Proteomics and Metabolomics as Tools to Unravel Novel Culprits and Mechanisms of Uremic Toxicity: Instrument or Hype?

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Summary: The development of proteomic and metabolomic technologies holds the promise to significantly impact patient management by improving diagnosis, unraveling more appropriate therapeutic targets, and enabling more precise prognosis of disease development. Proteomics and metabolomics have been applied with the aim of improving dialysis, defining uremic toxins, and unraveling their origin. Ideally, these technologies should inform us which proteomic or metabolomic compounds are subject to significant alterations of concentration or structure as a result of failing kidney function, and thus can be considered as potential uremic toxins. After a few years of applying these technologies in the area of uremic toxicity studies we are now in a position where we can estimate how and what they can contribute to the field. In this review we critically examine the current literature on the application of proteomics and metabolomics in the context of dialysis and uremic toxins. We highlight the most promising findings, indicate where we see the current need, and which future developments consequently are to be expected, given the technological constraints that undoubtedly exist.
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Uremic toxins are a group of poorly defined molecules that are eliminated in healthy individuals via the kidney, and that accumulate in patients with end-stage renal disease (ESRD). Several molecules have been described as uremic toxins, for more details see the recent review by Duranton et al.[1] The different classes of uremic toxins and their representatives also are discussed in more detail in articles 1 through 5 of this issue.[2–6] However, it is unknown how comprehensive the list of uremic solutes summarized by Duranton et al[1] in fact is, and for many of them the presumed toxic effects in vivo are extensively evaluated and described. There is hope that both questions may be answered by generating an exhaustive list of compounds found in plasma of healthy individuals, and in patients with late-stage chronic kidney disease or ESRD patients. The compounds that differ between these two populations constitute the pool of potential candidate uremic toxins. The observed associations of several of these compounds with specific pathophysiology (eg, cardiovascular complications) is the first step toward defining their toxicity. With these goals in mind, it is obvious that samples must be evaluated (ideally plasma) that are collected from patients and controls (see later), to obtain information on the compounds involved, in a hypothesis-free approach. As such, proteomics and metabolomics have been applied in this context. After about 10 years of research, it is time to reflect on the results and to re-evaluate the findings and the strategies used.

METABOLOMIC TOOLS

The recent growth of metabolomics has depended greatly on nuclear magnetic resonance (NMR) spectroscopy (mostly 1H-NMR)[7] and the development of mass spectrometry (MS).[8] In general, MS, particularly liquid chromatography (LC), coupled online via electrospray ionization (ESI) to high-resolution Fourier transform ion cyclotron resonance MS, and NMR spectroscopy (mostly 1H-NMR) are the two major spectroscopic techniques used in metabolic analysis (Fig. 1). They both have specific advantages and disadvantages,[9,10] as also described later.

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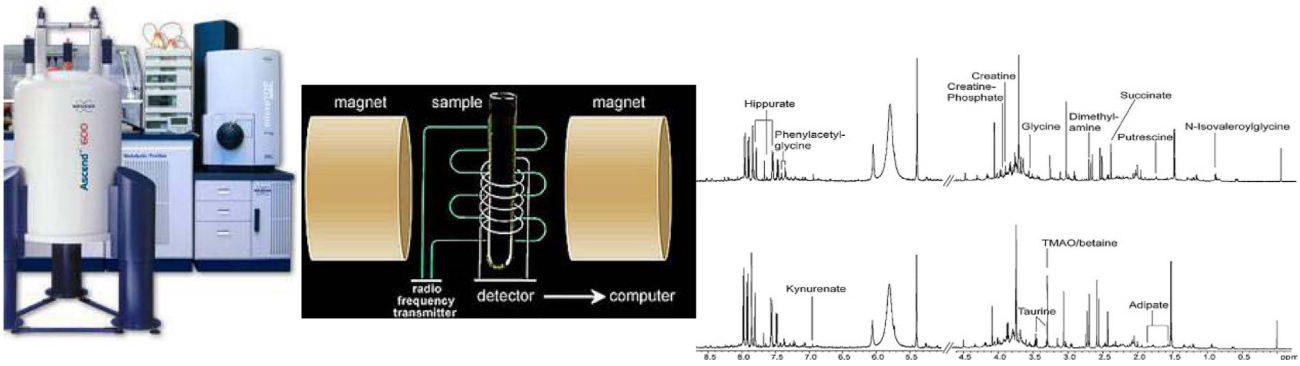

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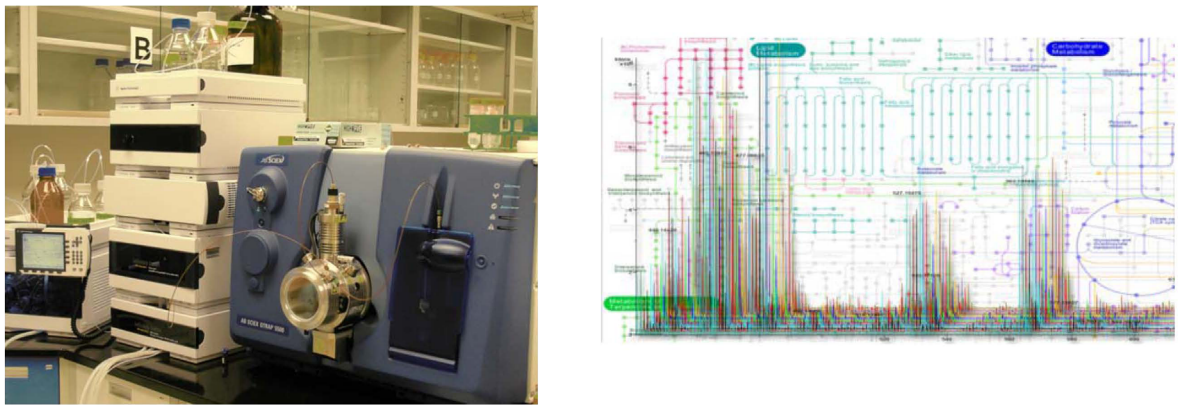

Figure 1. Commonly used metabolomic technologies. (A) Proton nuclear magnetic resonance (1H-NMR) is based on determining the resonance frequency in a strong magnetic field. This approach is of moderate sensitivity and resolution, but of low cost. (B) LC-MS–based approaches are of much higher sensitivity and resolution, however, also at a much higher cost.

MS determines the composition of molecules based on the mass-to-charge ratio in charged particles. MS-based metabolomics generally combines a first rapid global screening of untargeted metabolomics for searching candidate biomarkers using high-resolution MS, and, subsequently, a second determination screening of targeted metabolomics using tandem mass spectrometry (MS/MS). The advantages of MS (or MS/MS, these two instruments will to some degree be used synonymously in this article) are a wide dynamic range of detection, excellent sensitivity and selectivity, high throughput, reproducibility, and, depending on the instrument, high resolution. MS or MS/MS typically are interfaced with different separating devices, generally using ESI. Although ESI ideally is suited for polar charged molecules, nonpolar molecules may require chemical ionization. Several reports have been published using gas chromatography–mass spectrometry (GC/MS),[11] liquid chromatography–mass spectrometry,[12] and capillary electrophoresis–mass spectrometry (CE-MS)[13] for both untargeted and targeted metabolomics. In particular, time-of-flight and Fourier transform ion cyclotron resonance MS are useful for untargeted metabolomics,[14] and tandem quadruple MS is suitable for targeted metabolomics.[15] LC/MS is highly sensitive, typically at the high attomol level, and permits highly specific multiple metabolite assessments at low concentrations.[16] However, MS sensitivity is dependent on metabolite pKa and hydrophobicity,[17] and a widely adopted and validated methodology for sensitive, high-throughput discovery-based LC/MS metabolomics is still lacking. In part because of the heterogeneity in methods, the results from different groups using different experimental approaches are very divergent.

Furthermore, sample storage conditions and methods of extraction can affect and modify metabolite structure, confounding already complex data sets and introducing substantial additional variability. Despite the extensive use of MS to assess small molecules, a widely adopted and validated methodology for sensitive, high-throughput discovery-based LC/MS metabolomics is still lacking, and most compounds detected in MS-based metabolomics approaches are unknown/unidentified. Nevertheless, discovery metabolomics showed a wealth of possibilities in pharmaceutical and biomedical research.[18] To date, LC/MS–based

metabolic profiling methodologies are undergoing validation for reproducibility.[19] Applications that allow absolute quantification and reproducibility are established for targeted metabolomics (ie, for the analyses of a set of selected [usually 20-200] metabolites).[20,21]

Another MS-based approach, GC/MS, generally requires derivatization of compounds. Although such derivatization methods exist, these can be quite time consuming, costly, associated with the risk of metabolite loss, and samples treated by this approach consequently cannot be used for a comprehensive assessment of the metabolome.[22]

The other major technology applied in metabolomics is NMR. NMR-based studies generally are performed as follows: quenching/extraction of metabolites → data collection → data processing/analysis.[23] NMR exploits the behavior of molecules when placed in a magnetic field, allowing the identification of different nuclei based on their resonant frequency. Compared with MS with detection limits in the attomol range, NMR is of much lower sensitivity (in the order of 10 μmol/L), lower resolution, and requires more expensive instrumentation. In addition, 1H-NMR spectra are sensitive to pH, ionic content, and temperature, and may require solvent suppression. The major advantages of NMR include its low cost (after the initial investment, which is significantly higher than for MS-based metabolomics), and the fact that it is not restricted to liquid, but can be used to evaluate metabolites in solid samples with minimal sample preparation (eg, using high resolution magic angle spinning [HR-MAS] techniques).[23] 31P-NMR of tissue specimens and cultured cells reflect products of energy or phospholipid metabolism, whereas 13C-NMR measures dynamic carbon fluxes, such as those occurring in glucose metabolism. 13C-NMR can be performed on tissues and cell extracts after incubation with a 13C-labeled precursor, but is less sensitive than GC/MS–based 13C assays. An important advantage of NMR is that metabolic markers discovered and analyzed in vitro can be measured in vivo (in situ) when sufficiently abundant, using localized magnetic resonance spectroscopy imaging. Magnetic resonance spectroscopy imaging is an additional technology related to magnetic resonance imaging whereby metabolites instead of anatomy are imaged. In essence, magnetic resonance spectroscopy imaging is a composite of traditional NMR spectroscopy and magnetic resonance imaging that allows for noninvasive in vivo visualization and determination of spatial distribution of a specific metabolite in patients without exposure to ionizing radiation.

PROTEOMIC TOOLS

Over the past 2 decades, proteomics slowly has changed from a mostly qualitative science (describing compounds present in typically one specific sample) to a quantitative one (comparing relative abundance and frequency of multiple proteins in different samples). Mass spectrometry is the most frequently used technique for large-scale proteome characterization, which can be applied to the identification, as well as relative and absolute quantification, of proteins in complex samples.

Obtaining an accurate quantification of protein content of a sample is crucial in the biomarker discovery phase. Common approaches of MS-based proteomics include two-dimensional electrophoresis (2DE) followed by tryptic digestion of the proteins of interest and identification based on the sequence and mass of

Table 1. Summary of Advantages and Drawbacks of Different Proteomic Approaches Commonly Used

Proteomic/Metabolomic Approach	Advantages	Drawbacks
2DE	Well-established technique	Not applicable to low-molecular-weight or hydrophobic proteins
	Relatively low cost	Cannot be automated
		Time consuming
LC-MS/MS	Good resolution, sensitivity, and reproducibility	Need for optimization by an expert in the field
	High loading capacity	High running cost
		Carryover effects
		Low throughput
Targeted LC-MS/MS	High throughput	Only a selected set of metabolites
	Absolute quantification	
	automation	
MALDI-MS	High-throughput profiling, automation	Low resolution
	Low cost	Low sequence coverage
CE-MS	High-throughput profiling, automation	Not applicable to proteins with molecular masses $\geq$20 kDa
	Fast separation, high resolution, robustness, and low cost performance	Low loading capacity

those tryptic fragments. Alternatively, the entire sample can be digested with trypsin (in cases after fractionation, for example, using strong cation exchange chromatography), and the tryptic fragments are analyzed using liquid chromatography coupled to tandem MS (LC-MS/MS) or to MS (LC-MS). Alternative to online coupling via ESI, offline coupling using matrix-assisted laser desorption/ionization (MALDI) can be used. A further strategy, mostly focusing on naturally occurring peptides, that sees increasing use owing to its high resolving power, is CE-MS. A few years ago, another technology, termed *surface enhanced laser desorption ionization*, was widely used, but this approach mostly has been abandoned because of its low resolution and lack of reproducibility.[24]

The advantages and limitations of the different technologies recently were reviewed extensively[25–29] (Table 1) and are only outlined briefly here. The different approaches also are shown graphically in Figure 2.

Although 2DE has been the workhorse of proteomics research in the past, several drawbacks have reduced its use,[30–32] as follows: (1) it offers no possibility to cope with the broad dynamic range of complex samples; (2) many proteins such as hydrophobic membrane proteins or low abundance proteins precipitate during separation and hence are not detected by 2DE; (3) high salt content interferes with electrophoresis; and (4) 2DE is a time-consuming and technically demanding technique of moderate reproducibility. However, until now it has been the only

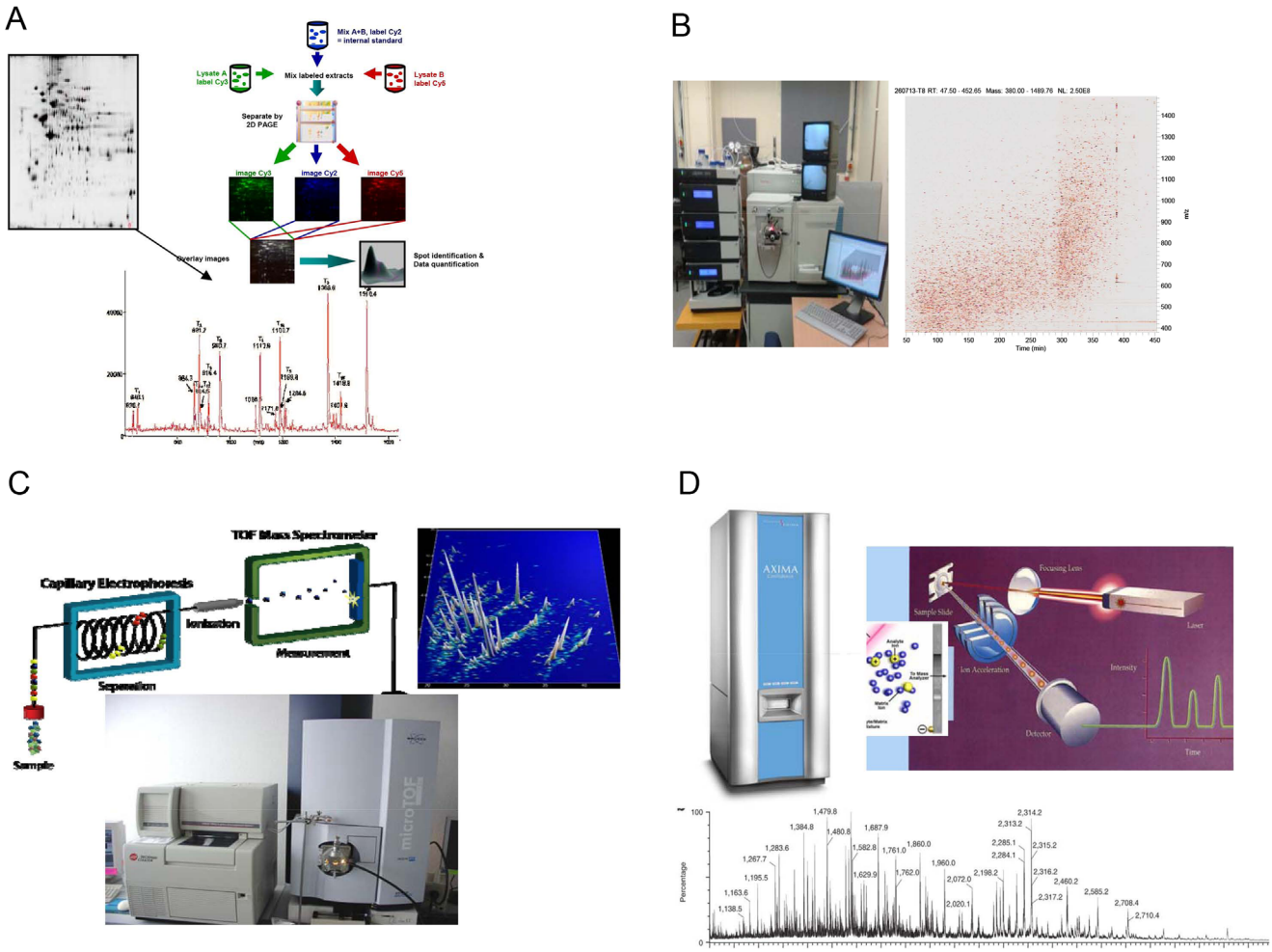

Figure 2. Generally used proteomic technologies. (A) 2DE-MS. Proteins are separated in two dimensions according to the isoelectric point and molecular mass. Protein spots of interest are excised and digested with trypsin, and the resulting peptides are analyzed by mass spectrometry. Derivation of samples (eg, case and control) before analysis with fluorescence dyes allows comparative analysis in the same gel. (B) LC-MS. Proteins are digested and fractionated according to their hydrophobicity using high-resolution reversed-phase chromatography. The column is interfaced with high-resolution MS/MS instruments, enabling assessment of more than 50,000 peptides in one single analysis. (C) Like LC, capillary electrophoresis can be interfaced on line with mass spectrometers (CE-MS). Capillary electrophoresis separates polypeptides according to their charge and size. The approach is characterized by higher resolution and robustness than LC-MS, but lower capacity. (D) MALDI-MS. The protein sample is deposited together with specialized matrix solution on a plate (top left). By using a high-energy laser, proteins and peptides are ionized and subsequently analyzed in the MS. This approach is fast and inexpensive, but of lower resolution.

moderately robust approach that allows assessment of specific physical parameters (mass and isoelectric point), which enables us to distinguish between isoforms, posttranslational modifications, and so forth.

During the extensive development of the last generations of mass spectrometers coupled to highly efficient separating systems, the use of 2DE has been reduced and the main proteomics technology used today is LC-MS/MS, as is also the case in metabolomics (see earlier). The label-free, quantitative LC-MS/MS method enables comparison of a large number of samples. Because of its large dynamic range (generally about 4 orders of magnitude) and depth of analysis, LC-MS/MS enables detection and quantification of thousands of peptides in a complex sample. Unfortunately, it is a costly technique and requires a high level of expertise to obtain reproducible and reliable results, and especially the subsequent data interpretation can become very demanding. Hence, the use of (semi)quantitative LC-MS for comprehensive quantification of polypeptides for biomarker discovery is still limited to specialized laboratories.

As an alternative profiling approach, MALDI-MS has been used. This technology enables assessment of hundreds of peak signals that, taken together, may be a representation of a distinct pathophysiological status of the body. MALDI-MS has been used to detect the protein deregulation patterns, but its low resolution does not allow in-depth analysis of a complex proteome, hence its application in biomarker discovery studies is limited.[33] However, it is a well-suited technology for biomarker validation studies because of its comparatively low complexity and low cost per sample.[34]

CE-MS has been used successfully by using mostly urine as the biofluid of interest for the identification of low-molecular-weight biomarkers for several diseases.[35–40] CE-MS is a profiling method with a short route from the initial discovery in the analytical laboratory to implementation in clinical practice because it can be used in biomarker discovery, validation, and clinical application. CE-MS also offers some analytical advantages: fast separation, high resolution, robustness, and relative low cost. A recently published study comparing CE-MS and LC-MS methods has found some differences between the proteins identified by the two systems; CE-MS shows higher reproducibility and performs better in detecting small and highly charged compounds that generally do not bind to the typically used reverse-phase LC columns, although LC has the advantage of much higher loading capacity than CE, enabling assessment of less-abundant analytes with higher confidence.[41] Direct comparison of LC and CE has indicated high complementarity of the two approaches, and surprisingly low overlap of the results. Hence, similarly to metabolomics, a truly comprehensive assessment of the entire proteome can be accomplished only by using several different approaches.[42]

APPLYING PROTEOMICS AND METABOLOMICS FOR CLINICAL PURPOSES

Initially, both technologies were introduced and applied toward clinically relevant questions with high hopes, aiming for comprehensive "-omics" profiling for the identification of clinically useful biomarkers and for deciphering molecular pathology. However, these high hopes could not be fulfilled immediately, and several issues prevented an easy way to success. Among those were moderate reproducibility, large variability in sampling, and biological variability. To address these problems, guidelines for clinical proteomics were developed to ease performance of appropriate studies.[43] First initiatives towards these guidelines were mostly focusing on sample collection and analytical reproducibility, also have been initiated for metabolome analysis of biofluids.[44] Key elements of these guidelines, independent of the "ome" studied, include clearly defining the context of use and the use of appropriate platforms of known performance, strict adherence to stringent statistical testing, and the verification of results in an independent cohort. These principles are still fully valid today. A major practical problem of these considerations is the requirement of analyzing a substantial number of samples, at least more than 10 per group, but frequently exceeding 100 (Fig. 3). The requirement for larger cohorts, and the lack of validity of findings in small groups, was shown convincingly by Dakna et al.[45] They investigated the urinary proteome of healthy volunteers for sex-specific peptides. In this example, cohorts consisting of fewer than 20 subjects did not allow the identification of biomarkers that could be verified in an independent test set. Increasing the cohort size enabled detection of an increasing number of biomarkers that subsequently could be verified. This study also clearly showed the value of statistical testing, and the absolute requirement to adjust for multiple testing. As such, the efforts associated with any clinical metabolomics or proteomics studies are quite substantial with respect to resources, time, and cost. An appropriate approach to cope with these challenges recently was suggested by Vlahou[46]: the sharing of data, material, and resources, as has been shown in the genomics field. Unfortunately, most studies in the field of uremic toxins were performed before the publication of the guidelines and suggestions mentioned earlier.[43] Important and promising for large human studies are the recent observations on the time-dependent changes of the human metabolome showing that many metabolite patterns remain stable despite environmental (eg, nutritional) impact.[47]

Application in Uremic Toxicity

Goals in assessing the proteomics and metabolomics of uremic toxicity are to understand the mechanisms of

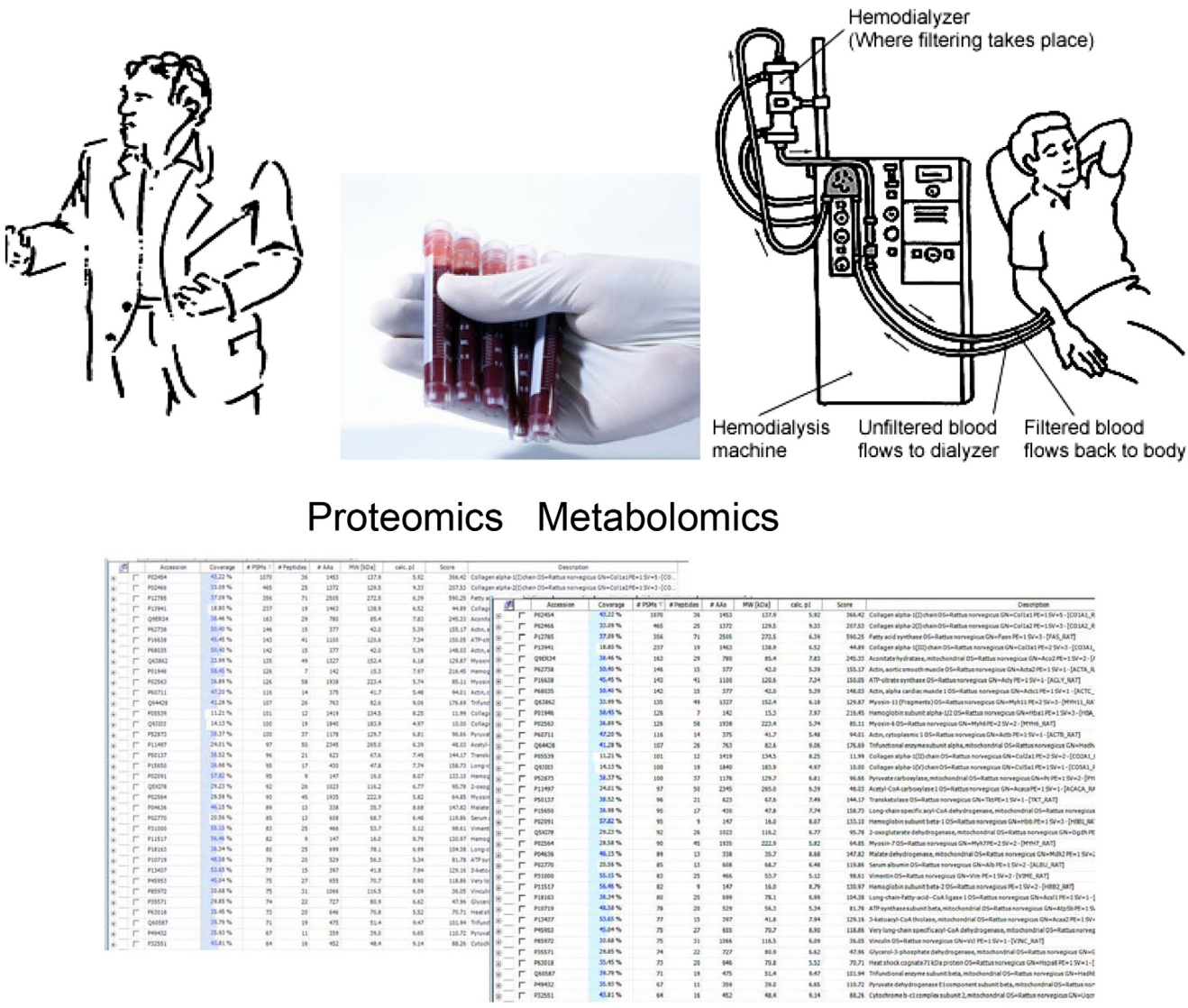

Figure 3. Suggestion for an appropriate study design to assess uremic toxins. Plasma samples from patients ($n > 20$), ideally before and after dialysis, and well-matched controls were collected. The samples were analyzed using the different proteomics and metabolomics technologies, to generate a list of compounds present in each sample. Comparison of the compound lists in the two groups will enable identification of the compounds that increase in the dialysis patients, and also inform about their removal in dialysis. As a next step, association of these compounds with specific events, as well as their biological relevance, can be tested.

changes associated with decreasing or failing kidney function, and ultimately enabling an improvement of the patient's situation. To date, a small number of studies have been reported by searching web-of-science for the terms "uremic toxin" and "proteom*" or "metabolom*" or "metabonom*" in the topic or title, resulting in 85 returns, and not all of them were relevant for the topic.

Proteomic analysis

Early reports on the application of proteomics in the field of dialysis and uremic toxicity came from Kaiser et al[48] and Ward and Brinkley.[49] Kaiser et al[48] used CE-MS in the investigation of dialysate obtained from hemodialysis (HD) using high- or low-flux membranes and showed that larger peptides and proteins were detectable in the dialysate only when high-flux membranes were used. Ward and Brinkley[49] used 2D gels and identified several plasma proteins in the dialysate, including some modified variants. Although these early reports served as proof of principle that proteins and peptides can be identified in dialysate with at least moderate reproducibility (increasing confidence in the data), they also suffered from several shortcomings: most of the proteins/peptides detected were not identified, statistical power was very low (owing to a very small number of samples), and, as became evident later, the proteome of the dialysate may not be highly informative because it is influenced substantially by the dialysis procedure (the simple equation of plasma

proteome before dialysis equals plasma proteome after dialysis plus dialysate being incorrect, in part likely owing to protein precipitation and degradation). However, at the time these reports were published, proteomics technology was not developed to a point that would allow assessing the plasma proteome, which is far more complex than dialysate, in a meaningful way.

Molina et al[50] described the proteomic characterization of hemodialysate in-depth and compared it with the plasma proteome. The investigators first used separation by sodium dodecyl sulfate gel (Ge), and then LC-MS/MS analysis of the tryptic digests of the single bands, a technology frequently also referred to as *GeLC-MS*. The investigators could identify 292 proteins in hemodialysate. Surprisingly, the majority of these had not been described in plasma. This likely reflects limitations in the possibilities for the identification of the proteome in the plasma, rather than the appearance of novel proteins in dialysate. Similar approaches to the investigation of dialysate were performed by other groups, including Weissinger et al[51] and Dihazi et al.[52] The aim in both studies was to assess differences in the removal of high- and low-molecular-weight proteins using high- and low-flux membranes, Weissinger et al[51] by using CE-MS, Dihazi et al[52] by using both surface-enhanced laser desorption ionization and 2D electrophoresis. Both articles identified differences between hemodialysate obtained from high- and low-flux membranes, albeit with essentially no overlap between the two studies, owing at least in part to the two different technologies used. However, these differences between high-flux and low-flux dialysates are not necessarily the only processes mandating changes in the plasma concentration of the described molecules because they also can be caused by differences in adsorption or differences in protease activity. No direct benefit was shown for patients from these studies. No attempt to link the observed peptides and proteins and the patient outcome has been made. Furthermore, it is still unclear whether any of the molecules detected by these proteomic studies do in fact represent toxins. However, the Membrane Permeability Outcome study showed better survival with high-flux membranes compared with low-flux membranes,[53] as discussed in depth by Liabeuf et al.[54]

A first attempt to investigate the serum proteome of dialysis patients was reported by Hallbauer et al,[55] using chromatographic fractionation followed by MALDI-time of flight (TOF) analysis of tryptic digests. Although the general approach of investigating the plasma proteome appears more appropriate than analyzing dialysate, the study suffered from several shortcomings such as a lack of appropriate controls, low-resolution analysis, and pooling of samples, hence no statistical assessment was possible.

By using 2DE and MALDI-TOF analysis of selected proteins, Lin et al[56] compared the plasma proteome from patients on long-term ($\sim$15 y) hemodialysis with the plasma proteome from patients on dialysis for approximately 5 years. Some differences between the two cohorts could be detected. Unfortunately, only 6 patients per group were used, which does not allow an appropriate statistical assessment of the data, and a comparison with controls (chronic kidney disease [CKD] patients not on hemodialysis) was missing. However, the investigators could identify vitamin D binding protein (DBP) as being increased in the patients on long-term dialysis. In the same article the investigators further reported that in a cohort of 60 patients with an average 4-year follow-up period, reduced levels of DBP were associated with increased mortality. This was in agreement with the initial observation that higher DBP levels are associated with longer survival on hemodialysis, and further supports the hypothesis that reduced levels of DBP may have a negative impact on survival.[56]

Based on the hypothesis that differences in the high-density lipoprotein complex may contribute to the increased risk of cardiovascular disease in dialysis patients, Mange et al[57] isolated high-density lipoprotein from 7 dialysis patients and 7 age-matched healthy volunteers. The investigators analyzed the high-density lipoprotein fractions using isobaric tag for relative and absolute quantitation (iTRAQ) labeling of tryptic digests, which allows analysis and identification of up to 8 samples in one MS experiment.[58] Then they separated the peptides with LC, followed by MALDI-MS analysis. Forty proteins were found to be altered, several apolipoproteins were up-regulated, and several proteins involved in the inflammatory response and complement activation were reduced in dialysis patients. The latter is a surprising finding in view of the proinflammatory status of dialysis patients and its presumed contribution to renal vascular disease, but may be related to their increased susceptibility to infection.

Post translational modifications

A further application of proteomics, and to some degree also metabolomics, is the detection of chemical modifications in proteins. These approaches were well described in a recent review by Galli,[59] in which the relevance of inflammation-associated protein damage, especially modification by glycation-generating advanced glycation end products (AGEs), was highlighted. The increase of AGE concentrations in CKD and in dialysis patients, illustrating uremic protein damage, may contribute to the pathophysiology of uremia. AGEs interfere, as a result of the modification introduced, with protein function. An increase in AGEs

in collagen may result in reduced elasticity and turnover, and accumulation of collagen in the tissue to a point at which it negatively affects physiology (eg, increasing arterial stiffness, compromising microvascular circulation, and glomerular filtration).[60] The hypothesis that AGEs significantly contribute to morbidity and possibly even mortality in CKD and ESRD is supported further by urinary proteomic data obtained from patients with CKD. One of the hallmarks in CKD progression is the reduction of urinary collagen (mostly type I) fragments. This highly significant change initially was described by Rossing et al[61] in patients with diabetic nephropathy. Although diabetic patients have increased levels of AGEs also in the absence of kidney disease, AGE levels are increased even further in diabetic nephropathy.[62] In accordance, several urinary collagen fragments are reduced significantly in diabetic patients who do not have any signs of kidney disease in comparison with control subjects,[63] and they are reduced further in patients with diabetic nephropathy. The investigators hypothesized that this reduction in urinary collagen fragments may be owing to reduced collagen degradation as a result of chemical modification by AGEs.[60] In subsequent studies the reduction in urinary collagen could be verified further in patients with CKD in general, irrespective of the etiology.[39] In more recent studies, urinary collagen fragments were shown to be associated with progression in albuminuria,[64] development of diabetic nephropathy,[65] and, at later stages, with ESRD and death.[66] Based on these data, it appears tempting to speculate that AGEs may in fact be a key molecule in uremic toxicity, although additional data, especially showing a direct link between reduction of urinary collagen, AGEs, and ESRD, have to be acquired.

Metabolomic analysis

Metabolomic profiling of kidney disease is one of the representative tools of researching biomarker candidates and determining them in biological samples.

Metabolomics recently resulted in the detection of several new biomarkers and insights into uremic mechanisms. In an effort to identify biomarkers for CKD, Toyohara at al[67] assessed the distribution of 312 cationic and 193 anionic compounds in the plasma of 41 CKD patients by using CE-MS. The investigators found a substantial number of metabolic biomarkers that were well associated with estimated glomerular filtration rate (eGFR), including several novel potential biomarkers for CKD. A further assessment of these metabolites in a larger cohort is still needed, but the data clearly indicate that a much larger number of CKD-associated metabolites exists, and several of these may be superior to creatinine in assessing kidney function.

One of the mechanisms of accumulation of uremic toxins in blood may be related to solute carrier organic anion transporter family, member 4C1 (SLCO4C1), one of the transporters of uremic toxin in the kidney.[68] In CKD, expression of SLCO4C1 was decreased, affecting the concentration of some specific compounds in rat and human plasma. Among these, the investigators proposed guanidine succinate (GSA), asymmetric dimethylarginine (ADMA), and transaconitate as potential novel biomarkers. After the first screening of untargeted metabolomics by CE-MS, they used a selective determination method, focusing on optimization of specific conditions for analyzing GSA, ADMA, and transaconitate and their structural analogues by LC/MS/MS. The separation problems of anionic compounds such as GSA and ADMA have been improved by using the following: (1) a strong cation exchange (SCX) column, (2) a silica column in hydrophilic interaction liquid chromatography (HILIC) mode,[69] and (3) the addition of an ion-pair reagent in the mobile phase to overcome the peak-tailing problem of cationic compounds.

Goek et al[20] showed a correlation between decreased eGFR and metabolite concentrations of 151 serum metabolites with targeted LC-MS/MS metabolomics. A cross sectional observational study of the general population was performed first in 3,011 samples from the Cooperative Health Research in the Region Augsburg cohort and then was replicated in 984 samples from the Twins UK[70] population, the biggest UK adult twin registry of 12,000 twins used to study the etiology of age-related complex traits and diseases. Reproducible associations with eGFR were observed for 22 metabolites and 516 metabolite ratios. The ratios refer to quotients resulting from dividing one concentration of a given metabolite by the concentration of another metabolite. These ratios are a way to normalize for the individual variability of the metabolome,[71] similar to the albumin/creatinine ratio used when assessing albuminuria.

Choi et al[72] performed a comparison of the metabolome in serum from patients on HD and from patients on peritoneal dialysis, using 1H-NMR–based metabolomics. The investigators found some differences in metabolites, specifically hypoxanthine and inosine, present only in HD, whereas peritoneal dialysis was associated with higher levels of lactate, glucose, maltose, pyruvate, succinate, alanine, and glutamate. Known uremic toxins such as urea, creatinine, myo-inositol, and trimethylamine-N-oxide were increased in both groups.

Aronov et al[73] used high-resolution LC-MS for the investigation of the distribution of more than 1,000 metabolite ions in the plasma of hemodialysis patients with an intact colon, and patients who had undergone a colectomy. The investigators reported on the reduction of several plasma compounds in the patients without a

colon. The most prominent compounds were p-cresyl sulfate and indoxyl sulfate, known uremic toxins that are produced by gut microbiome. Additional uremic toxins that were found to be reduced in patients without a colon included 5-hydroxyindole and α-N-phenylacetyl-glutamine. A large number of additional compounds were found to be distributed differentially, but unfortunately most of these compounds could not be identified. Despite this severe shortcoming, the article clearly showed the ability of metabolomics to identify potential uremic toxins. The role of the intestine in uremia is discussed in more detail in Jankowski et al and Meijers et al in this issue.[6,74]

Investigating samples from the population-based Cooperative Health Research in the Region of Augsburg study, Suhre et al[75] determined 420 unique small molecules in overnight-fasting blood using three different techniques, covering NMR and MS/MS. The investigators described several molecules to be altered significantly in diabetic subjects, including known uremic toxins such as indoxyl-sulfate and suggested that the latter may contribute to the increase in CKD and ESRD in the diabetic population.

Rhee et al[76] applied LC/MS/MS–based metabolite profiling to survey more than 350 small molecules in 44 fasting subjects with end-stage renal disease, before and after hemodialysis, and detected dicarboxylic acids (adipate, malonate, methylmalonate, and maleate), biogenic amines, nucleotide derivatives, phenols, and sphingomyelins as uremic retention solutes.

In total, NMR-based and MS-based metabolomics have improved and high-throughput analysis can be performed with confidence today. However, data analysis of untargeted metabolomics still is challenging because of shortcomings in databases and software solutions. Metabolites in blood also dramatically change depending on the clinical background, age, sex, lifestyle, therapeutic drugs, and so forth, and are affected by storage time and temperature. In addition, collection of serum samples, especially under different conditions, further adds to variability. Therefore, it is recommended to use plasma samples for metabolomics that are processed and frozen immediately after collection. This will reduce variability that is induced after sampling (eg, as a result of ongoing metabolic processes) or caused by the sampling procedure per se. Thus, for data analysis in clinically based untargeted and targeted metabolomics, we should take into account not only the presence or symptoms of kidney disease but also the sample quality and specific conditions that are highly relevant for adequate analysis.

CONCLUSIONS AND FUTURE PERSPECTIVES

The data currently available indicate a clear potential for proteomics and metabolomics in deciphering uremic toxicity, but at the same time they unfortunately also highlight that the studies published to date fall short of delivering the expected final outcomes: identification of the molecules relevant in uremic toxicity and assessment of their role in pathophysiology, ultimately enabling advancement in their removal. In general, the studies reported are on a high technical level. The most significant shortcomings appear to be the low number of samples that were analyzed, consequently decreasing the confidence in the observation. This problem could to some degree be solved by combining data from different studies. Unfortunately, there is a substantial lack of comparability between studies as a result of different technologies and different types of samples used. Because levels of uremic toxins likely are influenced by the complex and highly variable molecular changes associated with CKD, which is expected to be even more pronounced when examining patients with different etiologies of CKD and different comorbidities, a substantially large number of samples likely has to be analyzed to detect truly significant changes. Guided by the experience gained in clinical proteomics and metabolomics, and also as a result of technological advancement, it appears that we are now in a situation to perform the appropriate studies in this field, as also exemplified by the outline for such a study shown later. This optimistic view is based on substantial improvements in instrument sensitivity and accuracy, enabling identification of compounds with much higher confidence and at quantitative levels that were undetectable only a few years ago. Further, based on the past studies, the relevance of power calculations based on experimental data, strict statistical testing, including adjustments for multiple testing, and the requirement to verify the result in an independent test set has been established and widely accepted.[43,77]

As a first step, a comprehensive analysis of the plasma proteome and metabolome from dialysis patients, ideally before and after a dialysis session, as well as from appropriately matched controls with preserved kidney function, appears to be a promising and sensible way to go. Power calculations based on preliminary data have indicated that sample sizes in the range of 20 per group should suffice to enable detection of several truly significant differences. As a next step, the data can be placed in the context of biology and pathophysiology, as described for CKD and cardiovascular disease.[78,79] Such an approach, the combination of high-resolution proteomic and metabolomic data sets from human plasma in the context of dialysis, followed by a systems biology–driven in-depth evaluation, would present a first cornerstone and reference for others. As also outlined earlier, such projects can be accomplished successfully only in an interdisciplinary approach, and as a collaboration of several groups.

REFERENCES

1. Duranton F, Cohen G, De SR, Rodriguez M, Jankowski J, Vanholder R, et al. Normal and pathologic concentrations of uremic toxins. J Am Soc Nephrol. 2012;23:1258-70.
2. Duranton F, Depner TA, Argilés A. The saga of two centuries of urea: non-toxic toxin or vice versa? Semin Nephrol. 2014;34:87-96.
3. Schepers E, Speer T, Fliser D, Kielstein JT. The dimethylarginines ADMA and SDMA and other guanidines: the real water soluble small toxins? Semin Nephrol. 2014;34:97-105.
4. Sirich T, Meyer TW, Gondouin B, Brunet P, Niwa T. Protein-bound molecules: a large family with a bad character. Semin Nephrol. 2014;34:106-17.
5. Chmielewski M, Cohen G, Wiecek A, Carrero JJ. The peptidic middle molecules: is molecular weight doing the trick? Semin Nephrol. 2014;34:180-90.
6. Jankowski J, Westhof T, Vaziri ND, Ingrosso D, Perna A. Gasses as uremic toxins: is there something in the air? Semin Nephrol. 2014;34:135-50.
7. Nicholson JK, Holmes E, Kinross JM, Darzi AW, Takats Z, Lindon JC. Metabolic phenotyping in clinical and surgical environments. Nature. 2012;491:384-92.
8. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, et al. The human serum metabolome. PLoS One. 2011;6:e16957.
9. Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem. 2007;387:525-7.
10. Zhao YY. Metabolomics in chronic kidney disease. Clin Chim Acta. 2013;422:59-69.
11. A J, Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, et al. Extraction and GC/MS analysis of the human blood plasma metabolome. Anal Chem. 2005;77:8086-94.
12. Xiao JF, Varghese RS, Zhou B, Nezami Ranjbar MR, Zhao Y, Tsai TH, et al. LC-MS based serum metabolomics for identification of hepatocellular carcinoma biomarkers in Egyptian cohort. J Proteome Res. 2012;11:5914-23.
13. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J Proteome Res. 2003;2:488-94.
14. Glauser G, Veyrat N, Rochat B, Wolfender JL, Turlings TC. Ultra-high pressure liquid chromatography-mass spectrometry for plant metabolomics: a systematic comparison of high-resolution quadruple-time-of-flight and single stage Orbitrap mass spectrometers. J Chromatogr A. 2013;1292:151-9.
15. Becker S, Kortz L, Helmschrodt C, Thiery J, Ceglarek U. LC-MS-based metabolomics in the clinical laboratory. J Chromatogr B Analyt Technol Biomed Life Sci. 883-4:68-75.
16. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of metabolomics in oncology: a review. Clin Cancer Res. 2009; 15:431-40.
17. Want EJ, Nordstrom A, Morita H, Siuzdak G. From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. J Proteome Res. 2007;6:459-68.
18. Suhre K, Shin SY, Petersen AK, Mohney RP, Meredith D, Wagele B, et al. Human metabolic individuality in biomedical and pharmaceutical research. Nature. 2011;477:54-60.
19. Gika HG, Theodoridis GA, Wingate JE, Wilson ID. Within-day reproducibility of an HPLC-MS-based method for metabonomic analysis: application to human urine. J Proteome Res. 2007;6:3291-303.
20. Goek ON, Doring A, Gieger C, Heier M, Koenig W, Prehn C, et al. Serum metabolite concentrations and decreased GFR in the general population. Am J Kidney Dis. 2012;60:197-206.
21. Artati A, Prehn C, Moller G, Adamski J. Assay tools for metabolomics. In: Suhre K, ed. Genetics meets metabolomics: from experiment to systems biology. New York, Springer; 2012, pp 13–38.
22. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. Mass Spectrom Rev. 2007;26:51-78.
23. Serkova NJ, Niemann CU. Pattern recognition and biomarker validation using quantitative 1H-NMR-based metabolomics. Expert Rev Mol Diagn. 2006;6:717-31.
24. McLerran D, Grizzle WE, Feng Z, Thompson IM, Bigbee WL, Cazares LH, et al. SELDI-TOF MS whole serum proteomic profiling with IMAC surface does not reliably detect prostate cancer. Clin Chem. 2008;54:53-60.
25. Kolch W, Mischak H, Pitt AR. The molecular make-up of a tumour: proteomics in cancer research. Clin Sci (Lond). 2005;108:369-83.
26. Fliser D, Novak J, Thongboonkerd V, Argiles A, Jankowski V, Girolami M, et al. Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol. 2007;18:1057-71.
27. Mischak H, Coon JJ, Novak J, Weissinger EM, Schanstra JP, Dominiczak AF. Capillary electrophoresis-mass spectrometry as a powerful tool in biomarker discovery and clinical diagnosis: an update of recent developments. Mass Spectrom Rev. 2009;28:703-24.
28. Dakna M, He Z, Yu WC, Mischak H, Kolch W. Technical, bioinformatical and statistical aspects of liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) based clinical proteomics: a critical assessment. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:1250-8.
29. Mischak H, Massy ZA, Jankowski J. Proteomics in uremia and renal disease. Semin Dial. 2009;22:409-16.
30. Pieper R, Gatlin CL, Makusky AJ, Russo PS, Schatz CR, Miller SS, et al. The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Proteomics. 2003;3:1345-64.
31. You SA, Wang QK. Proteomics with two-dimensional gel electrophoresis and mass spectrometry analysis in cardiovascular research. Methods Mol Med. 2006;129:15-26.
32. Oh-Ishi M, Maeda T. Disease proteomics of high-molecular-mass proteins by two-dimensional gel electrophoresis with agarose gels in the first dimension (Agarose 2-DE). J Chromatogr B Analyt Technol Biomed Life Sci. 2007;849:211-22.
33. Molin L, Seraglia R, Lapolla A, Ragazzi E, Gonzalez J, Vlahou A, et al. A comparison between MALDI-MS and CE-MS data for biomarker assessment in chronic kidney diseases. J Proteomics. 2012;75:5888-97.
34. Albalat A, Stalmach A, Bitsika V, Siwy J, Schanstra JP, Petropoulos AD, et al. Improving peptide relative quantification in MALDI-TOF MS for biomarker assessment. Proteomics. 2013;13:2967-75.
35. Mischak H, Schiffer E, Zürbig P, Dakna M, Metzger J. Urinary proteome analysis using capillary electrophoresis coupled mass spectrometry: a powerful tool in clinical diagnosis, prognosis and therapy evaluation. J Med Biochem. 2009;28:223-34.
36. Mischak H, Schanstra JP. CE-MS in biomarker discovery, validation, and clinical application. Proteomics Clin Appl. 2011;5:9-23.
37. Albalat A, Mischak H, Mullen W. Clinical application of urinary proteomics/peptidomics. Expert Rev Proteomics. 2011;8:615-29.
38. Stalmach A, Albalat A, Mullen W, Mischak H. Recent advances in capillary electrophoresis coupled to mass spectrometry for clinical proteomic applications. Electrophoresis. 2013;34:1452-64.
39. Good DM, Zürbig P, Argiles A, Bauer HW, Behrens G, Coon JJ, et al. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics. 2010;9:2424-37.
40. Jantos-Siwy J, Schiffer E, Brand K, Schumann G, Rossing K, Delles C, et al. Quantitative urinary proteome analysis for

biomarker evaluation in chronic kidney disease. J Proteome Res. 2009;8:268-81.
41. Mullen W, Albalat A, Gonzalez J, Zerefos P, Siwy J, Franke J, et al. Performance of different separation methods interfaced in the same MS-reflection TOF detector: a comparison of performance between CE versus HPLC for biomarker analysis. Electrophoresis. 2012;33:567-74.
42. Mischak H, Kolch W, Aivalotis M, Bouyssie D, Court M, Dihazi H, et al. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. Proteomics Clin Appl. 2010;4:464-78.
43. Mischak H, Allmaier G, Apweiler R, Attwood T, Baumann M, Benigni A, et al. Recommendations for biomarker identification and qualification in clinical proteomics. Sci Transl Med. 2010;2:46ps42.
44. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc. 2011;6:1060-83.
45. Dakna M, Harris K, Kalousis A, Carpentier S, Kolch W, Schanstra JP, et al. Addressing the challenge of defining valid proteomic biomarkers and classifiers. BMC Bioinformatics. 2010;11:594.
46. Vlahou A. Back to the future in bladder cancer research. Expert Rev Proteomics. 2011;8:295-7.
47. Floegel A, Drogan D, Wang-Sattler R, Prehn C, Illig T, Adamski J, et al. Reliability of serum metabolite concentrations over a 4-month period using a targeted metabolomic approach. PLoS One. 2011;6:e21103.
48. Kaiser T, Hermann A, Kielstein JT, Wittke S, Bartel S, Krebs R, et al. Capillary electrophoresis coupled to mass spectrometry to establish polypeptide patterns in dialysis fluids. J Chromatogr A. 2003;1013:157-71.
49. Ward RA, Brinkley KA. A proteomic analysis of proteins removed by ultrafiltration during extracorporeal renal replacement therapy. Contrib Nephrol. 2004;141:280-91.
50. Molina H, Bunkenborg J, Reddy GH, Muthusamy B, Scheel PJ, Pandey A. A proteomic analysis of human hemodialysis fluid. Mol Cell Proteomics. 2005;4:637-50.
51. Weissinger EM, Kaiser T, Meert N, De Smet R, Walden M, Mischak H, et al. Proteomics: a novel tool to unravel the pathophysiology of uraemia. Nephrol Dial Transplant. 2004;19:3068-77.
52. Dihazi H, Muller CA, Mattes H, Muller GA. Proteomic analysis to improve adequacy of hemo- and peritoneal dialysis: removal of small and high molecular weight proteins with high- and low-flux filters or a peritoneal membrane. Proteomics Clin Appl. 2008;2:1167-82.
53. Tattersall J, Canaud B, Heimburger O, Pedrini L, Schneditz D, Van BW. High-flux or low-flux dialysis: a position statement following publication of the Membrane Permeability Outcome study. Nephrol Dial Transplant. 2010;25:1230-2.
54. Liabeuf S, Neirynck N, Drüeke TB, Vanholder R, Massy ZA. Clinical studies and uremia: what did we learn recently? Semin Nephrol. 2014;34:164-79.
55. Hallbauer J, Kreusch S, Klemm A, Wolf G, Rhode H. Long-term serum proteomes are quite similar under high- and low-flux hemodialysis treatment. Proteomics Clin Appl. 2010;4:953-61.
56. Lin YP, Yang CY, Liao CC, Yu WC, Chi CW, Lin CH. Plasma protein characteristics of long-term hemodialysis survivors. PLoS One. 2012;7:e40232.
57. Mange A, Goux A, Badiou S, Patrier L, Canaud B, Maudelonde T, et al. HDL proteome in hemodialysis patients: a quantitative nanoflow liquid chromatography-tandem mass spectrometry approach. PLoS One. 2012;7:e34107.
58. Zieske LR. A perspective on the use of iTRAQ (TM) reagent technology for protein complex and profiling studies. J Exp Bot. 2006;57:1501-8.
59. Galli F. Protein damage and inflammation in uraemia and dialysis patients. Nephrol Dial Transplant. 2007;22 (Suppl 5):v20-36.
60. Rossing K, Mischak H, Rossing P, Schanstra JP, Wiseman A, Maahs DM. The urinary proteome in diabetes and diabetes-associated complications: new ways to assess disease progression and evaluate therapy. Proteomics Clin Appl. 2008;2:997-1007.
61. Rossing K, Mischak H, Dakna M, Zürbig P, Novak J, Julian BA, et al. Urinary proteomics in diabetes and CKD. J Am Soc Nephrol. 2008;19:1283-90.
62. Tan AL, Forbes JM, Cooper ME. AGE, RAGE, and ROS in diabetic nephropathy. Semin Nephrol. 2007;27:130-43.
63. Maahs DM, Siwy J, Argiles A, Cerna M, Delles C, Dominiczak AF, et al. Urinary collagen fragments are significantly altered in diabetes: a link to pathophysiology. PLoS One. 2010;5:e13051.
64. Roscioni SS, de Zeeuw D, Hellemons ME, Mischak H, Zurbig P, Bakker SJ, et al. A urinary peptide biomarker set predicts worsening of albuminuria in type 2 diabetes mellitus. Diabetologia. 2013;56:259-67.
65. Zürbig P, Jerums G, Hovind P, MacIsaac R, Mischak H, Nielsen SE, et al. Urinary proteomics for early diagnosis in diabetic nephropathy. Diabetes. 2012;61:3304-13.
66. Argiles A, Siwy J, Duranton F, Gayrard N, Dakna M, Lundin U, et al. CKD273, a new proteomics classifier assessing CKD and its prognosis. PLoS One. 2013;8:e62837.
67. Toyohara T, Akiyama Y, Suzuki T, Takeuchi Y, Mishima E, Tanemoto M, et al. Metabolomic profiling of uremic solutes in CKD patients. Hypertens Res. 2010;33:944-52.
68. Toyohara T, Suzuki T, Morimoto R, Akiyama Y, Souma T, Shiwaku HO, et al. SLCO4C1 transporter eliminates uremic toxins and attenuates hypertension and renal inflammation. J Am Soc Nephrol. 2009;20:2546-55.
69. Saigusa D, Suzuki N, Takahashi M, Shiba K, Tanaka S, Abe T, et al. Simultaneous determination of guanidinosuccinic acid and guanidinoacetic acid in urine using high performance liquid chromatography/tandem mass spectrometry. Anal Chim Acta. 2010;677:169-75.
70. Spector TD, Williams FM. The UK adult twin registry (TwinsUK). Twin Res Hum Genet. 2006;9:899-906.
71. Illig T, Gieger C, Zhai G, Romisch-Margl W, Wang-Sattler R, Prehn C, et al. A genome-wide perspective of genetic variation in human metabolism. Nat Genet. 2010;42:137-41.
72. Choi JY, Yoon YJ, Choi HJ, Park SH, Kim CD, Kim IS, et al. Dialysis modality-dependent changes in serum metabolites: accumulation of inosine and hypoxanthine in patients on haemodialysis. Nephrol Dial Transplant. 2011;26:1304-13.
73. Aronov PA, Luo FJ, Plummer NS, Quan Z, Holmes S, Hostetter TH, et al. Colonic contribution to uremic solutes. J Am Soc Nephrol. 2011;22:1769-76.
74. Meijers B, Glorieux G, Poesen R, Bakker S. Non-extracorporeal methods for decreasing uremic solute concentration: a future way to go? Semin Nephrol. 2014;34:228-43.
75. Suhre K, Meisinger C, Doring A, Altmaier E, Belcredi P, Gieger C, et al. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. PLoS One. 2010;5:e13953.
76. Rhee EP, Souza A, Farrell L, Pollak MR, Lewis GD, Steele DJ, et al. Metabolite profiling identifies markers of uremia. J Am Soc Nephrol. 2010;21:1041-51.
77. Mischak H, Ioannidis JP, Argiles A, Attwood TK, Bongcam-Rudloff E, Broenstrup M, et al. Implementation of proteomic biomarkers: making it work. Eur J Clin Invest. 2012;42:1027-36.
78. Perco P, Wilflingseder J, Bernthaler A, Wiesinger M, Rudnicki M, Wimmer B, et al. Biomarker candidates for cardiovascular disease and bone metabolism disorders in chronic kidney disease: a systems biology perspective. J Cell Mol Med. 2008;12:1177-87.
79. Molina F, Dehmer M, Perco P, Graber A, Girolami M, Spasovski G, et al. Systems biology: opening new avenues in clinical research. Nephrol Dial Transplant. 2010;25:1015-8.