

**INVITED PAPER**

**URINE PROTEOMICS IN CLINICAL APPLICATIONS:  
TECHNOLOGIES, PRINCIPAL CONSIDERATIONS AND CLINICAL  
IMPLEMENTATION**

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**Abstract:** The technology platforms for proteome analysis have advanced considerably over the last few years. Due to these improvements the number of studies on the analysis of the proteome/peptidome with the aim of defining biomarkers has escalated. In this review, we will summarise the technical aspects that relate to the proteomics field targeting the discovery of biomarkers for disease diagnosis. We will describe the course from biomarker discovery or ‘potential’ biomarkers to those that are clinically important. We also present several examples of successful proteomic studies that have defined ‘biomarker patterns’ for clinical applications, focussed on urine as a material source and capillary electrophoresis coupled mass spectrometry as a technology. Finally, current challenges and considerations for future studies will be discussed.

**Key words:** proteomics, urine biomarkers, clinical applications.

*Introduction*

Body fluids contain a vast amount of information on the (patho)physiological state of an organism. This has been known for centuries but it is over the last two decades that several "omics" technologies have dramatically evolved, driven by the advances in analytical technologies. The major constituents of body fluids that contain information on the physiological state of an organism are proteins and peptides. For this reason many proteins and/or peptides, the

study of which is known as proteomics and peptidomics, have been used as single biomarkers for disease in clinical applications with different degrees of success. The main limitation of this approach is that in many cases single biomarkers lack specificity. As an illustration, currently the main biomarker used for the clinical diagnosis of prostate cancer is prostate-specific antigen (PSA). However, the specificity of this marker is uncertain when levels of PSA are moderately increased (4–10 ng/mL). This leads to many unnecessary biopsies and a high rate of false positive prostate cancer diagnoses, that could be reduced if the specificity of the biomarker used was higher [1]. Therein proteomics have moved towards the identification and validation of panels of biomarkers rather than trying to identify a unique ideal diagnostic marker that might not exist [2]. Although initial expectations in this field were high, progress has been limited due to several reasons, which include a lack of comparability between studies, poor reproducibility, over-interpretation of data, questionable study designs, the use of inappropriate technologies and a lack of knowledge regarding data analysis and evaluation. Nevertheless, the experience gained in the past have helped scientists to understand that panels of biomarkers cannot be defined by an ill-defined ‘pattern’ and that a ‘potential’ biomarker needs to be well defined if it is to be used as a clinical diagnostic tool. In addition to these issues, a substantial obstacle might lie within the most commonly targeted proteome itself: the blood proteome. The blood proteome is highly complex with an extensive dynamic range that cannot be analysed successfully using today’s MS-technologies [3]. This has provoked a shift of biomarker research from blood to other more amenable and stable body fluids such as urine, which has been successfully applied in a number of clinical settings that will be summarised here, indicating the potential of this approach.

In this review we will define the course and obstacles from biomarker discovery or ‘potential’ biomarkers to clinically valuable biomarkers. We will present a number of successful studies that have defined ‘biomarker patterns’ in urine for clinical applications and will discuss main considerations for future studies.

### *Technical aspects in biomarker discovery*

#### *Sample source*

The sample sources available for proteomic studies can be classified into tissue and body fluids (mainly blood, cerebrospinal fluid or CSF and urine).

*Tissue* – The use of tissue is a promising approach for the identification of potential biomarkers especially when directly affected tissue is compared to normal/healthy tissue. The main limitation of this methodology is that tissue is

generally not accessible, especially for monitoring purposes. However, if biomarkers can be identified in tissues it is then possible to target the analysis of them in the blood using immunoassays [4]. Following this 2-step approach Roessler and colleagues successfully identified proteasome activator complex subunit 3 (PSME3) as a biomarker for colorectal carcinoma [5] which was then detected using immunological techniques in serum using an independent dataset. This general concept was further applied in other studies such as the one by Lou *et al.* [6] where Cathepsin D was identified as a biomarker of lung cancer and the identification of CRAMP, stathmin, EF-1alpha and chitinase as biomarkers for human aging by Jiang *et al.* [7].

*Blood* – As already mentioned, blood was initially one of the main targets in proteome/biomarker research. The role of blood as a transport medium for molecules from and to tissues made this body fluid an unquestionable initial attractive source. However, due to its complexity and dynamical range between low-level and high abundance proteins, the complete assessment of human plasma proteome is not possible with the current MS-technology. Efforts to overcome its analytical intricacy, due to its dynamical range, have been attempted using affinity columns and immunoaffinity depletion [8–10]. Other associated problems with blood are its invasive collection, scrupulous pre-analytical handling and the fact that it is prone to analytical artefacts.

New approaches using a selection of a sub-proteome (i.e. glycosylated or phosphorylated proteins) as reviewed by Temporini and colleagues [11] or the use of combinatorial peptide ligand beads [12] might lead to useful plasma and/or serum biomarkers in the future.

*Cerebrospinal fluid (CSF)* – Although CSF is not readily accessible, its collection for clinical diagnostic indications is not inconsiderable. Several of the disadvantages of blood apparently do not apply to CSF. Due to its contact with the brain and the central nervous system this body fluid should contain valuable biomarkers of neurological and neurodegenerative diseases. Consequently, information regarding the discovery of biomarkers from CSF is increasing and potential biomarkers are being described [13–15].

*Urine* – Urine has gained considerable interest and some of the previously thought obstacles have not been actual facts. The main advantages of urine are that it can be obtained in large quantities and that medically trained personnel are not required for its collection. Furthermore, and in contrast to the situation when working with blood, urine does not contain significant numbers of cells, lipids and large proteins, herein rendering to a less complex analysis. Furthermore, urine has appeared to be considerably stable. Urine stability could be related to the fact that it is ‘stored’ in the bladder for a period of time (several hours) which might provide sufficient time for complete proteolytic processing by endogenous proteases. Previous studies have shown that the low molecular

mass proteome of the urine does not undergo any significant change if urine is stored for up to 3 days at 4°C or 6 h at room temperature [16, 17]. Furthermore, urine samples can be stored at -20°C for several years without a significant detrimental effect (Mischak *et al.*, data not published). However, urine samples are influenced by intrinsic variability due to diet and exercise. To minimise this variability and produce consistent urine proteomic data it is recommended to collect the second urine of the day [18] and to compensate concentration variability using urinary peptides such as creatinin generally present in human urine that can work as housekeeping peptides [19]. Taking all these factors into consideration, urine appears to be an excellent choice as a source for proteomics/biomarkers research. Biomarkers from urine would reflect information on the (patho)physiological condition of organs in direct contact with urine, such as kidney, bladder and also the vascular system.

### *Instrumentation*

Approaches for the examination of the proteome consist of a separation step, followed by ionisation and subsequent mass spectrometry (MS) analysis. In general, all pre-MS separation techniques can be combined with any mass spectrometric approach. MS analysers presently used for proteome analysis are quadrupole (Q), ion-trap, time of flight (TOF) and Fourier transform-ion cyclotron resonance (FT-ICR) or their combinations (see review by [20, 21]). The highest resolution and best mass accuracy is currently achieved by using FT-ICR instruments (< 1ppm) although any other MS capable of delivering a resolution > 5,000 and a mass deviation < 50ppm would be suitable for many proteomic studies.

Ionisation of the analytes prior to their entry into the MS analysers can be achieved using matrix-assisted laser desorption ionisation (MALDI) or electro-spray ionisation (ESI). For MALDI, the sample is mixed with a matrix solution that transfers the laser energy to the peptides, enabling ionization. Generally, the sample is dried on the surface of a target plate, and introduced into the high-vacuum of the mass spectrometer. Evidently, MALDI can only be employed off-line. This type of ionization generates mostly single charged ions which make the spectra simpler for interpretation. However, MALDI is more prone to signal suppression as analytes compete for the available energy.

ESI generates multiple charged ions by applying high voltage at atmospheric pressure to the effluent from e.g. an LC column. The high surface charge on the liquid droplets formed eases desolvatisation, and the "naked" ions are guided into the MS by the electric field. ESI spectra are much more complex as the same peptide/protein contains multiple charged ions and different ionisation states, but ESI is also less susceptible to signal suppression. These more complex spectra are very informative for molecular mass determination necessary

for biomarker discovery but they also require sophisticated software solutions for the interpretation of the data.

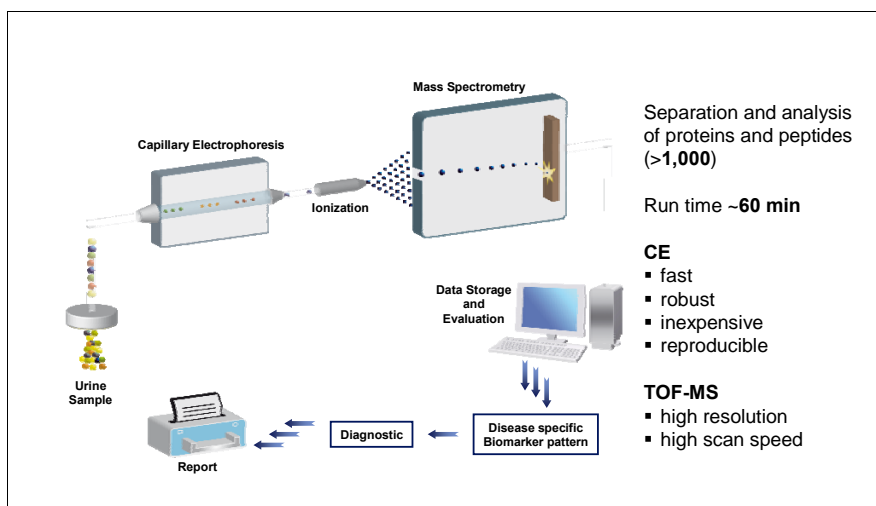
Below, we will summarise the main properties of the different separation techniques which can be broadly divided into gel-based and non-gel based processes, although more detailed information can be found in several reviews [2, 22].

*Two-Dimensional Gel-Electrophoresis followed by Mass Spectrometry (2DGE-MS)* – 2DGE-MS is the most commonly used method to separate and identify proteins > 20 kDa. This technique enables assessment of the mass of potential biomarkers in its native form which is one of the key issues in the definition and characterisation of a biomarker. However, it is technically demanding and comparison of multiple datasets is challenging due to inter-gel variability. New advances such as the development of two-dimensional differential in gel electrophoresis (2D-DIGE) has however significantly improved the accuracy and sensitivity of this technique, and has considerably reduced inter-gel variability and the number of gels required making this technique more attractive for proteome profiling [23–25]. While both approaches may enable identification of valuable biomarkers, they are too time-consuming to be applied in a clinical laboratory setting. Consequently, other technologies have to be employed for the subsequent analysis of these biomarkers in larger cohorts, and especially to be employed in a clinical setting.

*Surface-enhanced laser desorption/ionisation coupled mass spectrometry (SELDI)* – SELDI is based in the differential adsorption of proteins onto different active surfaces (i.e. hydrophilic matrix, reversed phase material or affinity reagents). The majority of proteins are removed with subsequent washing steps, and matrix solution is applied. As in MALDI, the matrix absorbs energy and allows laser ionisation prior to MS detection. The advantages of SELDI include high-throughput capabilities and low sample volumes. However, its limitations are difficulties in standardisation as SELDI outcome can be affected by many factors such as the type of surface coating, pH, salt condition and protein concentration. Nevertheless, this technique has been used for the identification on potential biomarkers in different diseases [26, 27]. Unfortunately, the results generally could not be reproduced; hence this technology is not in general use anymore.

*Capillary electrophoresis coupled to Mass Spectrometry (CE-MS)* – CE is able to separate analytes from a complex mixture based on differential migration through a liquid-filled capillary in a strong electric field. CE can be coupled with both MALDI and ESI as reviewed by Stutz [28]. For proteome analysis, CE is normally coupled on-line to an electrospray source which can then be interfaced with any MS via sheathless or sheath-flow coupling, the latter being

preferred for its stability [29, 30]. In this system, analysis of complex samples containing up to several thousands of peptides can be performed in less than 1 h. Its main advantages are high reproducibility, robustness and resolution [21, 31] and its main disadvantages include its limited capacity to separate large proteins and the small sample loading capacity which compromises MS/MS sequencing. Regardless of this restriction, CE-MS is being successfully applied for the analysis of the low molecular proteome aiming at the identification of biomarkers [32, 33] which will be outlined in more detail later in this review. A schematic representation of a CE-MS system is shown in Figure 1.



*Figure 1 – Schematic representation of on-line coupling of CE for peptide separation and MS for mass detection, accomplished by a coaxial sheath-flow system. The main advantages of CE-MS are listed to the left of the figure. These include a short turn-around time, high resolution and the detection of at least 1000 peptides per analysis*

*Liquid chromatography coupled to Mass Spectrometry (LC-MS) – LC-MS has developed into a powerful technique for the quantification and identification of complex proteomic samples in isolation or as complementary technique to other methods. High resolution quantitative approaches have been reported using stable isotopes and also by comparing peak intensities between multiple runs obtained by continuous detection in MS mode [34]. The disadvantages of LC-MS include moderate reproducibility of the LC separation, and substantial sensitivity towards interfering compounds like salts, lipids, or larger (> 5 kDa) proteins. Nevertheless, LC-MS or 2D LC/MS (MudPIT) is gaining relevance in clinical proteomics [35–37].*

*Evaluation of LC-MS and CE-MS data*

Data evaluation and processing of proteomics is a challenging matter. There are several reviews concerning this subject that the reader is encouraged to consult and only the main points will be outlined in this review [38, 39]. However, it is important to acknowledge that there has been a dramatic increase in the number of tools to assist scientists in the data evaluation of proteomics. The first requirement for proteome data evaluation is an appropriate software package that allows the charge of a particular peak to be determined, to identify and combine peaks of the same mass but different charge-states, and finally that is able to perform normalisation of the migration/retention times and amplitudes to compensate for any differences between measurements. Such software allows the generation of a list of features, peptides and proteins that are found to be present at a certain (relative) concentration in a given sample.

Identification based on MS/MS analysis is achieved using specialized tandem MS instruments. The first instrumental unit serves essentially as a filter to isolate the ion of interest, and, after adding energy that results in fragmentation mostly at the peptide bond, a second instrumental unit is employed to analyse the resulting fragments, that can be used to assign sequence. For more details information, we refer to [21]. The complex spectra are evaluated using specialized software to compare the experimental results with protein databases. However, due to limitations in the scoring system of current search engines not all fragmented peptides can be identified with high confidence. For this reason, and especially when dealing with huge datasets, it is important to separate high-confidence from low-confidence assignments using an adequate filtering mechanism.

After this initial processing of peak spectra and the tentative identification of proteins or peptides, the next step is to use the datasets to conduct comparative studies on the basis of multivariate statistical analyses. At this point it is important to point out that the fact that a 'potential' biomarker shows statistical significance does not mean that it will perform well as a class-discriminating item, as was clearly demonstrated recently by Dakna *et al.* (Dakna *et al.*, 2010). These considerations will be further discussed below.

*From 'potential' discovered biomarkers to clinically valuable biomarkers*

A successful clinical proteomics workflow comprises the collection and examination of the proteome of a target sample source in order to identify proteins and/or peptides that are significantly altered in disease. As schematically shown in Figure 2, such attempts require the combination of demographic and clinical information, MS data, sequence, and the application of powerful statistical methods. Validation of these initially identified 'potential' biomarkers using blinded test sets is essential. The final step of the study is deciphering of the association of these biomarkers with the pathophysiology of the disease in a

Systems Medicine approach, ultimately aiming at the development of improved diagnostic procedures and therapeutic drugs (Figure 3).

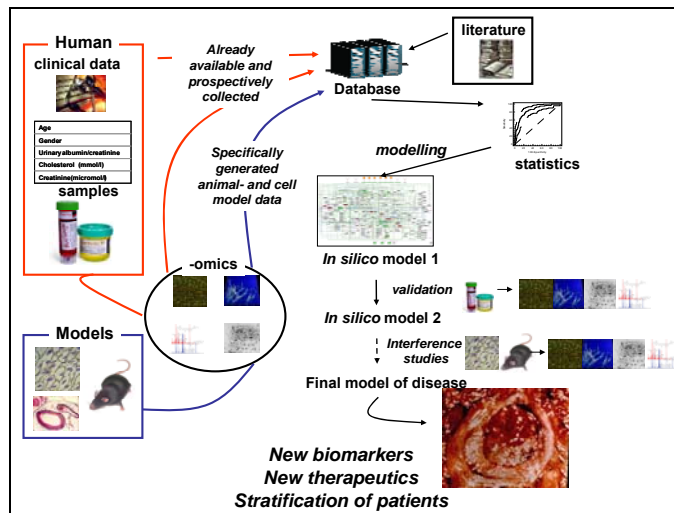


Figure 2 – Establishment of a database as an information system of patient proteome and clinical data. Storage and retrieval of peptide profiles, peptide sequences, and patient clinical records allowing sample selection and differential proteomic profiling for the purpose of biomarker discovery and patient classification. Reprinted with permission from [62]

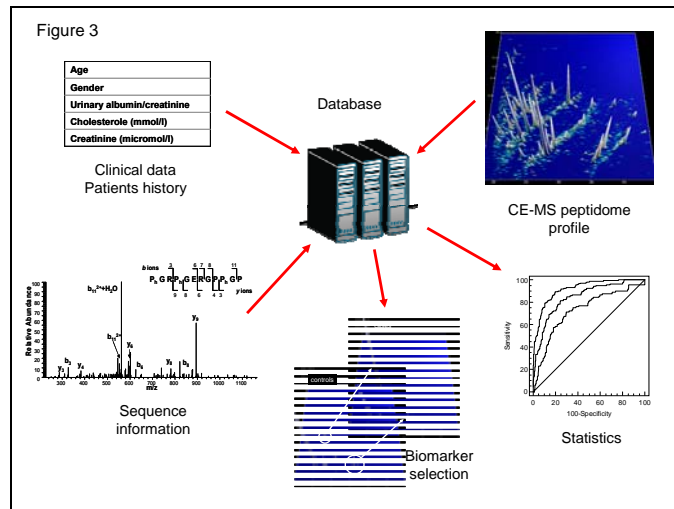


Figure 3 – Existing data (clinical, – omics and literature) are being identified and combined in a specialized database. All data will be analysed with appropriate bioinformatics and statistics, fed into the systems biology modelling process to produce the initial in silico model of a specific disease. This initial model is subsequently refined



*by the mandatory validation in human disease, as well as in animal and cellular models. Multiple iterative steps will be undertaken to reach the appropriate final disease model, which will be validated using interference studies. Ultimately, this approach will result in the identification of molecular pathology (description of molecules and their involvement in the disease), generating new biomarkers and therapeutics and enabling personalized medicine. Adopted with permission from [105]*

#### *An initial interdisciplinary effort*

The initial challenges in the development of proteomic biomarkers for clinical applications were outlined in a recent manuscript [33] and can be summarised as:

- requirement of a clearly defined clinical question,
- the need for definition of the purpose of the biomarker and finally
- an appropriate study design.

These issues should be addressed by using an interdisciplinary approach involving laboratory, clinical, population and statistician scientists. A recent consensus statement on clinical proteomics has emphasised the importance of having well-characterised, large set of samples together with transparent statistical analysis and a clear and concise way of reporting the results. In this sense, standards for reporting of research that would be relevant to many proteomic studies include STARD (for diagnostic tests) and REMARK (for tumour markers) [40, 41]. Other aspects to consider at this point are the use of standard protocols for the collection and extraction of the target material when possible in order to minimise naturally occurring variability. In the case of urine, standardised protocols for collection and extraction for proteome studies as well as reference standard samples have been developed and should be applied to allow comparison between different studies and research groups [42]. Furthermore, sufficient information should be provided regarding the experimental methodology and appropriate statistical approaches. This statistical analysis should account for technical, biological variability, confounding factors and other anticipated sources of bias and should be adjusted for multiple testing in an adequate way to reduce false associations [43, 44].

#### *Clinical validation*

One of the main limitations in a vast majority of the studies published in proteomics/biomarkers research is the absence of validation of the identified ‘potential’ biomarkers. The observation of a significant association in a given data set does not ensure *per se* that the findings will apply in the same way in a different set of samples. Most statistical methods assume an even distribution of features across the data, that the findings can be generalised and that the found association exists only with the investigated condition. Unfortunately, these simplifications are not always shown to be right and for this reason the confirmation of the initial results in an independent test set is essential. Although

many studies have been published without a validation test, thus compromising their validity, several studies have introduced this validation step promoting the transition of 'potential' to 'clinically valuable' biomarkers, e.g.: [17, 45–55]. This validation process should be performed when possible with samples from multiple sites in a blinded fashion.

#### *Biomarker identification and quantification*

Initially proteins and/or peptides that work efficiently as biomarkers for diagnostic purposes do not necessarily need to be identified. However, their identification allows displaying the biomarkers on other technological platforms, and the investigation of their pathophysiological role. To this end, tandem mass spectrometry (MS/MS) is currently applied to determine amino acid sequence using different fragmentation techniques. Fragmentation can be achieved by e.g. collision induced dissociation (CID), electron-capture dissociation (ECD) or by electron-transfer dissociation (ETD). In general terms, CID supplies sufficient internal energy to induce covalent bond breakage but does not provide sufficient backbone fragmentation to allow sequence identification of peptides that are too large or that contain certain PTMs. These limitations can be partially overcome by producing shorter peptides using enzymatic digestions but are still very problematic when dealing with peptides containing PTMs. The fact that these PTMs might be disease-specific and could serve as biomarkers [56] then limits the applicability of CID fragmentation in this field. On the contrary, ECD fragmentation results in a complementary cleavage of the backbone N-C $\alpha$  bond with minimal loss of PTMs. This fragmentation technique coupled with FT-ICR MS has been successfully applied to identify urinary peptides larger than 8 kDa [57] and has also allowed the localisation of glycosylation sites in various glycoproteins [58, 59]. Regardless of all these technical advances, sequencing is still highly challenging especially when dealing with naturally occurring proteins and/or peptides that contain PTMs. The most common technique applied for sequencing is LC coupled to an MS/M instrument. Interfacing CE with an MS/MS instrument has been challenging due to the fact that in CE only limited amounts of samples can be loaded onto the capillary which leads to low-intensity peaks that cannot be successfully analysed by the MS/MS.

Quantification of protein and/or peptides present in a sample is another challenging matter in the proteomics field given the current available technologies. In this respect, several MS-based quantification methods have been tested with different degrees of success. One of the most simple and cost-effective techniques has been the quantification based on signal/intensity/ion counting [60]. While this method does not permit absolute analyte quantification, studies in this field have shown that the relative quantification obtained is highly comparable to that obtained using absolute quantification and therefore it should be applicable to do relative quantification using a selection of a set of naturally occurring, highly abundant collagen fragments as in internal standards in future studies [19].

### Clinical application

Other considerations for the establishment of new proteomic biomarkers in clinical applications are the assessment of the improvement and impact that the biomarker will have and its applicability in routine care. It is therefore important to determine in which way the biomarker will be most useful and if the defined initial clinical question is been successfully solved with the new proteomic biomarker. At this stage other relevant hurdles will be encountered such as the commercialisation process and its associated complications such as reassessing its usefulness in the event that the commercialised prototype has introduced some changes. Finally, further studies should start evaluating the impact of the newly developed diagnostic biomarkers on the outcomes of the tested individuals.

### Use of urinary biomarkers in clinical proteomics using CE-MS

As urine is currently one of the most successful target body fluids in clinical proteomics this review will summarise the main clinically relevant proteomic findings in this field using CE-MS as a working analytical platform. In Figure 4 a graphic depiction of the different diseases and pathologies investigated to date is given. For more detailed information the reader is directed to other reviews focussed on this topic [42, 61, 62]. However, before summarising this information it has to be mentioned that well-characterised male and female urine samples have been characterised using different methods [42] and that this information is freely available on request.

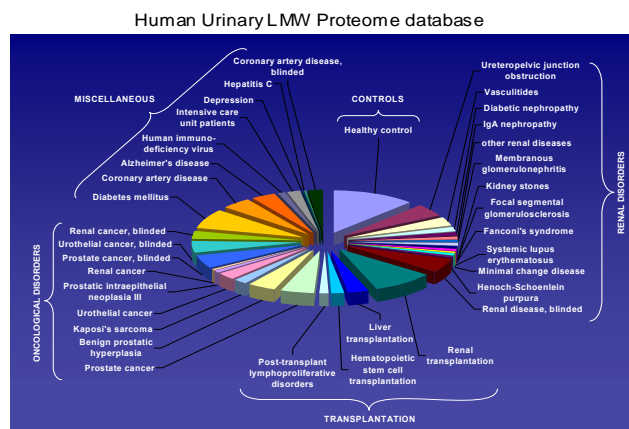


Figure 4 – Graphic depiction of comparable datasets obtained by CE-MS (analyzed with identical pre-analytical preparation, instruments, and analytical parameters).

To date, a total of over 20000 datasets are available. The size is representative of the number of samples obtained from subjects with a specific diseases/(pathological) conditions. Reprinted with permission from [101]

### Renal diseases

**Chronic kidney disease** – For a comprehensive review of urinary biomarkers for chronic kidney disease, we would like to refer to e.g. [2, 63]. While essentially all previous studies showed the potential of urinary proteomics in detecting CKD, they were performed on a limited number of patients. However, a first study that included data from almost 1000 patients and controls was published recently. Good *et al.* [55] used CE-MS to identify a biomarker pattern that allowed the classification of patients with or without CKD with very high confidence. In the identification phase the authors of this study analysed 340 urine samples from patients with various biopsy-proven CKDs and 552 urine samples from healthy individuals and diseased patients without any evidence of renal diseases. This analysis allowed the identification of 273 potential biomarkers (displayed in Figure 5) that were then sequenced and combined using SVM to create a specific urinary biomarker array. The identified biomarker pattern was validated in a blinded heterogeneous cohort consisting of 114 samples in total and it obtained a sensitivity score of 85% and a specificity score of 97.8%. Among the biomarkers identified for DN and CKD in these and other studies, the reduction of specific collagen fragments in urine of patients with CKD is extremely prominent. These findings, that indicate collagen degradation being attenuated in CKD [64], indicate that urinary proteome analysis may also help to obtain a better understanding of the pathology of these and other related diseases such as diabetes.

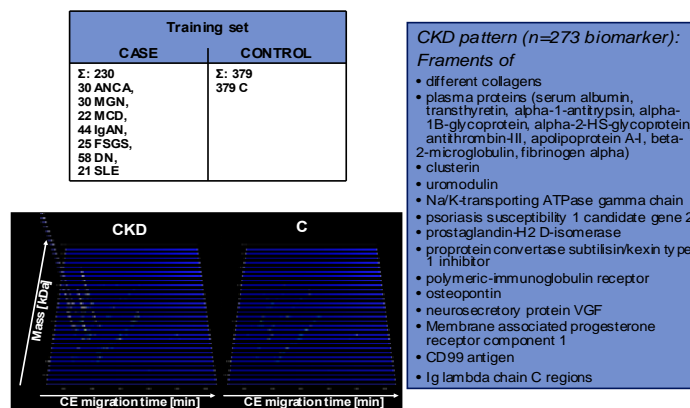


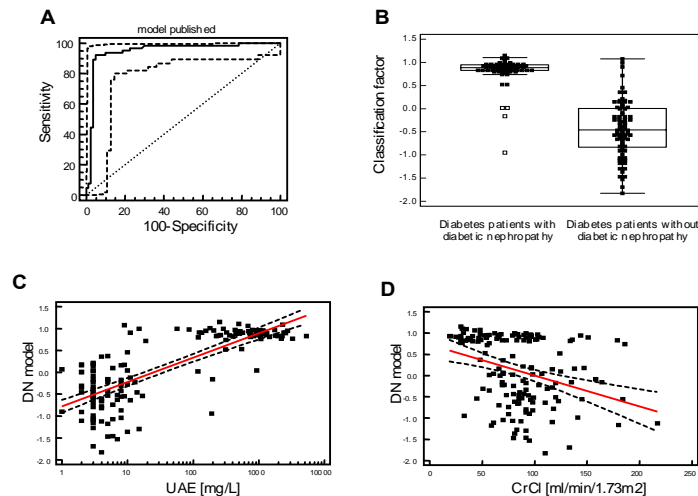
Figure 5 – Upper left: list of patients and controls included in the training data used for the discovery of biomarkers of CKD. The patient cohort included a wide range of aetiologies that can lead to CKD. Right panel: List of urinary peptides that present in the 273 CKD pattern. Lower left: Graphic representation of the 273 differentially expressed sequenced CKD peptides. Data from [55]

*Diabetic nephropathy* – The most common causes of chronic kidney disease (CKD), which in many can lead to an end-stage renal disease (ESRD) in North America, Europe and Japan are diabetic nephropathy (DN), hypertension and glomerulonephritis [65]. Of these diseases, DN is the most common and serious complication of diabetic patients (type 1 and type 2) affecting up to 40% of diabetic patients. Currently, DN is diagnosed based on an increased urinary albumin excretion (30–300 mg/day) together with other risk factors such as increased arterial blood pressure and poor glycaemic control. However, in many cases renal function is already reduced when microalbuminuria starts to increase while in other patients elevated microalbuminuria does not result in DN [66]. As a consequence the need was recognized to find more specific and sensitive biomarkers for DN. Although in this review we will broadly summarise studies regarding the identification of proteomic biomarkers for this disease in urine a detailed review on DN biomarkers has been published by Ameer *et al.* [67]. In urine samples, Varghese and colleagues [46] using 2DE-MS were able to identify a set of biomarkers that allowed the separation of patients with DN and other chronic renal diseases (focal segmental glomerulosclerosis, membranous glomerulonephritis and Lupus nephritis). The reported proteins in that study were also accounted for by Rao *et al.* [9] confirming the suitability of such identified markers although further work is needed to determine if the detected changes can be utilised as early indicators for the diagnosis of DN.

Rossing *et al.* [54] have identified biomarkers for DN in type I diabetic patients. Using data from in total 320 subjects, a panel of 102 urinary biomarkers differed significantly between patients with normoalbuminuria and nephropathy, and a model that included 65 of these correctly identified diabetic nephropathy with 97% sensitivity and specificity. This panel of biomarkers also enabled prognosis: the identification of patients who had microalbuminuria and diabetes and progressed toward overt diabetic nephropathy over 3 yrs. Many of the biomarkers were fragments of collagen type I, and quantities were reduced in patients with diabetes or diabetic nephropathy. These results were recently validated in an independent multicentric study [68], as shown in Figure 6. The data also indicate that analysis of the urinary proteome may allow early detection of diabetic nephropathy and may provide prognostic information. The results were further supported by recent reports by Merchant *et al.* [69] and by Lapolla *et al.* [70].

*Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)* – Alternative clinical tools for the diagnosis and especially the monitoring of ANCA-associated vasculitis are of high relevance, given that this disease is currently diagnosed by renal biopsies that do not easily allow the monitoring of patients. Investigating urinary proteome analysis may enable non-invasive moni-

toring, Haubitz and colleagues [50] used a set of urine samples from healthy individuals, patients with renal and non-renal diseases and patients with AAV at diagnosis and in remission, and studied their urine proteome. This discovery phase resulted in the identification of a pattern of 113 potential biomarkers, 53 of which could be sequenced. 18 of the sequenced biomarkers were combined with a biomarker model and further examined in a validation test. The authors could demonstrate correct classification of patients with AAV and differentiation from patients with other renal diseases with a sensitivity of 90% and a specificity of 86–90%. Marked substantial differences between the different pathologies are evident in the urinary proteome, as shown in Figure 7. Longitudinally collected urine samples from 10 patients with AAV in therapy were also evaluated, clearly demonstrating that urinary proteomics enables monitoring of the disease, hence it can be employed as a non-invasive technique to display therapeutic success.



*Figure 6 – Statistical analysis of the results of the blinded, multicentric validation of a biomarker pattern specific for diabetic nephropathy. A) ROC curve and B) Box-Whisker-plot for classification of the ‘case and control’ patient collective with the ‘DN’ pattern are shown. Correlation analysis. Scatter diagrams of correlation from proteomic biomarker pattern with urinary albumin excretion (UAE), (C) and creatinine clearance, (D) The red line shows the regression line with 95% confidence interval (dashed line). Reprinted with permission from [68]*

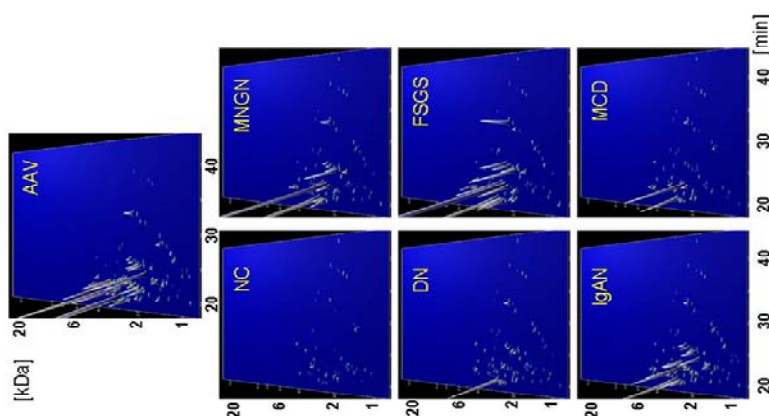


Figure 7 – Compiled protein patterns of the CE-MS analysis of urine samples from patients and controls examined in [50]. Shown are the compiled data on the 113 peptides that reveal significant differences between AAV and controls ( $p$  value < 0.05 after adjustment for multiple testing), in the patients with active vasculitis and patients with other chronic renal disease or healthy controls. (NC, apparently healthy normal control; DN, diabetic nephropathy.) The molecular mass on a logarithmic scale (0.8–25 kDa; indicated on the left) is plotted against normalized migration time (18–45 min; indicated on the bottom). Signal intensity is encoded by peak height and color. Differences between AAV and the other chronic renal diseases are evident when examining these 113 peptides. Reprinted with permission from [50]

*Autosomal dominant polycystic kidney disease (ADPKD)* – Kistler *et al.* [51] analysed the urine proteome of 41 young patients with ADPKD with preserved renal function. Using capillary electrophoresis and mass spectrometry, the authors could identify 197 peptides with significantly altered urinary excretion; and 38 of them could be sequenced. Again, most of the identified peptides were collagen fragments, suggesting a high turnover of extracellular matrix proteins associated with ADPKD. Uromodulin peptides, previously implicated in tubular injury, were also found significantly altered in the urine of ADPKD patients. These urinary peptides were found to distinguish patients from controls with a high degree of accuracy. The sensitivity and specificity of this marker set remained high in an independent validation cohort of 24 patients with ADPKD and 35 healthy controls (sensitivity of 87.5% and specificity of 97.5%), and even in comparisons with patients with a variety of other renal diseases or patients with kidney or bladder cancer. While these findings could be validated in a recent study (Kistler *et al.*, unpublished), to date no prognostic value of the biomarkers could be demonstrated.

*Paediatric renal disease* – In newborns and children the use non-invasive prognosis is highly desirable. Neonatal ureteropelvic junction (UPJ) obstruction is a common condition that needs close and currently invasive monitoring. Decramer *et al.* [49] performed urinary proteome analysis which identified 53 specific biomarkers for different grades of UPJ obstruction. This study was further validated in an additional blinded prospective study [71] that indicated that the proteome pattern established by Decramer and co-workers was accurate in children of < 1 year (sensitivity of 83% and specificity of 92%) but lost its sensitivity (20%) and specificity (66%) in older patients. This biomarker pattern is now under evaluation in an international multi-centre prospective study on 358 UPJ patients for further validation in independent paediatric units. Other paediatric diseases such a renal Fanconi syndrome (FS) have been studied by Drube and co-workers [72] with positive outcomes, although in a small scale study.

*Acute kidney injury* – Acute kidney injury (AKI) is a frequent complication in hospitalised patients; a recent meta-analysis highlighted the increased mortality associated with AKI, independent of other factors. Over the last decade, the incidence of AKI has increased from 60 to 500 events/100,000 population [73]. Currently no effective therapy of AKI is available. To improve the associated serious prognosis, efforts are focussed on the early detection of AKI. Recent definitions of AKI, namely the Risk, Injury, Failure or Loss of renal function and End-stage kidney disease (RIFLE) classification or the Acute Kidney Injury Network criteria, incorporate serum creatinine and urine output as the principal markers to define and detect AKI. However, these and other clinically available and widely used markers (blood urea nitrogen, tubular enzymuria or proteinuria, elevated serum creatinine or oliguria) detect AKI only at later stages [73]. A specific and reliable assay that enables monitoring of patients for developing AKI would be of great clinical interest. Numerous new markers such as neutrophil-gelatinase associated lipocalin, interleukin-18, cystatin C and kidney injury molecule 1 have been studied and proposed as early detection markers of AKI [74–77]. They all performed well in initial pilot trials, but the results could generally not be confirmed in later, larger multi-centre trials [78, 79]. Zhou *et al.* [80] analysed urinary exosomes from a rat model of cisplatin-induced AKI, by Difference Gel Electrophoresis. Fetuin-A was found at increased levels in the AKI versus control animals. This finding was further validated in animals with bilateral renal inschaemia and reperfusion injury as well as in a very small cohort ICU patients with AKI.

Metzger *et al.* [81] applied CE-MS to identify urinary biomarkers predictive for AKI in a training set of 87 urine samples, collected on different days from ICU patients who later developed AKI or maintained normal renal function. An AKI-specific biomarker pattern consisting of 20 urinary polypeptides was developed. The biomarkers were specific peptides derived from 6 proteins.



Fragments of albumin, alpha-1-antitrypsin and beta-2-microglobulin were up-regulated; peptides derived from fibrinogen alpha and collagen type I and III were down-regulated in AKI. Good diagnostic performance was obtained with the area under the ROC curve (AUC), being 0.84 and 0.90, during validation in two blinded sets of prospectively longitudinal collected samples from 20 ICU patients, and from 30 patients after haematopoietic stem cell transplantation. Additional 28 healthy controls all scored negative. The proteomic profile could detect AKI at least 4–5 days in advance of any significant rise of serum creatinine. In contrast, cystatin C, KIM-1, IL-18 and NGAL showed no significant association with AKI in this cohort. However, an evident shortcoming of this approach is the requirement to transfer the biomarkers onto another analytical platform, to enable efficient analysis with the required short time-interval of about 1 hour.

*Renal transplant rejection.* Acute rejection is the main complication observed in renal transplantation. Approximately 15–30% of the transplanted patients suffer from one or multiple acute rejections which occur mainly in the first year of transplantation [82]. Timely detection and appropriate therapy of acute rejection episodes is important to conserve allograft function. However, detection of acute rejection at an early stage is challenging. Regular monitoring for increases in serum creatinine enables detection only at an advanced stage where the graft is already impaired by the rejection process.

Protocol biopsies have been used to detect acute rejection in an earlier, subclinical stage, where functional impairment is not yet present [83]. An inherent limitation of this approach is the invasive procedure, and even with multiple biopsies it is impossible to capture every rejection episode. Therefore, non-invasive tests in blood or urine were sought which may be able to detect acute rejection, e.g. [84]. Most of these approaches used single markers or combinations of a few markers. However, due to low specificity and sensitivity of these markers, none of these tests is established in the clinical routine and post-transplant care of the patients. Several groups have reported success using mass spectrometry of urinary peptides to detect acute rejection [85–87]. Overall good performance was reported in these studies, with AUC values ranging from 0.85–0.97. However, verification of these results in larger patient cohorts is still outstanding.

Acute tubulointerstitial rejection is often associated with acute tubular injury [88]. Therefore, an important clinical question is whether proteomic markers identified in acute rejection are indicative of the rejection process itself or they non-specifically reflect tubular injury. Schaub *et al.* (2005) described cleaved urinary  $\beta$ 2-microglobulin as a potential marker for acute tubular injury in renal allografts [85]. However, fragments of  $\beta$ 2-microglobulin correlate with tubular injury in samples with and without rejection, but not with rejection itself, and  $\beta$ 2-microglobulin fragments were recently described as markers for acute kidney injury in non-transplanted patients [81].

Interestingly recent studies [89] and Gwinner *et al.*, submitted, support that the levels of specific collagen fragments are altered in the urine of patients with acute T-cell mediated tubulointerstitial rejection. Aligning these fragments to other, commonly present urinary Col1A1 peptides, the collagen fragments that are increased in rejection possess a characteristic sequence motif at their c-terminus indicative of increased extracellular matrix degradation by metalloproteinases. By immunohistological staining of biopsy sections, a significant number of MMP-positive polymorphonuclear cells close to the endothelium as an early event of the rejection process could be detected. Metalloproteinase expression at peritubular capillaries may be an indicator for trans-endothelial migration of infiltrating cells into the interstitium and into the tubules as an early process in acute rejection.

#### *Urological cancer*

*Urothelial carcinoma* – In 2006, Theodorescu *et al.* [17] described a series of biomarkers for the diagnostic of urothelial carcinoma using CE-MS. This author used a 2-step approach for the identification (training set formed by 46 patients with urothelial carcinoma and 33 healthy subjects) and then the validation of the identified biomarker pattern (366 urine samples of different condition). The identified biomarker pattern was able to correctly classify all urothelial carcinoma and all healthy patients from a blinded test set.

Recently, this approach was extended to the non-invasive staging of bladder cancer. A biomarker profile consisting of fragments of Uromodulin, Collagen alpha 1 (I) and (III), and of membrane-associated progesterone receptor component 1 was predictive of muscle invasive disease [90]. In a blinded cohort of 130 urine samples, the biomarker panel together with clinical data showed a sensitivity of 92% (95% CI 82–97) and a specificity of 68% (95% CI 55–79). These data support the thesis that urinary peptides appear promising in identification of patients harbouring muscle-invasive bladder cancer and may also give novel insights into the biology of bladder tumour progression.

*Prostate cancer (PCa)* – Since the introduction of prostate specific antigen (PSA) as a screening tool for prostate cancer the number of diagnosed cases has increased considerably [91]. However, PSA cannot distinguish between benign prostatic hyperplasia (BPH) and malign prostatic conditions (PCa) [92]. As prostate cancer can manifest in some patients in a very aggressive form there is a need for discriminating those patients from the ones that do not require further intervention [93]. In a study by Theodorescu and colleagues [94] a model of 10 biomarkers using the first-void urine was established. This biomarker pattern resulted in the prediction of 88.9% of the PCa and 66.7% of the BPH patients in a subsequent validation trial.

*Non-renal and urological diseases*

Initially, urine has been targeted as a body fluid containing information on the tissues with which it is in direct contact. However, as plasma is filtered by the kidney there has been an increasing interest in exploring the hypothesis that urine could be a useful sample source for biomarker of disease of more distant organs. This hypothesis has been confirmed in a number of studies on patients with different non-renal conditions including patients after allogeneic haematopoietic stem cell transplantation (HSCT) [95, 96]. Urine samples from 40 patients after HSCT (35 allogeneic, 5 autologous) and five patients with sepsis were collected for CE-MS analysis. A pattern that consisted of 16 differentially excreted polypeptides discriminated patients with early graft versus host disease (GVHD) from patients without complications with 82% specificity and 100% sensitivity. In a subsequent blinded multi-centre validation study on 100 patients with more than 600 samples collected prospectively, the results were confirmed, although with reduced specificity and sensitivity [97]. Initial results of preemptive therapy based on proteome profiling clearly indicate a benefit for the yet limited number of patients (Weissinger *et al.*, submitted).

Coronary artery disease (CAD) is a leading cause of morbidity and mortality worldwide. Early diagnosis of CAD would allow for better and more effective prevention compared to current practice. Recently, urine from 88 CAD patients and 282 controls was examined by CE-MS [98]. This resulted in the identification of 15 urinary peptides that defined a characteristic CAD biomarker panel. This panel was evaluated in a blinded study on 59 individuals, where CAD patients were identified with 90% sensitivity and specificity. The CAD biomarker panel significantly changed after therapeutic intervention towards the signature of healthy humans. In another recent study, patients with CAD could be distinguished from patients presenting symptoms of CAD, but without clinical evidence in the coronary angiography [99]. The value of urinary proteomics in identification of CAD could further be validated in prospectively collected samples from patients with type I diabetes [100]. That study also highlighted another benefit of the urinary proteome analysis: the “proteomic fingerprint” can be analysed for several different biomarker panels that are indicative of different pathological conditions, as shown in Figure 7. Further supporting and extending these findings, Delles *et al.* [52] were able to define a pattern of 238 CAD-specific polypeptides by comparing 586 spot urine samples from 408 subjects. This pattern identified patients with CAD in a blinded cohort of 138 urine samples (71 patients with CAD and 67 healthy individuals) with high sensitivity and specificity (AUC 0.87). The sequences of the discriminatory polypeptides include fragments of alpha-1-antitrypsin, collagen types 1 and 3, granin-like neuroendocrine peptide precursor, membrane-associated progesterone

receptor component 1, sodium/potassium-transporting ATPase gamma chain, and fibrinogen-alpha-chain. Several biomarkers changed significantly towards the healthy signature following 2-years treatment with irbesartan, whereas short term treatment with irbesartan did not significantly affect the polypeptide pattern.

Although there is no clinical need for biomarkers for diabetes, investigation of proteomic changes associated with diabetes may help in further understanding the pathophysiology and consequences of diabetes. Based on a comparison of 205 samples, a panel of 261 potential biomarkers was defined by Rossing *et al.* [54]. This panel was validated by Snell-Bergeon *et al.* [100] in a cohort of 38 prospectively collected samples from the coronary artery calcification in type 1 diabetes (CACTI) study. The results again indicate that a panel of a large number of significant biomarkers enables high accuracy in assessment of disease. In a recent larger study on 902 subjects from 10 different clinical centres, this biomarker pattern could be further validated, resulting in 94% accuracy in this multicentric assessment [53]. In addition, the authors demonstrated significant differences between diabetes type I and type II in the urinary proteome. These findings may enable identifying potential pathophysiological changes early in disease that are specific for type I or type II diabetes.

#### *Proteomic changes during ageing*

Ageing results in morphological changes of the kidney, and a significant reduction of the glomerular filtration rate can be observed in the elderly. In order to gain insight into this process [32] examined urine samples from 324 healthy individuals aged 2–73 years using CE-MS. The authors identified 325 urinary peptides that showed statistically significant age-related changes. Most of the changes were observed during puberty, coinciding with the completion of renal development. 49 peptides could be correlated with ageing in adults. Interestingly, several of these peptides were also found to be biomarkers of chronic renal diseases. Sequence information of those ageing markers suggested that a prominent result of human aging is a reduced turnover of extracellular matrix, which leads to increased fibrosis.

#### *Urinary Biomarkers in the context of pathophysiology*

To date, sequences are available for more than 1500 different urinary peptides ([101] and Mullen *et al.*, unpublished). Not unexpectedly, most of those peptides are derived from abundant proteins in the body: collagen – mainly type I, II and III, albumin, beta 2-macroglobulin and uromodulin. These results are the basis for the hypothesis that urinary peptides of diagnostic value

are not merely degradation products of abundant larger proteins, but a result of distinct, disease-specific processes; in many cases, due to significant changes in the activity of proteases as suggested by Haubitz [102]. That hypothesis is further strengthened by the detection of specific collagen fragments that correlated with the disease-specific activity of matrix metalloproteases (Metzger *et al.*, submitted). In addition, the increase of collagen and extracellular matrix proteins is observed in patients with diabetes and DN, while collagen degradation products are significantly reduced in diabetic urine [54, 69], possibly indicating reduced activity of proteases and protection of the extracellular matrix from proteolysis by advanced glycation end products [64].

A similar scenario might be applicable to other abundant proteins. Thorough examination of the sequences of the urinary peptides and comparison with protease specificities might provide additional support for the above hypothesis, and could lead to a better insight into the regulation and pathophysiological role of specific proteases in many diseases.

A related hypothesis can be proposed: the urinary peptidome displaying, to a large degree, the turnover of the extracellular matrix. Changes in turnover should result in indicative changes in urinary peptides, which serve as a very specific, non-invasive indicator for disease-specific alterations in ECM turnover. Such changes can be due to, e.g., invasion of tumours (ECM must be "dissolved" in order to make room for the growing tumour), fibrosis (reduced ECM degradation), increased arterial stiffness (change in ECM composition), or changes in endothelium.

#### *Conclusions and considerations for the use of proteomic biomarkers in clinical applications: Future studies*

The contribution of proteomics to the prognosis, diagnosis and therapy evaluation in clinical applications has been significant but modest in comparison to the first expectations and the technological progress that has occurred in the last 25 years. As a general rule an interdisciplinary approach with a clearly defined objective, a rigorous study design, a transparent statistical analysis and a concise way of reporting the results are key points that all studies should adhere to. Experience also shows that a single molecule that clearly defines one disease, the 'perfect biomarker', does not exist. A panel of biomarkers is apparently better suited as shown by the examples summarised in this review. These panels of biomarkers need to be clearly defined, sequenced and validated using a blinded set of samples. In addition, progress in this field will only be possible with a joint effort from all the scientists working in this field by setting a global

standardisation of protocols for the planning, execution and reporting of clinical proteomic studies and the adoption of standardised methods for the identification of disease-specific biomarkers [33]. In this way, comparable datasets which allow the definition of biomarkers between healthy controls and specific disease and other closely related pathological conditions could be extended [101]. This would make the comparison between studies less intricate, which is one of the main aims of the EuroKUP COST action ([www.eurokup.org](http://www.eurokup.org)) [103]. In addition, future implementation of proteome profiling in clinical applications will also rely on the optimisation and minimisation of the time required for the analysis [104] and also on the assessment of the gain for the patients and health economic benefits which should be evaluated by doing prospective studies.

Despite this apparent long list of hurdles or considerations, application of biomarkers for the diagnosis of several diseases has been reported and some examples summarised in this review show that proteomic profiling can deliver clinically relevant information and that there is a vast potential for further development in this field.

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HM is the founder and co-owner of Mosaiques Diagnostics, who developed the CE-MS technology for clinical application.

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#### Резиме

### УРИНАРНИ ПРОТЕОМИ ВО КЛИНИЧКИ ПРИМЕНИ: ТЕХНОЛОГИИ, ГЛАВНИ РАЗМИСЛУВАЊА И КЛИНИЧКА ПРИМЕНА

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Технолошките платформи за протеомска анализа значително напреднаа во последните неколку години. Поради овие подобрувања ескалираше бројот на студии за анализа на протеомот/пептидомот со цел дефинирање на биомаркерите. Во овој преглед ќе направиме резиме на техничките аспекти што се однесуваат на полето на протеомиката насочени кон откривање биомаркери за дијагноза на болест. Ќе го опишеме текот од откривањето биомаркер или „потенцијални“ биомаркери до тие што се клинички важни. Исто така, претставуваме неколку примери на успешни протеомски студии што ги дефинирале „моделите на биомаркери“ за клинички примени, фокусирани на урината како извор на материјал и ка-

пиларна електрофореза "coupled mass spectrometry" како технологија. На крајот, ќе се дискутираат тековните предизвици и размислувања за идните студии.

**Клучни зборови:** протеомика, уринарни биомаркери, клинички примени.

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