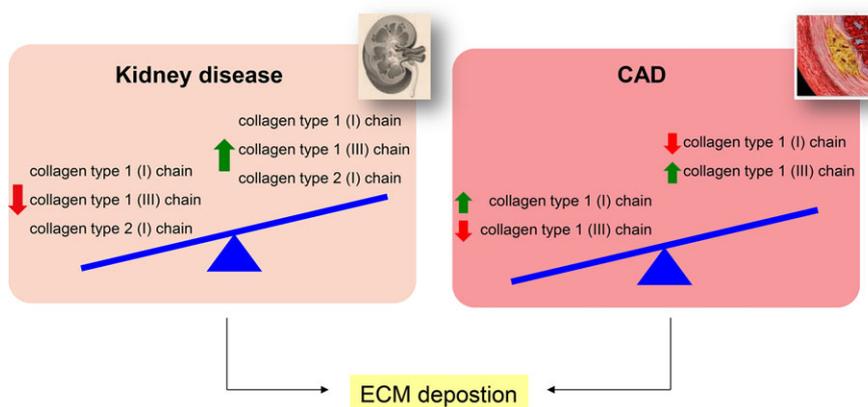


Figure 4. Collagen balance. Due to the important role of ECM in chronic disease, the differential excretion/regulation of different collagen fragments is shown for two major pathological conditions—CAD and kidney disease. The direction of the balance indicates the highest number of peptides found in samples. For example, collagen type 1, a major constituent of CKD, was found to be downregulated, which reflects on decreased activity of ECM and collagenases.

regression model was established combining the classification factors of bile and urine proteome analysis. This enabled CC-diagnosis with an accuracy >90% in a set of 36 CC patients, of whom 10 had concomitant PSC, 33 patients with PSC, and 18 patients with other BBD [120].

4 Summary and outlook

The studies summarized in this review illustrate that CZE-MS is a powerful platform for proteomic disease biomarker identification. The studies further indicate that the multi-dimensional protein/peptide biomarker patterns identified through CZE-MS-based proteomic analysis of noninvasively obtained urine samples carry the potential to improve clinical diagnostics and prognostics for many common as well as rather rare diseases. In addition, the disease-specific peptide patterns may provide information on involved pathological processes.

Based on the published data available, CZE-MS has allowed the reproducible analysis of >20 000 independent samples and enabled the establishment of the by far largest comparable proteomic dataset currently available. Also as a result of its robustness, CZE-MS is already being applied in clinical diagnostics of several diseases, and in large multicentric clinical trials. The latter appears to be the most relevant cornerstone in the path toward clinical application: the demonstration of a significant patient-relevant benefit in a randomized controlled clinical trial will likely result in the broad application of clinical proteomics as a routine diagnostic tool.

In this respect, some of the studies reviewed even raise hope that the proteomic analysis of urine by CZE-MS may rather sooner than later be established as a modality for routine clinical diagnostics providing a more accurate earlier diagnosis of chronic diseases like CKD and HF that pose a growing public health problem. As such, one could envision performing urinary proteome analysis as part of the regular health check-up, and examine for significant changes indicative for a variety of diseases, as indicated in Fig. 2.

The identification of disease-indicative changes could enable early life-style [121] and/or therapeutic interventions, thereby preventing disease, or at least significantly delaying onset.

The application of CZE-MS-based proteome analysis on urine for diagnostic and prognostic purposes appears to be especially suitable for diseases with alterations of the ECM as part of their primary pathology due to the predominance of collagen fragments, especially collagen type I and III, in biomarker patterns of most of the reviewed urinary proteomic studies (Fig. 4 and Supporting Information Table 1). It further appears that the observed differential excretion of distinct collagen fragments is disease specific. Besides collagen fragments, protein/peptide biomarkers identified in urine can provide information on pathophysiological processes during disease progression, comprising, e.g., alpha-1 antitrypsin, fibrinogen, albumin, and uromodulin. As these peptides result from diverse proteolytic activities, the peptides identified by CZE-MS may also provide information on disease-associated alterations in proteolytic processes (protease and protease inhibitors) [122] through the statistical analysis of relative abundance of potential cleavage sites.

However, the reviewed studies also revealed that at this point in time, limitations associated with standardization and implementation in clinical routine analysis still exist. While utilization of CZE-MS-based proteomic analysis for routine diagnostic purposes appears possible for certain applications, additional studies will be necessary to proof clinical validity and utility [123].

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