Urinary proteomic strategies in biomarkers discovery of renal diseases

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❚ **Abstract**

Proteomic analysis have been commonly used in numerous studies in the field of renal medicine. However, invasive tissue sampling and also its small quantity are the main obstacles in this type of analysis. Urine might be a suitable alternative for kidney puncture as it can be collected in a non-invasive way, in large amounts, and it also contains proteins, peptides and amino acids. Therefore, attempts have been made to identify urinary biomarkers for renal diseases, such as: IgA nephropathy, membranous nephropathy, idiopathic nephritic syndrome, steroid-resistant nephritic syndrome, steroid-sensitive nephritic syndrome, diabetic nephropathy, uretopelvic junction obstruction, radiation nephropathy and chronic kidney disease. The search for novel protein markers, which may enable early detection of the above-mentioned kidney diseases, requires the application of several, different proteomic techniques. This review is intended to discuss the present state of knowledge within this subject.

❚ **Keywords**

chromatography; electrophoresis; mass spectrometry; proteomics; biomarkers; kidney; renal diseases; urine

INTRODUCTION

The term 'proteome' was first coined in 1994 by the Australian scientist Marc Wilkins, a geneticist, and refers to the set of PROTEins encoded by the genOME [1]. Proteomics is defined as the systematic analysis of proteins in order to determine their unique identity, quantity, functions and interactions [2]. Proteomic analyses have been successfully used in a large number of studies including biological and medical. Most of these studies have focused on protein changes occurring in tissue samples and/or in body fluids (e.g. serum, plasma, urine, saliva) in response to cancer or other diseases [3]. The final results of proteomic analysis can reveal the possible role of selected proteins in disease mechanisms. Thus, it may lead to biomarker discovery, identification of new therapeutic targets and also drug discovery [4].

Proteomic approaches display a superiority over genomic methods, which only enable determination of the static image of gene expression. Functional genomic-based analysis of mRNA also does not reflect the full pattern of protein expression. Illness as a dynamic process modifies the switching on and off of the expression of specific genes, resulting in a qualitative changes in the relationship between the produced and secreted proteins. Moreover, intracellular localization, modifications, and interactions between proteins can be studied only at the protein level [5]. Many techniques have been developed to study changes in the single protein expression in various biological materials, including Western blot, which is the most widely used approach for this purpose. However, simultaneous analysis of a large number of proteins requires an application of much more sophisticated proteomic tools [6].

Regardless of the proteomic techniques employed, few general steps can be distinguished through analysis. The first step of proteomic analysis is protein extraction from the biological sample, which is followed by its separation by either gel or non-gel techniques, e.g. by liquid chromatography (LC). It seems that two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the most popular method used in order to obtain urine protein profiles. However, despite its advantage manifested in high reproducibility, it also has some limitations, including difficulties in the separation of highly hydrophobic proteins, as well as proteins and polypeptides with molecular weight <10 kDa. It is also time-consuming and labour–intensive. Capillary electrophoresis (CE) has a higher sensitivity than 2-D PAGE, but it is not suitable for analysis of proteins with molecular masses above 20 kDa. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is another analytical technique suitable for separation of urine proteins. This method is much more sensitive than 2-D PAGE, but simultaneously, it is time-consuming and too sensitive, as it interferes with compounds in the urine [7]. An important element of proteomic analysis is the proper choice of detection method employed to visualize previously separated proteins. Commonly, proteins are stained either with Commassie blue (equal stain intensity, low sensitivity), or silver nitrate (higher sensitivity, lower dynamic range). Other detection methods based on fluorescent dyes (e.g. Sypro Ruby, Deep Purple, Flamingo) are as sensitive as silver nitrate, and also have a wider dynamic range [5]. In methods of protein separation coupled to mass spectrometry (e.g. LC-MS/MS, CE/ESI-TOF-MS), the staining step is omitted.

Separated and stained proteins are subsequently identified by various mass spectrometers, e.g. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). The SELDI-TOF MS (Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry) is easier

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to operate compared to LC-MS/MS, and it also requires a smaller amount of sample (<10 μl). However, this technique is applicable only for some groups of proteins, and it is also characterized by low reproducibility [8,9]. According to Xin *et al.* [10] the most effective proteomic method is the combination of capillary electrophoresis with an ESI-FT-ICR (Electrospray Ionization Fourier Transform Ion Cyclotron Resonance) mass spectrometry. Nevertheless, as reported by Liu *et al.* [11], the most appropriate method of urine proteome analysis is protein microarray technology. This high-throughput approach requires a low sample volume, and moreover, enables the detection of proteins with low molecular masses (potential biomarkers), which is practically impossible when using other (commonly used) approaches, e.g. 2-D PAGE [12]. As protein microarrays are based on interactions with specific antibodies, its availability is the main limitation of this method.

Invasive renal tissue sampling, and also its small quantity, are the main obstacles in this type of analysis. Urine proteomics has become one of the most attractive sub-disciplines in clinical proteomics, in particular for the purpose of discovering biomarkers and clinical diagnostics [7]. Urine proteomics, however, encounter several barriers, such as low concentration of total protein, and high concentration of salts and other ingredients that hinder analysis, especially at the stage of protein separation [13]. Large inter-individual differences in the composition of urine, as well as the high dynamics of changes in urine composition between different seasons of the material collection, are other limitations.

Due to differences in sensitivity and availability of various proteomic techniques, considerable efforts have been made to find a suitable method to analyze the expression of specific urine marker proteins. Urinary proteome contains not only filtered proteins, but also proteins of renal origin, the identification of which could be very helpful in the analysis of pathological processes occurring in the kidneys and other organs [14]. To allow specific and early diagnosis of disease, at least some of these proteins should be biomarkers that are independent from proteinuria [8].

URINE PROTEIN MARKERS IN RENAL DISEASES

One of the major tasks of clinical proteomics is the identification of biomarkers in urine, which enable early diagnosis of disease, and/or may be used in the prevention of renal diseases [14]. Renal damage in clinical practice is detected by several easy and fast tests, including: urine albumin concentration, urine total protein concentration or plasma creatinine concentration. Nevertheless, these indicators are not specific, and additionally, their noticeable alterations occur only in the late-stage of the pathological state [15]. Thus, proteomic analysis of the urine proteins, which aims to identify early biomarkers of kidney diseases, has become a global trend in renal research.

IgA nephropathy. One of the most common glomerular disease is IgA nephropathy (IgAN), also called Berger's disease, which leads to glomerular inflammation. IgAN is characterized by deposition of immunoglobulin A in the glomerulus. Furthermore, IgA nephropathy is also associated with other diseases, such as proteinuria, immune disorders, infections and cancers [16]. The patients suffering from IgAN

(15-40%) will probably show end-stage renal disease [17]. So far, the only way to diagnose IgAN is renal biopsy [18].

In studies on IgA nephropathy in humans, Park *et al.* [19], using two-dimensional electrophoresis for separation of urine proteins, achieved on the 2-DE gels 311 protein spots, from which 59 were identified (intracellular, cytoplasmic, nuclear, membrane, secreted, plasmic, extracellular, mitochondrial and lysosomal) with the aid of a MALDI-TOF mass spectrometer. The authors reported that 35 proteins, which expression in patients with Berger's disease, down-regulated when compared with controls, were not identified due to the low sensitivity of MALDI-TOF MS.

Moreover, Wu *et al.* [20], in studies aimed at discovering IgA nephropathy urine biomarkers, separated proteins and polypeptides using magnetic beads, and subsequently identified them by the aid of MALDI-TOF MS. The authors reported that a fragment of uromodulin (peptide m/z 1913.14) may be an important biomarker of kidney disease in non-invasive clinical studies.

In the urine there is a wide range of dynamically changing polypeptides that can be used as a potential source of biomarkers. Chalmers *et al.* [21] described many ways to identify polypeptides of renal origin that are present in excreted urine. In order to identify biomarkers, the authors used a capillary electrophoresis coupled to a mass spectrometer with electrospray ionization technique and time-of-flight detector (CE/ESI-TOF-MS). Four polypeptide biomarkers of renal damage caused by IgA nephropathy were found: 1829-Da, 2081-Da, 4240-Da; 8.8-kDa, which were then analyzed for their precise characterization. For a detailed characterization of 1829-Da and 2081-Da biomarkers, an electron capture detector (ECD) was used. As a result, precise molecular weight of 1829.0827 Da and 2081.011 Da, respectively, were determined. The molecular mass of the 4240-Da biomarker was measured at 4240.1592 Da, by combining the techniques of preparative capillary electrophoresis and liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS). The authors determined a neutral mass of monoisotopic 8,8-kDa biomarker at 8763,49-Da by on-line CE/ESI-FT-ICR MS (capillary electrophoresis coupled to electrospray Fourier transform ion cyclotron resonance mass spectrometry). Chalmers *et al.* [21] highlighted that solid analysis of polypeptide biomarkers require the use of highly specific separation and identification techniques adapted to the molecular weight of polypeptides and their sources.

Similarly Haubitz *et al.* [22] used the on-line CE/ESI-TOF MS technique to identify biomarkers of IgAN and membranous nephropathy (MN). Using this method, the authors obtained from 300 – 2,000 polypeptides and proteins within the molecular weight range 800 – 7,000 Da from a single urine sample. Among these, Haubitz *et al.* [22] detected 12 polypeptide biomarkers characteristic for membranous nephropathy and IgA nephropathy. These markers did not allow distinguishing IgAN from MN disease, therefore they can only be considered as biomarkers of kidney damage. Among 12 biomarkers, 3 are the most characteristic for IgAN (2752.4 Da with CE migration time of 15.5 min., 2,427.1 Da with CE migration time of 16.4 min., 2,057.3 Da with CE migration time of 19.1 min.) were selected, and then subjected to sequential analysis using MALDI-TOF/TOF mass spectrometry. All 3 polypeptides were identified as fragments of serum albumin. Additionally, the authors analyzed the resulting data in order to determine

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biomarkers (independent from the degree of proteinuria), which may allow distinguishing between IgA nephropathy and membranous nephropathy. Further analysis allowed determination of 28 polypeptides, which differ between these 2 diseases. The authors described 9 polypeptides characteristic for IgAN and 17 polypeptide biomarkers of MN. Using urine samples from patients with membranous nephropathy, Neuhoff et al. [23] compared the usefulness of SELDI-TOF mass spectrometry and capillary electrophoresis coupled to mass spectrometer ESI-TOF (CE/ESI-TOF MS) in biomarker discovery. The authors reported that analysis of SELDI-TOF generates less complex polypeptide maps of weaker resolution than CE/ESI-TOF MS. Moreover, SELDI TOF analysis is more dependent on sample concentration, and installation sensitivity is lower than CE/ESI-TOF-MS. Neuhoff et al. [23] reported that in order to obtain the same amount of proteins at different expression level by SELDI-TOF analysis, different modifications should be carried out, e.g. protein fractionation, dilution, and the use of different surfaces of chips. However, the great predominance of use of the SELDI-TOF method is a very small volume of sample (even 50nL of urine). Nevertheless, the method of CE/ESI-TOF MS generates a huge amount of information, is not interfered with by the sample concentration, or type of surface used. The authors described the technique of capillary electrophoresis coupled to ESI-TOF mass spectrometer as the most appropriate method for analysis of a mixture of several polypeptides.

Haubitz et al. [22] highlighted, that two-dimensional electrophoresis is not a suitable method for searching for biomarkers of several kidney diseases, such as IgA nephropathy and membranous glomerulonephritis (membranous nephropathy), mainly due to its time-consuming procedure and the impossibility of potential biomarker separation with a molecular mass below 10 kDa. According to the authors, identification using mass spectrometer with SELDI type of ionization should also be avoided, this is because of its limited resolution and loss of many protein/polypeptides during the step involving mixing sample with the matrix.

Khositseth et al. [16] described the case of 14-year-old boy suffering from IgA nephropathy associated with Hodgkin's disease. The authors created a 2-D map of the patient's urine proteins, which was then compared with a map of the urine proteins of healthy individuals. Images of these protein maps varied noticeably. However, Khositseth et al. [16] did not identify proteins which differed between these 2 images. The authors identified 14 proteins (collagen α 1 (IV) chain precursor, cadherin-11 precursor, albumin, transferrin precursor, kininogen, albumin complexed with decanoic acid, α-1 antitrypsin, Ig heavy chain, leucine-rich α-2 glycoprotein, inter-α-trypsin inhibitor heavy chain, α-1 microglobulin, Ig light chain, complement C1q tumor necrosis factor-related protein, β-2 microglobulin), the expression of which changed with the length of patient therapy. According to the authors, proteomic tools can be successfully used not only to diagnose the disease, but also to monitor effectiveness of the treatment.

Moon et al. [24] found 4 unique urinary exosomal proteins related to IgA nephropathy (ceruloplasmin, serotransferrin, α-2-macroglobulin, α-1-antitrypsin). These proteins were identified by LC-MS/MS, and additionally confirmed by Western blot analysis. The protein α-1-antitripsin (Serpina1) had previously been reported by Miyata et al. [25] to be

upregulated in IgAN. However, Moon et al. [24] showed that the clinical application of these 4 biomarker candidates requires further study in a larger group of patients.

The strategy investigated in the above-described study could be a model approach in order to discover potential biomarker candidates of human renal diseases without typical animal models [24].

Steroid-resistant and steroid-sensitive nephrotic syndrome. Hodgkin's disease may also be the cause of idiopathic nephrotic syndrome (NS) in children. Varghese et al. [26], using 2D-PAGE, separated urine proteins to search for NS biomarkers. The 21 excised protein spots with altered expression, in comparison to the control, were subjected to in-gel tryptic digestion and identified using a mass spectrometer MALDI-TOF, MALDI-TOF/TOF, and Finnigan LTQ linear ion trap MS. As a result, 11 plasma proteins were identified: orosomucoid, transferrin, α-1 microglobulin, zinc α-2 glycoprotein, α-1 antitrypsin, complement factor B, haptoglobin, transthyretin, plasma retinol binding protein, albumin, and hemopexin. The authors postulate that these proteins may be important biomarkers of nephrotic syndrome. Accurate identification of biomarkers of this disease and its subsequent treatment is important, as the remission of nephrotic syndrome leads to remission of Hodgkin's disease [27].

Steroid-resistant nephrotic syndrome (SRNS) is a type of NS. Khurana et al. [28], with the aid of SELDI-TOF MS, identified 5 potential urine biomarkers of this disease In the obtained mass spectrum, the following peaks were characteristic for this disease: 3917.07, 4155.53, 6329.68, 7036.96, and 11117.4. According to the authors, the peak of 11117.4 was considered to be a biomarker of SRNS. In order to identify this peak, polypeptides were again extracted from the urine samples by C18 columns, and were then sequenced by a tandem mass spectrometer (MALDI-TOF/TOF). This peak was identified by Khurana et al. [28] as ß2-microglobulin.

Woroniecki et al. [29] also used a SELDI-TOF mass spectrometer to discover biomarkers of SRNS and SSNS (steroid-sensitive nephrotic syndrome) in patients. The authors discovered a urine protein with a molecular mass of 4144 Da, which has been identified as the most important protein distinguishing between the profile of the SSNS group from that with SRNS.

Diabetic nephropathy. One of the most common renal diseases is diabetic nephropathy. It is estimated that approximately 10-50% of type 2 diabetes patients will develop kidney failure [30, 31], and many of them will further progress to end-stage renal disease (ESRD) by a relentless decline in the glomerular filtration rate [32]. Recent studies have demonstrated that early detection of diabetic nephropathy reduces the possibility of developing ESRD, which results in the necessity of a kidney transplantation. Thus, discovery of noninvasive biomarkers may lead to faster diagnosis and earlier treatment of patients at high risk for type 2 diabetic nephropathy [32]. One of the first applications of CE-MS for the analysis of a specific urinary proteins was in patients with type 2 diabetes [8].

Jain et al. [33] used 2D-PAGE in order to identify urine biomarkers of diabetic nephropathy in patients with type 2 diabetes. The proteins with altered expression, in comparison to proteins from the control group, were sequenced using

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MALDI-TOF MS. The authors reported that identified proteins (zinc α-2 glycoprotein, α-1 acid glycoprotein, α-1 microglobulin, IgG), together with albumin, may serve as biomarkers for diabetic nephropathy. Presence of the above-mentioned proteins in the urine was also confirmed by Western blot.

The results achieved by Jain *et al.* [33] were confirmed by Narita *et al.* [34], who observed an increase in the concentration of IgG in the urine of individuals with type 2 diabetes. The authors reported that the increase of IgG concentration was parallel with the increase of ceruloplasmin and transferrin concentration in the excreted urine.

In studies focused on diabetic nephropathy, Rossing *et al.* [35] used capillary electrophoresis coupled with a tandem mass spectrometry (MALDI-TOF/TOF). A total of 4,551 peptides were detected in the urine samples. The authors limited the number of polypeptides to 758 and analysed only those that occurred in at least half of the individuals. 523 polypeptides were present in the urine of patients with diabetic nephropathy without normoalbuminuria, 580 polypeptides in patients with diabetic nephropathy and normoalbuminuria, 639 polypeptides in patients with microalbuminuria, and 507 polypeptides in patients with macroalbuminuria. Rossing *et al.* [35] obtained a map of 113 polypeptides, which differed between patients with macroalbuminuria and patients with normoalbuminuria. 11 of these polypeptides were identified as: Tamm-Horsfall protein, collagen, and fragments of albumin.

Rao *et al.* [36] also used two-dimensional differential in-gel electrophoresis (2-D DIGE) to separate and visualize urine proteins from patients suffering from diabetes with diabetic nephropathy. Identification of the excised protein spots was carried out using LC/ESI-MS/MS. 195 spots were identified, representing 62 unique proteins. According to the authors, in patients with diabetic nephropathy. the following proteins were up-regulated: α-1B-glycoprotein, zinc-α-2-glycoprotein 1, vitamin D-binding protein, α-2-HS-glycoprotein, calgranulin B, α-1-antitrypsin and hemopexin, while retinol binding protein, α-1-microglobulin/bikunin prekursor, apoA-1 and transthyretin, were down-regulated.

The 2D-DIGE method was also applied by Sharma *et al.* [37] who observed 99 protein spots with altered expression, of which 63 spots were up-regulated and 36 down-regulated. Differentially expressed proteins were identified by the SELDI-TOF mass spectrometer. The authors did not show a complete list of identified potential biomarkers, but reported that expression of α-1-antitrypsin was 19-fold higher in patients with diabetic nephropathy, in comparison to the control group.

Wu *et al.* [33] analysed 95 urine samples from type 2 diabetic patients with normoalbuminuria (n=30), microalbuminuria (n=25), macroalbuminuria (n=20), and healthy controls (n=20), by ProteinChip and SELDI-TOF MS. The authors reported that over 300 proteins or peptides in a mass range of 1-80 kDa were obtained using ProteinChip. 40 peaks were selected with significant differences between diabetic patients and healthy controls [32]. Furthermore, Wu *et al.* [32] claimed that 4 peaks with m/z values of 2797.03, 4545.77, 4984.03 and 9083.71 Da were found to be highly expressed between patients with normoalbuminuria and patients with microalbuminuria. These were further identified by the authors as the novel potential biomarkers for early type 2

diabetic nephropathy with sensitivity of 88% and specificity of 96.7%.

Mischak *et al.* [38] described a strategy for searching for biomarkers of a diabetic nephropathy in urine. The authors applied on-line CE/ESI-MS, and then sequenced the resulted, relevant polypeptides using MALDI-TOF/TOF and MS/MS. Polypeptides with molecular masses of 1,232.7 Da, 1,679.9 Da, and 2,228.2 Da were identified as INSL3 (insulin-like peptide 3), THP (Tamm-Horsfall protein), and a fragment of albumin.

According to the results of the study conducted by Matheson *et al.* [39] the most relevant diabetic nephropathy biomarkers are: alanine aminopeptidase, albumin, alkaline phosphatase, α 1-microglobulin, β2-glycoprotein-1/apolipoprotein H, β2-microglobulin, β-Igh3, cathepsin B, ceruloplasmin, dipeptidil aminopeptidase IV, epidermal growth factor, fibronectin, γ-glutamyltransferase, glycosaminoglycan, immunoglobulin G, lactate dehydrogenase, laminin, lipocalin-type prostaglandine D synthase, N-acetyl-β-Dglucosaminidase, retinol-binding protein, Tamm-Horsfall protein/uromodulin, transferring, and type IV collagen.

Ureteropelvic junction obstruction. Identification of biomarkers in urine has special significance in the case of diseases which are currently diagnosed only in the late stages. For instance, ureteropelvic junction obstruction is usually diagnosed using ultrasound, renal scintigraphy, and dynamic urography.

In order to identify biomarkers for ureteropelvic junction obstruction, Decramer *et al.* [40] used 2 different proteomic strategies:

1) separation by 2D-PAGE and identification by SELDI-MS;

2) on-line capillary electrophoresis coupled to ESI-TOF mass spectrometer.

The authors showed that the use of the second strategy resulted in identification of many more polypeptides which may be potential biomarkers for this disease. 53 highlighted polypeptides with altered expression may allow diagnosis of ureteropelvic junction obstruction at early stage of development. 3 of them were identified as α3 chain of type 9 collagen, a fragment of the type V preprocollagen α2 chain and type V collagen protein.

Radiation nephropathy. Radiation nephropathy is a rare type of nephropathy. The threat of a radiological detonation by terrorists or an accident at nuclear facilities has become a subject of general concern [41,42]. Thus, Sharma *et al.* [43] tested the potential of high throughput proteomics to identify protein biomarkers of radiation injury after total body X-ray irradiation (TBI) in a rat model. The authors separated the proteins using 2D-PAGE and stained the gels with silver nitrate in order to verify differentially expressed proteins. In urine from irradiated animals, 188 proteins had 2-fold or greater abundance when compared with control animals; 76 proteins were found to be 2-fold decreased in the irradiated animals when compared with the control animals. Further analyses were proceeded by nanospray-LC-MS/MS using an LTQ linear ion trap mass spectrometer. Nanospray-LC-MS/MS was used to determine the presence of markers of acute and/or initial kidney injury caused by radiation. According to the authors, decreased urinary albumin suggested a number of changes in the renal physiology. Therefore, the urinary

protein profile reflects the complexity of systemic and renal changes within 1 day after TBI.

Chronic kidney disease. The above-described diseases may cause chronic kidney disease (CKD). Good *et al.* [15] used CE-MS/MS and on-line CE coupled to FT-ICR-MS to compare the urine peptidome of healthy people and those with CKD. The authors analyzed the peptidome in the urine samples containing up to 5,010 peptides, of which 444 were sequenced. Good *et al.* [15] identified 273 potential biomarkers of CKD by CE-MS/MS and LC-MS/MS. The authors demonstrated a lower efficiency of the method of CE-FT-ICR MS, by which only 107 potential biomarkers of CKD were identified.

In comparison, Good *et al.* [15] described the advantage of the method of coupling capillary electrophoresis to tandem mass spectrometry (CE-MS/MS), in which the loss of small and highly charged peptides is reduced, which happens in the most commonly used reversed-phase columns during LC-MS/MS procedure. Proteins and polypeptides with a molecular mass greater than 5 kDa are often not properly eluted, and their number may not be precisely determined. In addition, LC-MS/MS has a lower repeatability than CE-MS/MS. Interfering substances found in large amounts in the urine do not affect the CE-MS/MS analysis as much as LC-MS/MS.

CONCLUSION

The idea of using proteomic methods for medical diagnostics of renal diseases is based on the assumption that the simultaneous analysis of a large number of parameters, particularly the expression level of several selected proteins and polypeptides, allows the creation of sensitive and specific indicators of health status. The conception of protein identification by the MS methods is the same, in spite of biochemical properties and physiological significance of the analyzed proteins, identified by their molecular weights. Nevertheless, in order to optimize the identification of biomarkers, there is an emerging need to search for new proteomic strategies strictly adapted to the specific renal diseases. The urine is an easily accessible fluid which contains proteins, peptides and amino acids. Moreover, it may reflect physiological and/or pathophysiological processes occurring within the body [44]. Urine, as a material collected in a non-invasive way, enables the development of a 'liquid biopsy' as a diagnostic alternative to invasive procedures which may cause many complications. However, despite the large number of various proteomic strategies, these methods are still far from showing their full potential as a routine clinical tool [6,8].

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