Title

Implementation of CE-MS identified proteome based biomarker panels in drug development and patient management

Abstract

The recent advancements in clinical proteomics enabled identification of biomarker panels for a large range of diseases. A number of CE-MS identified biomarker panels were verified and implemented in clinical studies. Despite multiple challenges, accumulating evidence supports the value and the need for proteome-based biomarker panels.

In this perspective, we provide an overview of clinical studies indicating the added value of CE-MS biomarker panels over traditional diagnostics and monitoring methods. We outline apparent advantages of applying novel proteomic biomarker panels for disease diagnosis, prognosis, staging, drug development and patient management. Facing the plethora of benefits associated with the use of CE-MS biomarker panels, we envision their implementation into the medical practice in the near future.

Defined key terms

Clinical proteomics: Sub-discipline of proteomics concerned with application of proteomic technologies on clinical specimens to obtain information on protein networks and the identification of protein-based biomarkers. Urine and blood, due to their minimally invasive method of collection, are the most commonly used body fluids for clinical proteomics.

"Humanised" biomarker panels: Biomarker panels composed of multiple molecules that are orthologous (share sequence similarity) between human and animals. Such humanised biomarker panels are of high value for translational medicine.

Personalized medicine: Medical approach for disease treatment and prevention that takes into account biological differences and variations among individuals, in order to develop the best suited for a given individual therapy. Biomarker panels facilitate the personalized medicine approach by pointing the individual differences between patients.

Patient stratification: Process of subdividing patients into groups differing in terms of biological make up and expected clinical response. Patient stratification is a common concept in clinical trials, which aims to select the optimal population for drug evaluation**.** As an example, implementation of biomarker panels in nephrology allows patient stratification with greater accuracy than commonly used methods based on functional parameters such as albuminuria and estimated glomerular filtration rate.

Omic platforms: High throughput platforms encompassing proteomics, metabolomics, genomics, and transcriptomics allowing the analysis of the global molecular content of a sample for the targeted omics trait.

Introduction

Drug development and patient management are crucial to any healthcare system. These two aspects are interconnected and influence each other. One of the best examples of their mutual dependence is the development of the personalised medicine approach, which aims to deliver “the right drug at the right dose to the right patient” [1]. The concept of personalised medicine was already evident to Hippocrates (460 – 370 BC), who apparently said: “It’s far more important to know what person has the disease than what disease the person has”.

Essentially all drugs either directly or indirectly target the function of specific proteins. Along the same lines: any pathophysiologic change (that will ultimately result in a specific disease) is associated with and dependent on specific changes on the protein level (Figure 1). Therefore, protein-based biomarkers appear to be best suited to inform about disease, the optimal treatment options, and drug effects on patients subjected to therapies [2]. In contrast, histological assessment (e.g. in tissue biopsies) can inform about structural damage, but generally holds no information on molecular pathophysiology or the respective drug targets.

A state of an organism (healthy or diseased) relies on the interplay among different biological levels (genomics, transcriptomics, proteomics, and metabolomics). Proteomics, defined as the analysis of the total protein content of a sample or system, is a prominent multiparametric approach, providing information beyond what genomics or transcriptomics can deliver. When describing the different omics platforms an analogy to a fireplace can be made (Figure 2)[3]. Genomics could be considered as the logs of wood, containing all potential, but no information about actual execution. However, it can be assessed fairly well

and accurately. Proteomics can be considered as a fire, integrating the potential with the environmental impact. It is highly variable, difficult to assess in detail, but contains all information about the current status. Metabolomics is analogous to the ashes, representing the result of the fire (protein) action, clearly containing information about the action of the fire and thus potentially leading to the identification of metabolite-based biomarkers of disease, but most likely not representing active or early disease stages. In addition, any interference targeting the ashes would generally be too late to be of relevance in the context of the fire.

Irrespective of the omics-trait, combination of multiple biomarkers has shown to have improved performance (specificity and sensitivity) over single biomarkers [4]. This appears to be a result of compensating for the biological variability generally associated with any biomarker. This variability can be counteracted either by multiple measurements of the same biomarker, or by the simultaneous measurement of multiple markers. The latter has the additional value that bias that may be associated with a single biomarker, even if measured repeatedly, can be corrected for by the additional markers that generally should not display an identical bias.

These observations have resulted in the development of multi-marker panels and classifiers. While initially such classifiers were flawed as a result of data overfitting [3,5], and due to inadequate technology and data interpretation, these issues have been addressed in several guidance manuscripts [6,7]. Hence, the implementation of omics-based biomarker panels into the clinical practice, drug development, and patient management, becomes more feasible every day.

In this perspective, we discuss the value and consequence, but also technical issues associated with the implementation of multi-biomarker panels into the medical practice in the near future, either to complement or to replace currently used standards. This assumption is based on the growing evidence demonstrating the advantages of biomarker panels over currently applied traditional tools.

Proteomic platforms in biomarker research

Different proteomics platforms are currently employed, the most common ones are LC-MS, 2DE-MS, and CE-MS. The pros and contras of the different platforms have been described in detail in several recent reviews [8-11]. In Table 1 the main advantages and disadvantages of the most common techniques are listed. The high complexity of the mammalian proteome imposes the need for pre-fractionation prior to mass spectrometry (MS) analysis e.g. by using two-dimensional gel electrophoresis (2DE), liquid chromatography (LC) or capillary electrophoresis (CE). The separation phase is coupled to a mass spectrometer, either on- or off-line. 2DE-MS has the advantage over the other techniques of obtaining additional information on the full protein such as molecular mass and isoelectric points. The major drawback of 2DE-MS is its low throughput and low resolution compared to other technologies. Shot-gun LC-MS is most often used for discovery studies and displays high resolution. The comparison of LC and CE-MS indicated that small and highly charged molecules are rather unable to bind to the LC column, but can be detected with CE-MS [12]. Due to the long run-times (often 4-8 h to obtain high resolution) LC-MS has rather low-throughput and was found less robust and reproducible than CE-MS in direct comparison [12,13]. Further comparison between LC and CE-MS did not reveal a big difference in the variation in migration time and peak area repeatability (these need to be corrected for both,

CE and LC-MS). Yet, LC-MS is more commonly used than CE-MS in research. This appears at least to some degree due to the fact that no vendor currently offers a complete working CE-MS solution fit for clinical application. However, CE-MS has demonstrated its value in biomarker discovery and validation, the by far largest proteomics studies (including >10 000 subjects, [14]) reported are based on CE-MS, CE-MS is used in PRIORITY, the currently largest clinical proteomics study including over 3200 subjects ([15], NCT02040441,) and it is used in clinical decisions.

MRM (multiple reaction monitoring), targeting multiple protein-based biomarkers in complex samples, is a promising LC-MS-based alternative for ELISA and is specifically used in biomarker validation studies. It is high-throughput and allows absolute quantification. It is however due to the use of peptides standards still costly. CE-MS is focussing on native peptides and due to its reproducibility and comparability between datasets can be used for discovery, validation and clinical application.

The combination of the separation phase with high-resolution mass spectrometers enables to theoretically resolve over 1 million compounds. Irrespective of the approach used, the platform and the utilized protocols have to be well-characterized in the context of analytical variability and measurement precision, prior to the implementation of the analytical strategy [6].

Table 1. Main proteomic approaches applied in the field of biomarker research.

Approach	Advantages	Disadvantages	Application	Other characteristics
2DE-MS	Retains Information at protein level	Low throughput and low resolution	Biomarker discovery	Enables relative quantification

LC-MS (shot-gun)	High resolution	Low throughput	Biomarker discovery	Data analysis is often complex; currently the most widely used platform for biomarker discovery
LC-MS (MRM)	High throughput, enables absolute quantification	Proteotypic peptides required, not applicable in discovery	Biomarker validation	Promising alternative to ELISA for targeted quantification
CE-MS	High resolution for the native peptidome, highly reproducible	Low loading capacity	Biomarker discovery, validation and implementation	Established technology for a wide range of urinary-peptide-profiling applications; clinical implementation feasible

Abbreviations: CE-MS, capillary electrophoresis coupled to mass-spectrometry; 2DE-MS, 2-dimensional gel electrophoresis followed by mass-spectrometry; LC-MS, liquid chromatography coupled to mass-spectrometry.

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Capillary electrophoresis coupled mass spectrometry (CE-MS) technology and its clinical application

CE-MS has been applied in clinical proteomics for over 10 years and enabled identification of multiple proteomic-based biomarker panels [3,16]. Capillary electrophoresis can be hyphenated to a number of MS technologies including time of flight (TOF), ion-traps (Orbitraps) and triple quadrupoles mass spectrometers [17,18]. However CE-ESI-(electron spray ionization)-TOF has been the most widely used approach and the only one currently contributing to patient management. This may be owed to the TOF representing the optimal compromise between resolution and sensitivity, versus cost. We will therefore in the remainder of review focus on CE-ESI-TOF (for convenience called CE-MS in the rest of the manuscript). Over time, CE-MS method was demonstrated to be a powerful analytical tool for the identification, characterization and quantification of protein and peptide biomarkers in urine. The work flow involving the CE-MS technology with associated computational

approach is depicted on Figure 3. The sample preparation and the CE-MS analytical cycle are performed according to a standardised protocol enabling good reproducibility and comparability, also when urine samples of other species are to be analysed [19]. In the typical standard procedure, 700 µL of urine are defrosted with the addition of 0.1% PMSF saturated in ethanol and diluted with 700 µL of a solution containing 2 M urea, 10 mM NH_4OH and 0.02% SDS. The mixture is filtered through a 20 kDa MW cut-off ultracentrifugation filter device (Sartorius Stedim UK Ltd, United Kingdom) at 3,000 rcf for one hour at 4°C. A volume of 1.1 mL of the filtrate is subsequently loaded onto a PD-10 desalting column (GE Healthcare, Sweden) pre-equilibrated with 0.01% aqueous NH_4OH. The eluate is then freeze-dried and stored at 4°C prior to being resuspended in HPLC-grade water to a final protein concentration of 2 mg/mL for CE-MS analysis. For CE, typically 250 nl of a sample are injected hydrodynamically in a 90 cm long fused silica capillary of 75 µm inner diameter, which is filled with running buffer containing 79:20:1 (v/v) MilliQ water, acetonitrile, formic acid. Peptides are separated in an electric field of 300 V/cm. The capillary temperature is set to 35°C for the entire length of the capillary up to the ESI interface. Different interfaces between CE and ESI have been described in greater detail elsewhere [20].

The MS analysis is performed in positive electrospray mode using e.g. a microTOF-MS (Bruker Daltonic, Bremen, Germany). The ESI sprayer is grounded and the ion spray interface potential is set between 4 and 4.5 kV. The sheath liquid is applied coaxially at a rate of 200 nL/min. The data acquisition and the MS method are automatically controlled by the CE program via contact-close relays. Spectra are accumulated every 3 s, over an m/z range from 400 to 3000 for 60 min. CE-MS enables high throughput screening (1000-6000 analytes per sample) with high analytical specificity and sensitivity for small (< 20 kDa) proteins or

peptides (hence it is frequently referred to as a peptidomics approach) from biological fluids (mainly urine) or tissue supernatants [16]. The method is relatively fast (an analytical cycle takes approximately 60 min and sample preparation can be accomplished in 8 hrs), simple and reproducible. A standardised protocol and a set of peptides used for internal normalization contribute to the good reproducibility of the method [21,22]. The main clinical application was, and still is, in the assessment of the urinary proteome/peptidome. Urine appears to be especially well suited for clinical application, as the urine proteome/peptidome displays much higher stability than blood [2] . This is probably due to the fact, that urine before collection is "residing" in the bladder for several hours, where it exposed to 37^{o}C and to a number of proteolytic enzymes [3]. A major shortcoming is the variability in the protein and peptides concentrations arising as a result of fluctuations in daily fluid intake, circadian rhythms, and physical activity. These variations can be compensated by the use of various normalizing methods [23].

Several studies performed comparison of CE-MS with other proteomics technologies. When investigating chronic kidney disease (CKD), 273 peptides associated with CKD could be detected with CE-MS, while only three were found with MALDI-MS [24,25]. A direct comparison between CE-MS and MALDI-MS revealed better performance and greater reproducibility of CE-MS than MALDI-MS in CKD diagnosis [10]. In the utilised experimental set up, examining the peptidome of urine, a biologically and clinically relevant sample, CE-MS displayed superior resolution, robustness, and reproducibility, and lower variability, in comparison to LC-MS [26]. A disadvantage of CE-MS is its low loading capacity, which may impact on the assessment of very low abundant proteins and peptides. However, when comparing different platforms, CE-MS is currently the only technology that can be employed

for biomarker discovery, validation, and clinical application. This contrasts with LC-MS, which requires a change in technology (additional steps, such as multiple reaction monitoring (MRM) or antibodies) for validation or clinical application after the discovery phase. Furthermore, CE-MS is delivering tens of thousands of comparable datasets and thereby is greatly facilitating the evaluation of newly discovered biomarkers in closely related diseases for the assessment of the true specificity of “disease-related” biomarkers. In total, the approach has been used to date in over 50 different disease etiologies involving over 35 000 independent samples [101]. An overview of the different diseases investigated and the number of datasets available is presented in Figure 4. This database represents a highly valuable resource, as it enables comparative assessment (based on highly reproducible sample preparation and analytical technology) of multiple peptides in tens of thousands of datasets [101]. Finally, it is also the only proteome-based technology applied to studies with over 10000 subjects [14].

Proteome-based biomarker discovery with the CE-MS technology is a rapidly expanding area. Especially urinary proteomics is penetrating the field of clinical diagnostics, as urine is easily accessible and collected, available in large quantities, and of higher stability than other body fluids (blood, plasma, serum, or cerebrospinal fluid). Not surprising, the main application is in the field of nephrology. A literature search with keywords in Web-of Science with TOPIC: "capillary electrophoresis" AND "mass spectro*" OR CE-MS AND TOPIC: proteom* AND TOPIC: marker OR biomarker (DOCUMENT TYPES: ARTICLE OR REVIEW) over the timespan 2010-2015 yielded 125 manuscripts, with 91 related to renal disease. The CE-MS urinary proteomic approach enabled the discovery of at least 17 biomarker panels for diagnosis/prognosis of many common as well as rare diseases (Table 2) [16]. Several of these CE-MS-based biomarker panels are already registered and in use for clinical diagnosis in

Europe. Based on one urinary CE-MS analysis, multiple diseases can be assessed via application of designated biomarkers, enabling early detection and prediction of disease development several years in advance. For example:

- a classifier based on 273 urinary peptides “CKD273” was identified in the context of human chronic kidney diseases (CKDs) from different aetiologies (diabetic nephropathy (DN), IgA nephropathy, ANCA-associated vasculitis, focal segmental glomerulosclerosis, membranous glomerulonephritis, minimal change disease, and lupus nephritis) [25]. Subsequently this classifier was assessed for its predictive value. Several studies demonstrated that it allows: i) early detection of CKD or DN [27,28], with greater accuracy than the current clinical functional parameters (urinary albumin or plasma creatinine levels) and ii) prediction of CKD evolution from moderate to end-stage renal disease (ESRD)[29]. This biomarker is currently the most accurate biomarker for prediction of CKD progression, as it correlates with estimated glomerular filtration rate (GFR) decline (eGFR slope/year (%)), a standard measure reflecting renal function decline, to a greater extent than urinary albumin [30]. In a recent systematic review the performance of CKD273 is outlined in detail [31],
- heart failure (HF) biomarkers were identified using CE-MS based urinary proteomics and used to predict left ventricular dysfunction in asymptomatic hypertensive patients, indicating an advantage of implementing this approach as a screening method, prognostic tool and for subsequent monitoring purposes of individuals at risk [32],
- CE-MS identified biomarker panels allowed to assess the risk of coronary artery disease (CAD) [33]. In addition, these biomarkers indicated a beneficial effect of dietary products like polyphenols or olive oil [34,35].

241 Interestingly, the CE-MS studies for discovery and validation of urinary biomarkers also
242 indicated that assessment of chronic conditions such as CKD or coronary artery disease
243 (CAD) may require biomarkers comprised of a wider range of peptides than acute diseases
244 such as acute kidney injury (AKI) or preeclampsia, further underscoring the complexity of
245 chronic conditions [36-38].
246

247 **Table 2. Biomarker panels discovered and/or verified with CE-MS technology**

Disease area	Disease	No. of peptides in the biomarker panel	Sensitivity % (test set)	Specificity % (test set)	References
RENAL DISEASES	Diabetic nephropathy (DN)	65	97	97	Rossing et al. [39]
	Chronic kidney disease (CKD)	273	86	100	Good et al. [25,27-30]
	ANCA-associated Vasculitis	18	90	90	Haubitz et al. [40]
	IgA Nephropathy	25	90	90	Julien et al. [41]
	Autosomal polycystic kidney disease (ADPKD)	142	88	98	Kistler et al. [42]
	Acute kidney injury (AKI)	20	89	82	Metzger et al. [37]
	Posterior urethral valves (PUV)	12	88	95	Klein et al. [43]
	Ureteropelvic junction obstruction (UPJ)	53	94	80-100	Decramer et al. [44]
CARDIOVASULAR DISEASES	Coronary artery disease	15	98	83	Zimmerli et al. [45]
		17	81	92	von Zur Muhlen et al. [46]
		238	79	88	Delles et al. [47]
		35	56	93	Dwanson et al. [48]

	Heart Failure (Left ventricular dysfunction)	85	69	94	Kuznetsova et al. [32]
		85	56	93	Zhang et al. [49]
TRANSPLATATION AND OTHER RELATED DISEASES	Acute graft versus host disease (aGvHD) grade III and IV	31	83	77	Weissinger et al. [50]
	Acute renal allograft rejection	14	93	78	Metzger et al. [51]
	Bladder cancer (BCa)	22	100	63	Theodorescu et al. [52]
	Renal cell carcinoma (RCC)	86	80	87	Frantzi et al. [53]
	Prostate cancer	12	91	69	Theodorescu et al. [54]
	Cholangiocellular carcinoma	42	83	79	Metzger et al. [55]

CE-MS identified biomarker panels for drug research and development in preclinical studies

Preclinical animal models: Although animal models are frequently used in preclinical research, the translatability of results obtained in animal disease models to human is low, contributing to the high attrition rate of clinical trials investigating new drugs [56]. The availability of CE-MS identified biomarker panels for renal and cardiovascular diseases has great potential to improve on the selection of animal models for human disease. CE-MS analysis comparing the urinary proteome of humans and the Zucker diabetic fatty (ZDF) rat showed that this model does not reflect human DN on a molecular level [57], although routinely used as a model for this purpose. However, the ZDF rat model displays overlaps with human cardiovascular disease (CVD) biomarkers and may therefore be well suited for studying the CVD complications related to DN. Similarly, CE-MS analysis-based comparison of the urinary proteome of wild type (C57BL/6) mice harboring long term metabolic

syndrome with data on human DN indicated the absence of DN in this mouse model. Indeed, the renal injury in these mice with long-term metabolic syndrome induced by a high fat and fructose diet (HFFD) was minimal after 8 months of HFFD, as determined by detailed analysis of kidney structure and function utilizing GFR measurements and electron microscopy [58].

"Humanised" biomarker panels: These above mentioned studies indicate that CE-MS can be employed to assess the similarity of animal models and human disease. To close the gap between the preclinical and clinical stage and improve translation of results into the clinic, the CE-MS technology in combination with *in silico* models offers a simple yet effective solution: disease biomarker panels based on peptides orthologous between animal model and humans, ignoring the non-human animal disease related peptides. Observation in mouse models based on these "humanized" biomarker panels will have a great translational value. Recently such humanized biomarkers were identified using urinary peptidome data sets from two mouse models of type 2 diabetic (T2D)-DN and the human urinary proteome database. The identified orthologous biomarker panel for T2D-DN is more sensitive to reflect renal lesions in the investigated models than commonly used markers to detect renal injury, and this biomarker panel can be employed to assess benefit of therapeutic intervention in these preclinical mouse models (Schanstra et al., unpublished observations). In addition to the translational character of such humanized molecular models, the non-invasive character of the test allows on-route adjustment of drug concentrations and protocol lengths and thus contribute to the reduction in the number of animals required for experiments and thereby also to the reduction of animal handling costs.

Safety and toxicity: Another promising area for implementation of biomarker panels is in the safety/toxicity tests of lead compounds in preclinical studies. A plethora of drugs are known to induce renal damage and for this reason their administration has to be in certain cases discontinued [59]. Therefore the detection of nephrotoxic effects of the lead compounds in the premarketing, ideally even in the preclinical stage, is of great importance to ensure that only selected, most promising compounds reach the market and do not have to be withdrawn, because of adverse effects on the kidney.

Nephrotoxicity is an especially troubling issue for antibiotics, as they belong to the top ten medications, which damage the kidney [60]. Since the development pipeline for antibiotics was rejuvenated with recent initiatives encouraging their discovery (summarized elsewhere [102]), there is a growing need for nephrotoxicity tests for lead compounds with antibiotic properties. Implementation of the CE-MS technology for early detection of toxicity, already in preclinical stage, would enable the selection of nephrotoxicity-free compounds to enter the clinical studies on humans. Biomarker panels identified with CE-MS technology have demonstrated substantial potential for the determination of nephrotoxicity in animal models [61,62].

CE-MS identified biomarker panels in clinical studies: drug development and patient management

Drug development is becoming increasing complex, and most candidates never reach the market. For example, only 10% of oncological drugs that enter clinical development reach the stage of market approval and 9 out of 10 candidates do not complete the process for market authorisation, mainly because no therapeutic benefit could be demonstrated or as a result of unfavourable side effects [63]. The use of biomarker panels could improve the drug

development process not only at the above mentioned preclinical level, but also in early- and late-stage clinical trials.

Patient stratification: (urinary) biomarker panels could support selection and stratification of the trial population, and enriching for expected responders. Such approaches were first implemented in certain types of cancer, based on the availability of predictive biomarkers to detect aberrant gene expression products, affecting single genes (e.g. EGFR, HER-2, KIT, and BRCA1/BRCA2) or occurring due to gene fusion (eg. BCR-ABL), which drive the oncogenic phenotype [64-66]. In complex chronic diseases, such as renal or cardiovascular disease, single biomarkers or single gene mutations do not allow predicting the future disease course with high confidence. These diseases are heterogeneous in their pathophysiological and molecular background, thus require classifiers composed of multiple biomarker to account for their complexity and to provide a reliable measure of the severity of the pathological state. As demonstrated in several recent manuscripts, urinary biomarkers do not only allow assessing these diseases at an early stage, and enable prognosis, but they, as expected, also allow detection of the effects of therapeutic intervention [67]. For example, in diabetic nephropathy, irbesartan (angiotensin II receptor antagonist) initially developed for the treatment of hypertension, has been shown to delay progression of DN [68]. In a study with CE-MS- identified biomarkers for CKD, the renoprotective effects of irbesartan in microalbuminuric type 2 diabetic patients were clearly reflected in change of the pattern and score of the urinary biomarker panel for CKD [69]. Similarly, in the study of irbesartan in CAD patients, the CE-MS identified CAD biomarker pattern and overall score was significantly affected following two year treatment [47]. In another recent study, a significant beneficial

effect of olive oil consumption on cardiovascular disease (as assessed by CE-MS-based biomarkers) could be demonstrated [35].

Based on these results, the PRIORITY (Proteomic prediction and Renin angiotensin aldosterone system Inhibition prevention Of early diabetic nephRopathy In TYpe 2 diabetic patients with normoalbuminuria, PRIORITY, NCT02040441) study was initiated [15]. The study design, as depicted in Figure 5, aims at stratification of T2D patients for those that will develop CKD, using the well established CKD273 biomarker panel [25]. Patients positive in CKD273 screening will be randomized to low dose spironolactone (aldosterone antagonist) treatment or placebo. This large scale multicentric study in 13 different European centers aims at recruiting 3280 patients. If positive, this study will not only further demonstrate the predictive potential of the CKD273 classifier, but also the value of clinical proteomics in guiding early intervention.

Risk stratification: The data available also indicate that the CE-MS identified biomarker disease panels (Table 2) can be employed for monitoring (side) effects of novel drugs in clinical trials. As an example, we would like to highlight several recent trials in the context of CKD where an increase in mortality in the treatment group raised substantial concerns. Bardoxolone initially showed promising results (increased eGFR) in patients with T2D kidney disease [70,71]. However, already within the first four weeks after randomization in a phase 3 study (BEACON trial), the bardoxolone methyl group patients revealed a significant increase in hospitalization and death from heart failure [72]. Two years later post hoc analysis revealed that this was due to patients in the bardoxolone methyl treated group displaying an increased risk for heart failure already at the beginning of the trial [73,74]. This risk could have been assessed with CE-MS based urinary biomarkers *a priori*; patients

harbouring a signature for HF could have been excluded, and a potential benefit of bardoxolone could have been demonstrated. Similarly, in the Roadmap trial no convincing benefit, but a significant increase in mortality was observed [75]. Applying CE-MS analysis would not only have enabled enriching the population developing CKD (and hence likely demonstrating a convincing significant benefit), but also allowed excluding those patients that have an unfavorable risk profile (e.g. that are positive for CAD in the CE-MS based urine proteome analysis). The Altitude trial (NCT00549757) aimed at demonstrating a benefit of aliskiren, an aldosterone antagonist, as an add-on therapy to an angiotensin-converting-enzyme inhibitor or an angiotensin-receptor blocker [76] However, the trial was stopped, as a result of lack of efficacy, and safety issues. As above, in this trial application of urinary CE-MS analysis could have enabled stratifying for patients that would benefit from aliskiren intervention in a personalized/targeted medicine approach.

Collectively these trials can be seen as a classical example for displaying the unmet need for proper risk stratification, especially if translation of the findings from preclinical to clinical stage becomes problematic due to lack of suitable animal models. In case of bardoxolone, the results of the experiments in the investigated animal models did not indicate potential harmful effects of this compound on the cardiovascular system [77]. The experimental data obtained from the rat model of T2D kidney disease (ZDF rat) indicated absence of a benefit on kidney function and negative impact on liver. Yet, the effects observed in the human bardoxolone methyl trial were completely different. This is likely owed to the fact that the ZDF rat does not appear to be a suitable model for human DN , hence the observed impact of the investigated drug in man and ZDF rat is very different.

New study endpoints: Despite the increasing prevalence of CKD, there are fewer clinical trials investigating the effect of drugs on CKD progression than in other common diseases [78]. In January 2014, out of overall 4726 trials in medicine only 13 investigated DN, a leading cause of CKD across the world [79]. A reason may be the necessity of prospective studies with long follow-up periods to reach the currently accepted hard clinical endpoints (i.e. doubling of serum creatinine, end-stage renal disease (ESRD), or death) [68,80]. To improve on the management of CKD and stimulate clinical trials in the area, there is increased interest in exploring alternative clinical trial endpoints. In 2014, the European Medicines Agency (EMA) proposed that the primary efficacy endpoints for compound testing should be the prevention or delay of renal function decline defined as either time to occurrence or incidence rate of CKD stage 3 (eGFR 30-59 mL/min/1.73 m^2) or higher, with or without prevention of proteinuria/albuminuria [103]. The efficacy of focusing on such shorter-term targets in CKD is supported by a recent meta-analysis including 1.7 million participants where it was concluded that a 30% reduction in estimated glomerular filtration rate (eGFR) over two years was strongly and consistently associated with the risk of ESRD and mortality [81,82]. The CE-MS identified biomarker panel, CKD273, enables prognosis of a class change to CKD stage 3, as demonstrated in a prospectively collected cohort of over 1600 individuals [83]. Thereby, it is to our knowledge to date the only available biomarker panel to facilitate clinical trials with CKD stage 3 as a primary efficacy endpoint for compound testing, as proposed by EMA [103]. Drugs interfering with disease at an early stage (aim of the PRIORITY study) have a greater chance to prevent or slow down the progression of disease, as they can intervene with the molecular pathways governing the disease very early in disease development, before structural irreversible damage to the organs occurs. Biomarkers, such as CKD273 identified with CE-MS, are currently the only

means to evaluate drugs acting on asymptomatic CKD patients (e.g. PRIORITY), because such biomarkers reflect pathophysiological changes on the protein level in the course of disease and therefore are outcompeting the conventionally used/applied methods based on functional parameters (a significant reduction in eGFR, typical at and after CKD stage 3, reflects structural adverse alterations in the kidney).

CE-MS technology and biomarker panels meet patient needs, open a window of opportunities for pharmaceutical industry and reduces work load of health care professionals, while increasing patient compliance

Early diagnosis=improved outcome for patients: Use of the CE-MS technology has led to the discovery of protein-based biomarker panels that allow early detection of acute and chronic diseases. The major chronic diseases (e.g. diabetes, cardiovascular disease, kidney disease) are conventionally detected when symptoms appear (Figure 6). Unfortunately, at this point, in the majority of the cases the disease can already be qualified as advanced disease largely reducing the success of pharmacological treatment (e.g. a hypertrophied glomerulus in the kidney cannot be regenerated). At this late stage, disease progression can possibly only be delayed to some extend, although with moderate success, as also evident by the multiple trials that failed. For example most patients reaching CKD stage 3 will progress to a more advance stage and, in parallel, likely develop cardiovascular complications, leading to a significant reduction of the overall quality of life [82,84-86]. The option of accurate early disease detection also represents new opportunities for pharmaceutical and food industry as it enables earlier intervention and overall longer time-span for individuals to maintain high quality of life and healthy aging by providing adequate, ideally side-effects-free medication

due to the potential lower doses employed in early stage disease. Proteome analysis with CE-MS technology enables the identification of several diseases at an early stage, where intervention may still even be curative. Based on the individual CE-MS risk profile, personalized therapeutic approaches, that may well include dietary or lifestyle changes, could be implemented to enable “healthy ageing” [14].

Benefits for health care professionals and on patients’ compliance: The implementation of non- or minimally-invasive biomarker panels in the clinical routine carries the advantage of enabling earlier detection, frequently with higher accuracy than the current state-of-the art, and without the risks associated with interventional procedures, hence will also increases patient compliance. For example, in case of bladder cancer, the very high recurrence rate has resulted in patients typically undergoing repeated cystoscopy for surveillance [8]. During this procedure, which is highly unpleasant and requires local or general anaesthesia, urethral damage can occur, despite being performed by trained physicians. Subsequently patients may experience urinary incontinence, occasionally local abdominal pains, and suffer from infection. In addition to being obtained invasively, this approach is dependent on a trained physician and subject to observer bias. In contrast, biomarkers panels lack this observer variability and lead to quantitative and highly comparable results. CE-MS-based biomarker panels have been developed for the management of bladder cancer [87]. While urinary proteomics may not fully replace the conventional invasive methods in diagnosis, it likely will guide their application towards only those patients with a very high risk, similar to stratification of patients for intervention. This will increase patient quality-of-life and compliance and is predicted to be cost-effective [8,88]. This potential cost effectiveness is related to the fact that the biomarker panels can provide not only information about

presence or absence of the disease, but also its status and thereby exclude expensive procedures (magnetic resonance imaging, computed tomography) performed to e.g. determine metastasis in patients that have no risk of harbouring metastases.

A similar benefit is evident in managing prostate cancer (PC) [88]. The biomarker panel-guided diagnosis of PC can increase patients compliance, as it can prevent trans-rectal ultrasound (TRUS) guided prostate biopsy, performed in patients with high levels of prostate specific antigen (PSA) in the blood, a conventionally used parameter for PC diagnosis [89]. Since high PSA levels may also result from inflammation, age, sexual activity, and benign prostate hyperplasia [90-92], biomarker panels have a great potential to supplement the shortcomings of PSA determination as well [93].

Conclusion

The CE-MS technology has been used as a reproducible approach on over 35 000 human urine samples to date. It provided a large number of protein-based body-fluid biomarker panels for a wide variety of diseases, most of which have been validated in independent studies. In addition to disease detection and prognosis, the use of these panels is predicted to be beneficial in several stages of the drug discovery process:

I. Biomarker panels can guide selection of the optimal preclinical models, based on the similarity with human disease. This will improve the translatability of observations of effects of new drugs in preclinical models and at this early stage determine safety or toxicity of the new drug.

II. When moving to the clinical phase these biomarker panels can stratify/identify patient at risk of progression and thus significantly reduce the number of patients

required to be included in trials, whilst at the same time increasing the power of the study, as a result of a much higher number of investigable endpoints.

III. In addition, safety and toxicity can be addressed at an early stage by screening a number of biomarker panels for comorbidities of the disease under study.

IV. Since all CE-MS based biomarker panels have been identified in urine , a body fluid accessible non-invasively that can be easily resampled, patient adherence to such surveillance will be high and drug effects can be easily evaluated on-route.

Future perspective

Although the CE-MS technology has been around for over 15 years, the biomarker panels for various diseases identified with this technology have generally not been implemented in the clinics yet. However, due to the growing evidence supporting the added value of biomarker panels, it is foreseeable that they will soon become a more widely used tool of choice for early diagnosis, patients' stratification or drug evaluation.

Early diagnosis based on biomarkers panels will enable earlier clinical intervention, if possible, and therefore improve the chance of successful therapy, before irreversible changes to the organ structures take place. The prognostic value of the biomarker panels is expected to significantly impact on patient stratification, in particular to select the appropriate clinical trial population. This development will further pave the way towards personalized medicine. The usage of CE-MS-based urinary biomarker panels, as a surrogate marker for specific disease stages, will become more widespread in drug evaluation, based on the huge amount of data sets available for comparison (which is not available for any

other proteomics technique) and on the fact that urine is an easily accessible source of information.

CE-MS-based biomarker panels appear to have substantial potential to facilitate/guide drug development in preclinical stage. The application of orthologous biomarker panels will improve translatability of the results obtained from animal model-based preclinical research and thus decrease the number of unsuccessful trials. Taking into consideration all the advantages the biomarker panels bring to different health sectors, it is plausible that one day they will not only complement current gold standards, but in some cases, even replace them, as recently suggested in a slightly provocative article proclaiming urinary proteome analysis as “liquid kidney biopsy” [2].

Executive summary

- Among different proteomic technology platforms, the CE-MS technology has been shown to be well suited for biomarker research and on the way for implementation in the clinic.
- Biomarker panels have advantage over single biomarkers in terms of their stability, amount and quality of the information that they provide, and are therefore well suited to reflect the state and complexity of biological systems.
- CE-MS identified proteome based biomarker panels allow early diseases diagnosis and/or prognosis. Thus they can facilitate early therapeutic intervention and/or can guide patients to introduce life style changes.

- CE-MS is the only proteomics based technology has been utilized to analyze wide range of different diseases and to generate data base composed of over 35.000 highly comparable datasets [101].
- Urinary based proteomic biomarker panels can be implemented in medical research and practice, as well as in preclinical and clinical stages of drug development.
- Unique humanized biomarker panels developed using CE-MS carry advantage of enhancing the translatability of the results obtained in animal models to the clinic. They minimize the need for usage and sacrifice of experimental animals in preclinical research and enable longitudinal studies.
- The benefits of implementation of CE-MS identified proteome based biomarker panels in the clinics are vivid in the area of:
    1. patient stratification
    2. monitoring of disease progression and/or drug effects (including side effects)
    3. ensuring patient safety due to possibility of detecting comorbidities
    4. collection of statistically relevant results when conducting the trial
    5. practicability (non-invasive and less time consuming method for drug effects evaluation is patient-friendly and cost-effective)
    6. improved patient compliance
- Biomarker panels can facilitate the personalized medicine approach by pointing to the individual differences between patients.
- CE-MS identified proteome based biomarker panels have added value in medical research/practice and therefore have the potential to outcompete less accurate measures that are based on functional parameters.

Figure Legends

Figure 1. Proteins as sources of information for clinical research and drug development. Protein biomarkers and specific proteome pattern (lower right) reflect molecular pathways characteristic for a disease or a healthy state and represent the drug targets. This information can generally not be obtained from histological assessment (e.g. in tissue biopsies).
Adapted with permission from [2].

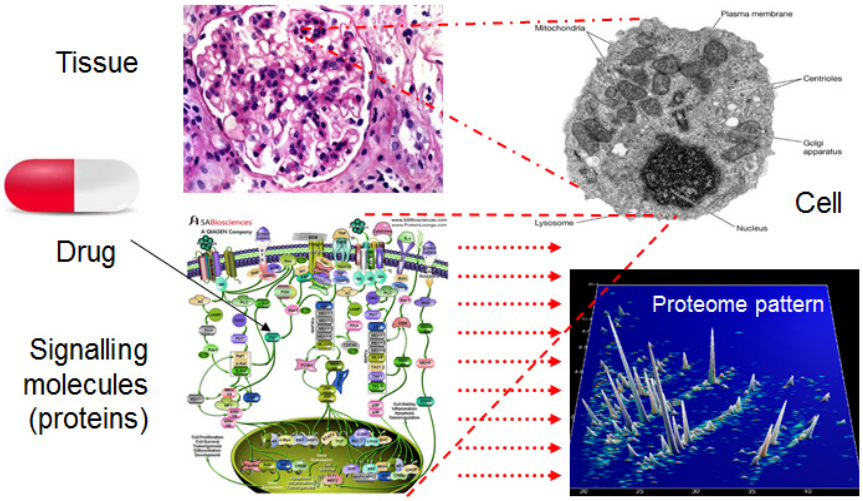


Figure 2. Omics technologies in analogy to a fireplace. Genomics comprises information about the entire genome, which can be well examined, yet it generally reflects the "potential" of an individual and remains unchanged during lifetime. In a way, it is analogous to the logs in a fireplace: logs have the potential to be set on fire under appropriate circumstances (oxygen, source of heat, dryness of the log etc.). The proteome delivers information on the current "status" of an organism, which is complex and continuously responding to various endogenous and exogenous stimuli. It difficult to assess comprehensively, undergoing constant changes, similarly to the fire in the fireplace. The action of the proteins results, at least to some degree, in metabolites. In analogy, the consequence of fire are ashes. Hence, ashes resemble metabolomics, small compounds, fairly stable, and allowing conclusions on previous action that led to their generation but generally not a trait that can be directly manipulated (via drugs) to change the strength of the fire. Reprinted with permission from [3].

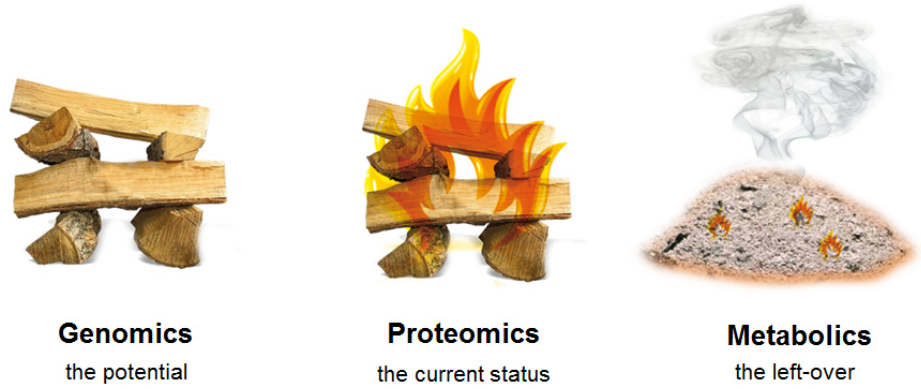


Figure 3. Schematic representation of the CE-MS-based platform and associated data base. Urinary peptides are injected in the CE and separated in a high-voltage field. The outlet of the CE is connected to the MS (time of flight (TOF) mass-spectrometer) using an electron spray interface (ESI) where mass and relative abundance of each peptide is analyzed. Individual peptide profiles can be compared to any peptide profile in the database either for classification or extraction of new biomarkers of yet undefined diseases. Adapted with permission from [94].

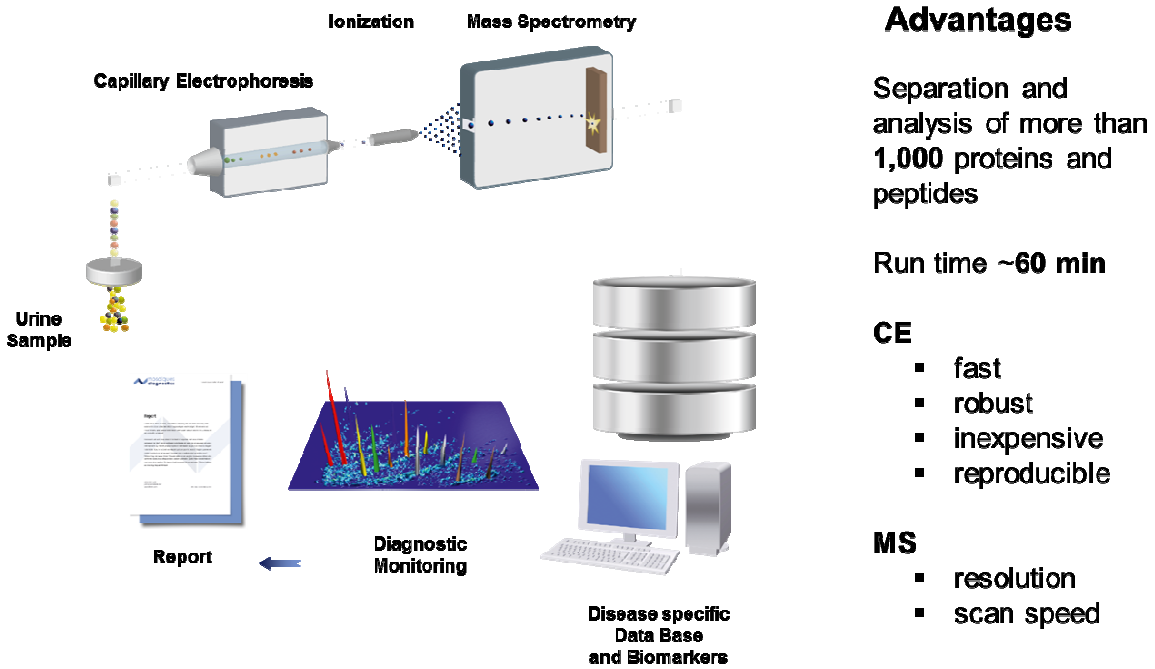


Figure 4. Content of the human urinary peptide database. To date, the human urinary peptide data base encompasses over 35 000 independent CE-MS human urine data sets. It covers a wide range of diseases including cardiovascular, renal and hematological disease and a large variety of cancers [101]. Adapted with permission from [13].

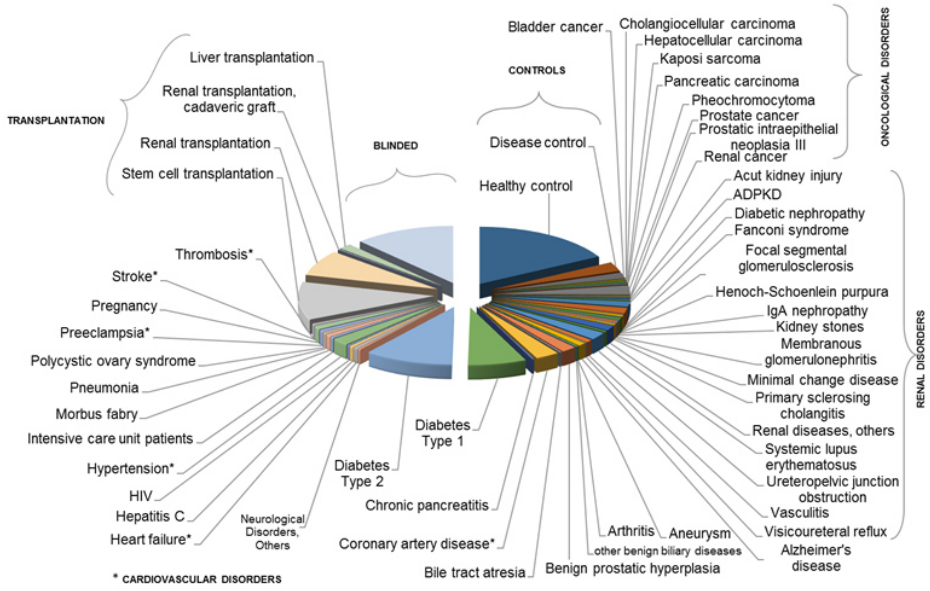


Figure 5. CE-MS biomarker guided patient stratification. In the PRIORITY clinical trial (NCT 02040441), the CE-MS identified biomarker panel CKD273 is implemented for patient stratification into the high (red box) and low (green box) risk groups for the development of CKD. The low risk group, which likely will not develop CKD, will not receive any additional treatment (as this may bring harm, but likely no benefit). The high-risk patients will be randomized to the therapy with the investigational drug (low dose spironolactone) or placebo. A total of 3280 patients are included in the study.
Adapted with permission from [95].

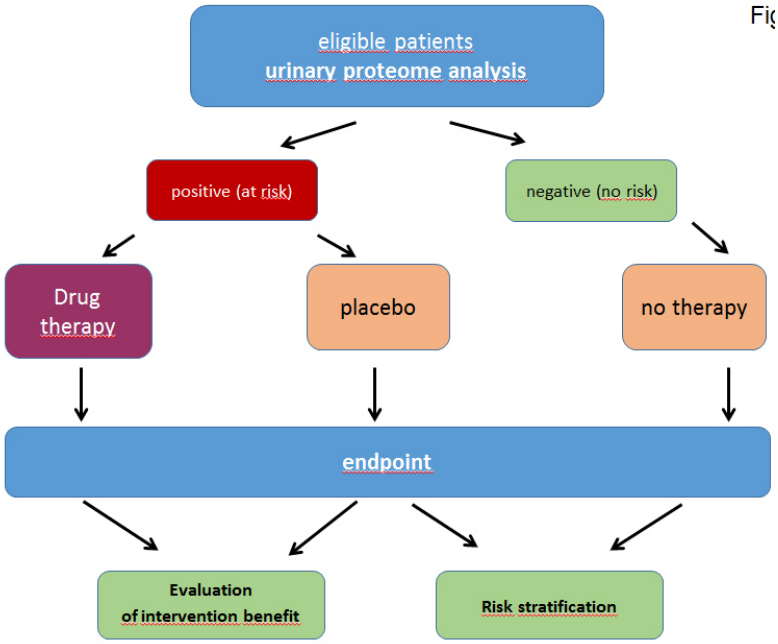
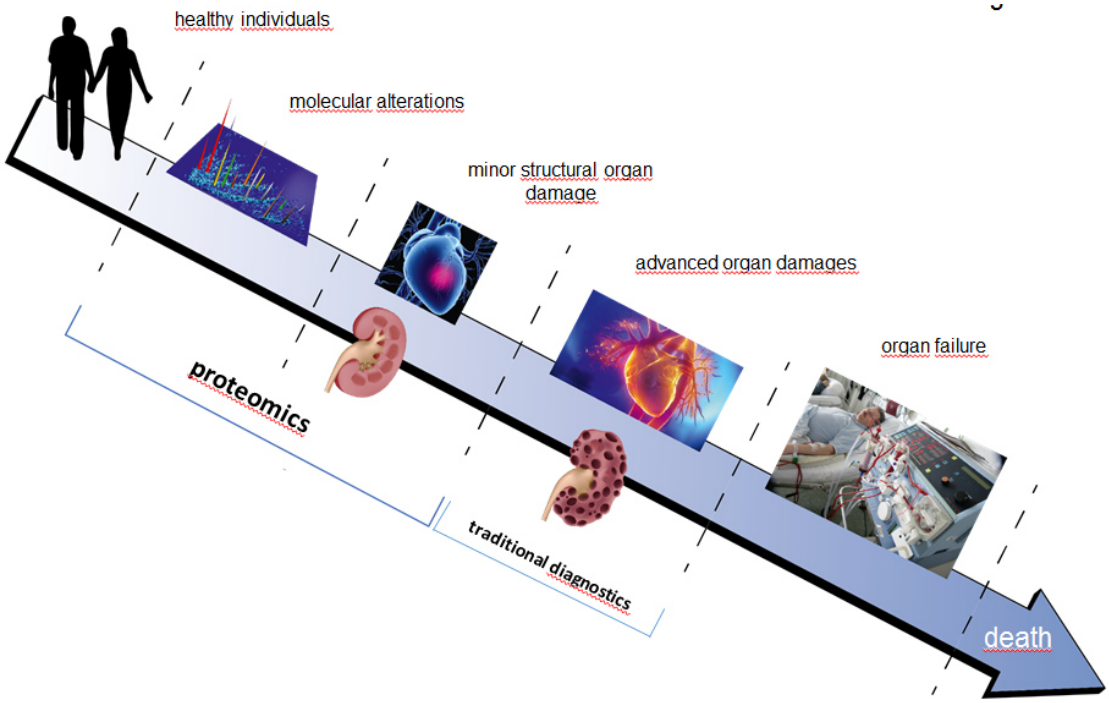


Figure 6. Early diagnosis and/or prognosis of diseases improves chances for a better outcome for the patients. The initiation of molecular processes that result in (chronic) diseases can be detected based on the decisive molecular changes, using proteomic technologies, substantially prior to advanced organ damage. This could allow earlier intervention where drugs are most effective.



Tables

Table 1 Main proteomic approaches applied in the field of biomarker research. Several proteomics platforms can be employed at different stages during the biomarker development, including discovery (2DE, LC-MS, CE-MS) and validation/ implementation phase (CE-MS, MRM).
Adapted with permission from [8].

Table 2 Biomarker panels discovered and/or verified with CE-MS technology. Biomarker panels for diagnosis and prognosis of many common as well as rare diseases have been identified using the CE-MS technology. Most of them are for chronic diseases including renal and cardiovascular diseases.

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102 http://www.pewtrusts.org/en/research-and-analysis/issue-briefs/2014/03/12/tracking-the-pipeline-of-antibiotics-in-development

103 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/06/WC500169469.pdf

Reference annotations

Ref 6 ●● Key paper identifying issues and suggesting changes in clinical proteomics studies for the generation of clinically valid biomarkers.

Ref 10 ● Key evidence for superiority of CE-MS over MALDI-MS for the identification of urinary biomarkers of CKD.

Ref 12 ● Comparison of CE-MS and LC-MS showing comparable detection of urinary peptide numbers but increased comparability between CE-MS datasets.

Ref 14 ●● Largest clinical proteomics study ever.

Ref 30 ●● First large scale study showing the capacity of urinary biomarkers to predict progression of CKD.

Ref 58 ● First evidence of the use of CE-MS urinary peptidomics in the selection of suitable preclinical animal models of disease.

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