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12. THE URINARY PROTEOMICS: A TOOL TO DISCOVER NEW AND POTENT BIOMARKERS FOR KIDNEY DAMAGE

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12.1 Introduction

The increasing number of patients suffering from chronic renal failure represents one of the major challenges to which nephrologists are faced worldwide today. For a better therapeutic outcome of this disease earlier detection is urgently warranted in routine clinical practice. Urine is a kind of messenger of the urinary system function. Kidney damage or dysfunction results in release of peptides and proteins in urine (Figure 12.1.), this renders urine analyses of wide clinical interest for evaluation of kidney and urinary tract disorders. Urinary diagnostic can help to detect diseases that do not produce striking signs or symptoms at an earlier stage. Following parameter are routinely analysed for urine: method of collection, urine specific gravity, colour, turbidity, pH, glucose, ketones, bilirubin icotest, blood and epithelial cells, and detection of proteins. Urinary proteins are of particular importance as their amount and composition reflect renal function and disorder (1). The estimation of protein amount in urine is of big importance for diagnostic as proteinuria is a marker for renal disfunction (2) and responsible for the progression of renal failure (3). Different methods were established to estimate the protein amount in urine, several of them found their way in routine diagnostic for evaluation of proteinuria. However, all these assays still not fulfil the conditions required for an adequate diagnostics. New techniques such as the analysis of the diseased renal proteome are highly promising to overcome some of these problems (4-9). Proteomics has enormous potential to improve the quality of urinoproteins based diagnostic, as well as providing practical insights that will impact medical practice and therapy. Beside direct analysis of renal tissue, mass spectrometric approaches to urinary peptide/protein profiling are promising to have potential value in the none-invasive diagnosis, monitoring or prediction of renal and urinary tract diseases.

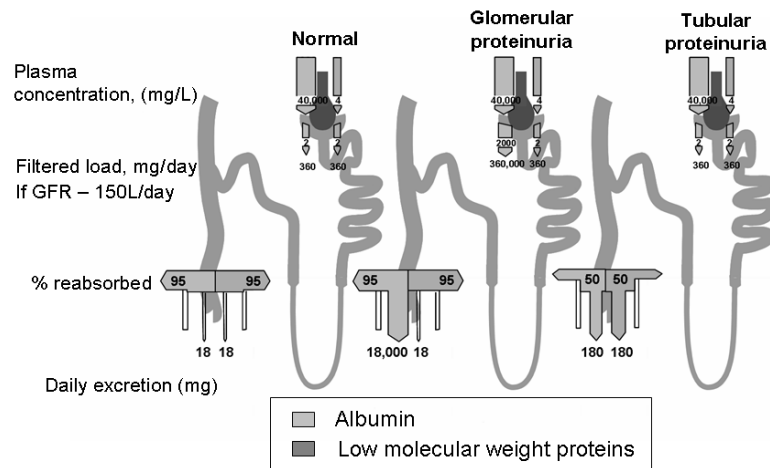


Figure 12.1. Origin of urine proteins: in the urinary system high molecular weight proteins (> 40 kDa) are held back in the glomerular part, whereas the low molecular weight proteins are absorbed in tubulus. Glomerular proteinuria led to increased release of high molecular proteins and tubular proteinuria is characterized by high excretion of low molecular weight proteins. Illustrated is the ratio of small and large proteins release in urine depending on the origin of the proteinuria. GFR: glomerular filtration rate.

12.2 Defining proteomics and clinical proteomics

Proteomics is the systematic study of proteomes, which describes the entire protein content of one or all cells of an organism as well as of bodily fluids such as blood, urine and sweat. While the genome of an organism is considered to be mostly static, the proteome shows dynamic properties with protein profiles changing in dependence of a variety of extra- and intracellular stimuli (i.e. cell cycle, temperature, differentiation, stress, apoptotic signals). Proteomics can be divided into three main areas: primarily, protein micro-characterization for large-scale identification of proteins and their post-translational modifications; secondly, differential display proteomics for comparisons of protein levels with potential application to a wide range of diseases; and thirdly studies of protein-protein interactions. Clinical proteomics is the part of proteomics that aims to characterize the interconnection between different tissues in organs or between organ and circulatory systems together, with clinical applications for diagnosis and therapy as ultimate target. Clinical proteomics include a large number of areas e.g. cancer proteomics, biomarker discovery, toxicoproteomics, pharmacoproteomics, stem cells proteomics, fluids proteomics... In clinical application, a comparative approach of normal and abnormal status of cells, tissues or bodily fluids is employed to identify proteins that exhibit quantitative changes in a disease-specific manner for use as diagnostic markers or therapeutic targets. Clinical proteomics still is a new promising analytic discipline with the following main aims: a) discovery of biomarkers allowing an early detection, risk management or therapeutic monitoring of diseases for the establishment of individualized treatment procedures, b) identification of protein targets for the development of new mechanistic intervention therapies with the promise of an improved clinical outcome.

12.3 Urinary proteomics and the advantages for clinical applications

Proteomics offer a new technology platform for identification and quantification of novel urinary biomarkers that may lead to the development of simple and more personalized diagnostic tests to be used in clinical practice for earlier disease detection and/or better therapeutic outcome (10). The proteomics techniques used to characterize urine can be divided in two groups: gel based urine proteome analysis and gel free urine proteome analysis (Figure 12.1., Table 12.1.) (11, 12).

The gel based techniques use two-dimensional gel electrophoresis. This method is powerful and widely used for the analysis of complex protein mixtures extracted from cells, tissues or biological fluids (13). The two-dimensional gel electrophoresis separates and characterizes proteins according to their charge/ion strength and molecular weight in two consecutive gel electrophoresis steps: Proteins are first separated by isoelectric focusing according to their isoelectric points and then distinguished according to their molecular weights in SDS-polyacrylamide gel electrophoresis. 2-D gel-electrophoresis is generally labour- and time-intensive and without strict standardization in the applied reagents, apparatus and software for the analysis usually not routinely applicable in clinical settings.

The gel free urine proteome analyses offer important conditions for the integration of proteomics in routine laboratories because of the reduced sample requirement and the high throughput and automation scale. For this reason, different methods have been developed which effectively couple high-end mass spectrometry to array formats, to capillary electrophoresis or to chromatography. The surface-enhanced laser desorption/ionization (SELDI) technique offers such an opportunity for urine analysis. Small amounts of native urine samples can be applied to the surface of a SELDI ProteinChip without prior concentration or precipitation of the urinary proteins (8, 14). The bound proteins may then be directly analysed by MALDI-TOF-MS (Figure 12.2.) (15, 16). Also CE-MS coupled to the high-resolution properties of capillary electrophoresis (CE) can be used combined with the powerful identification ability of the electrospray time-of-flight MS to profile and sequence urinary proteins. Liquid chromatography coupled to mass spectrometry (LC-MS) offers also a gel free alternative for sensitive urine proteome analysis. Thus, protein profiles or single identified proteins may be characterized as disease specific protein pattern or biomarkers which, however, have to be validated in controlled retro- and prospective clinical studies.

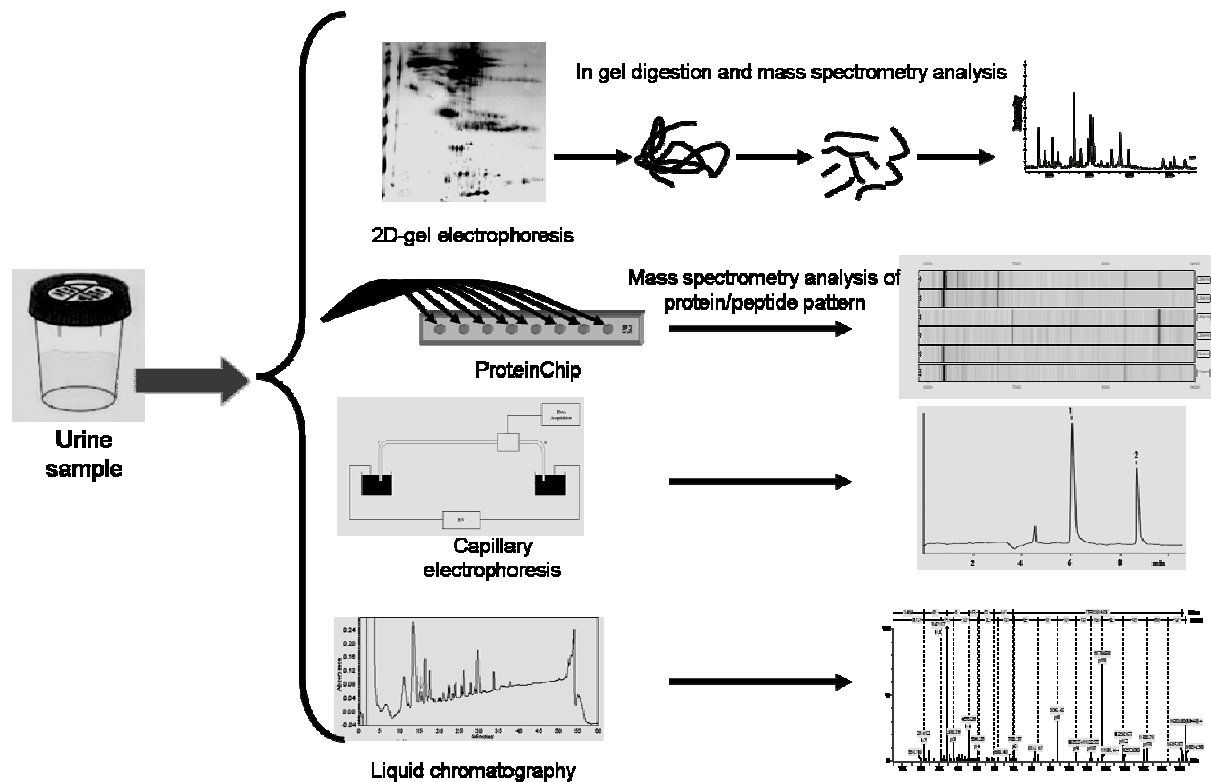


Figure 12.2. Gel based and gel free proteomics methods in urinary proteome analyses: Gel based urine analysis using 2D gel electrophoresis proteins will be separated according to their masses and pIs. After in-gel enzymatic digestion of the proteins the tryptic product can be analyzed by mass spectrometry. The identification can be performed by data bank search. Gel-free urinary proteome analysis. ProteinChip coupled to MALDI-TOF-MS (SELDI-TOF-MS) technology. Different types of ProteinChip surfaces are available. The chips are spotted with different chromatographic surfaces for urine protein binding. Bound proteins are then ionized with mass spectrometry resulting in protein profiles. CE-MS coupled the high-resolution properties of capillary electrophoresis (CE) and the powerful identification ability of the electrospray time-of-flight MS to profile urinary proteins. The resulting protein pattern can be used for diseases discrimination. Liquid chromatography coupled to mass spectrometry (LC-MS) offers also a gel free alternative for urine proteome analysis. Dihazi et al. (11)

	Gel based methods		Gel free methods		
	2D Gel electrophoresis	DIGE	SELDI	LC-MS	CE-MS
Advantages	<ul style="list-style-type: none"> – separation of large number of proteins – easy biomarker identification 	<ul style="list-style-type: none"> – quantification possible – separation of sample and control on the same gel – less amount of protein required – use of internal standard possible 	<ul style="list-style-type: none"> – possibility of quantification – automation – high-throughput analyses – less amount of protein required 	<ul style="list-style-type: none"> – possibility of quantification – automation – high-throughput analyses – less amount of protein required – sample fractionation possible LC 	<ul style="list-style-type: none"> – possibility of quantification – automation – high-throughput analyses – less amount of protein required – sample fractionation possible CE
Disadvantages	<ul style="list-style-type: none"> – required high protein amount – lack of automation – limitation for large and small proteins – lost of hydrophobic proteins – not useful as screening tool – lack of reproducibility – laborious 	<ul style="list-style-type: none"> – lack of automation – limitation for large and small proteins – lost of hydrophobic proteins – expensive – laborious 	<ul style="list-style-type: none"> – drawback in detecting low abundance proteins – lack in the detection of high molecular weigh proteins – protein identification is problematic – lack in identification of post-translational modifications – protein fractionation is necessary 	<ul style="list-style-type: none"> – drawback in detecting low abundance proteins – lack in the detection of high molecular weigh proteins 	<ul style="list-style-type: none"> – drawback in detecting low abundance proteins – lack in the detection of high molecular weigh proteins – protein identification problematic – deliver complex pattern – pattern recognition not a single biomarker

Table 12.1. Summary of the proteomic platforms used for urine analysis, their advantages in disadvantages.

Diagnostic tools using urine and non-invasive proteomic methods are particularly promising for the detection and differentiation of renal deterioration early before overt clinical symptoms during the various kidney specific or associated diseases. Furthermore proteomics methods have the potential advantage of lower costs and higher efficiency of patients care. Nevertheless, robustness, sensitivity, reliability and consistency of the test systems for the detection of changes in protein expression are crucial parameters in addition to labour and cost expenses for the acceptance of proteomics studies in specific clinical settings such as renal diagnostics. At present many proteomics techniques still suffer from insufficient standardization and only a few have the potential to fulfil essential criteria for future practical clinical application.

12.4 Trends in urine proteome analysis and biomarker discovery

Non-invasive accessibility of urine makes it attractive for the clinical proteomics. Different studies have already applied clinical proteomics to analyze the urinary proteome and tried to identify markers associated with renal diseases. The majority of these studies were carried out with a small number of individuals. Moreover these studies reported a peptide pattern or peptide/protein mass to charge (8, 9, 17-22). The identity of the discovered protein or peptide markers that discriminate renal disease is still lacking in most of these studies. Since the function of the protein marker can be very important for understanding the pathophysiology of the disease and might shed light on the involved pathways in the disease development. Regardless of the great promise of urine proteome analysis, the identification of urinary biomarkers by mass spectrometry technologies for an earlier diagnosis, prognosis or prediction of therapeutic responses in renal diseases has still many obstacles to cross. Additional to the technical aspects, handling conditions for urine are critical. The standardisation of urine collection is the first problem to be solved (Table 12.2.). In our days the midstream of the second morning urine was found to be optimal and was used with success in several studies (4, 23, 24). Urine collecting tubes should always include appropriate amount and composition of protease inhibitors to avoid protein degradation. After urine collection delays in analyzing the samples can result in artefacts, the interval of time between collection and analysis should be kept as short as possible. The delay in this handling step could have a high impact on the urine status and protein pattern. Protein degradation caused by proteases in urine, decreased clarity due to crystallisation of solutes, rising pH, loss of ketone bodies, loss of bilirubin, cell lysis leading to additional proteins in samples, overgrowth of contaminating microorganisms all these factors could be a source of artefacts in urine proteome analysis. The fragility of urine proteome renders the standardization of sample collection one of the main challenges facing the clinical proteomics and biomarker discovery. Recently published papers presented optimized protocols for urine handling for proteomics analysis (24-27). However, more intensive investigations are needed in this area to deliver optimal protocols for handling the fragile urinary proteome.

Important protein candidates for the therapy and for the understanding of the pathophysiology of renal disease are mostly in low amount in urine. Using depletion methods e.g., albumin/globulin depletion prior to proteome analysis make the access to low abundance proteins possible. Urine prefractionation can also be very helpful to prevent the complexity of the samples and to increase the analysis outcomes.

Additional to the biomarker identification, the quantification represent the next challenge to overcome. Traditionally urinary proteomics used gel based or mass spectrometry based methods (SELDI-TOF, LC-MS, CE-MS) for relative quantification. These approaches have their disadvantages. Quantification methods based on stable-isotope labeling coupled with mass spectrometry as the readout could offer promising alternatives. These alternatives are either peptide or protein based. The peptide based methods like the global internal standard technology (GIST) (28), or isobaric tags for relative and absolute quantification (iTRAQ) (29) have their drawback in the protein quantification, detection of posttranslational modifications, in detection of protein degradation, and in the reproducibility in the yield of the digestion which can result in errors in quantification. Among the protein

based approaches the isotope-coded affinity tags (ICAT) (30) was the first established mass spectrometry based quantification method. The ICAT have cysteine as target amino acid for labelling. The low abundance of cysteine in proteins results in decrease of the quantification output. In in-gel stable-isotope labeling (ISIL) (31), protein samples are labeled with stable isotopes in the gel matrix. The labeled proteins are digested, and analyzed by LC-MS. Isotope Coded Protein Label (ICPL) (32) is based on isotopic labelling of all free amino groups in proteins. Although these methods show their ability the perform relative and absolute peptide/protein quantification, most if not all are far from being applicable as a routine methods and it will be very challenging to implement effectively in routine urine analysis. In addition, information about the accuracy of these techniques in practice across multiple laboratories having various levels of expertise is still missing.

Urine collection method	Advantage	Disadvantage
24 h urine collection	<ul style="list-style-type: none"> – Monitoring of the kidney function over a long period 	<ul style="list-style-type: none"> – Lack of patients control – High protein degradation – Lack of reproducibility – Contamination (overgrowth of contaminating microorganisms) – Useful only when all urine is collected for 24 hours. – Standardisation for proteomics almost impossible
Random spot collection	<ul style="list-style-type: none"> – Flexibility: Can be taken at any time of the day or night – Less protein degradation – Better patient control – Easy to handle for proteomics 	<ul style="list-style-type: none"> – Does not represent the processes taking place over a 24-hour period – Proteome is depending on dietary and physical activity
First morning urine	<ul style="list-style-type: none"> – Free of dietary influences – Free of changes due to physical activity 	<ul style="list-style-type: none"> – Hypertonic – Concentrate urine – Bacterial contamination – Long residence time in bladder - Protein degradation in bladder – Protein pattern difficult to reproduce
Catheterizations of the bladder		<ul style="list-style-type: none"> – Invasive – Risk of introducing infection – Risk of traumatizing the urethra and bladder – Urine Proteome contamination with blood cell proteins
Second morning urine (midstream)	<ul style="list-style-type: none"> – Less protein degradation – Better patient control – High reproducibility – Free of dietary influences – Free of changes due to physical activity – Relatively stable proteome 	<ul style="list-style-type: none"> – Does not represent the processes taking place over a 24-hour period

Table 12.2. Urine collection methods advantages and disadvantages for urine proteome analysis

12.5 Conclusion

There is a strong need for inter-laboratory standardization of the techniques and of the interpretation of the results at the first place. These challenges can only be overcome by intensively collaborating teams of researcher scientists, clinicians and statisticians also with the support of HUPO (Human Proteome Organisation <http://www.hupo.org/>) and HKUPP (Website of the International Human Kidney & Urine Proteome Project <http://hkupp.kir.jp/>), which try to provide organized platforms of all information available on normal and diseased human proteomes at the international level.

The adequate diagnosis of complex diseases e.g., renal disease with a single biomarker seems to be an illusion. A multiple biomarker assay could deliver a better and a more individualized diagnosis and allow therapeutic strategies that delay or prevent the progression of the disease. Due the above named limitations and uncertainties, urinary proteomics at present cannot replace invasive standardized diagnostic procedures such as the renal biopsy, but holds great promise and potential for future highly improved diagnosis and care of the patient in nephrology (12).

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